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VOLUME 34

CELLULAR AND MOLECULAR BIOLOGY OF SODIUM TRANSPORT

Guest Editor

Stanley G. Schultz

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Cellular and Molecular Biology of Sodium Transport

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Preface

Knowledge of the cellular and molecular biology of sodium transport has advanced considerably since the early 1940s when Dean and Steinbach came to the then daring conclusion that there "...must be some mechanism for pumping out the sodium that wanders into the protoplasm" of animal cells, since 1949 when the first unequivocal demonstration of sodium "pumping" was made by Ussing, and since 1957 when Skou uncovered the molecular counterpart of this "Maxwell Demon," a milestone contribution.

During the past three decades, we have gained considerable insight into the working of Skou's ATPase, how its activity is influenced by intra- and extracellular substrates and activators, and, most recently, how the behavior of this rheogenic (or electrogenic) pump may be influenced by the transmembrane electrical potential difference. In addition, as the result of the application of relatively new and powerful techniques of cellular and molecular biology, we have gained important glimpses into the regulation of the synthesis, intracellular trafficking, expression, and turnover of this ATPase. To be sure, many of these matters are far from resolved, but, given our present technological armamentarium and knowledge base, a mere "foot in the doorway" portends great leaps forward in the not too distant future. Many of these and other advances in our understanding of the cellular regulation of sodium transport are discussed in this volume.

I am grateful to Joe Hoffman and Gerhard Giebisch for having given me the opportunity to organize this volume and the conference from which it was drawn. I would also like to thank the contributors who in the final analysis must be credited with the success of this undertaking.

The organizers would like to recognize the generous support of the following sponsors of the symposium: Abbott Laboratories, Hoechst-Roussell Pharmaceuticals, Inc., Hoffman-LaRoche, Inc., Merck Sharp and Dohme Research Laboratories, Miles Laboratories Inc., Packard Instruments, E.R. Squibb and Sons, Inc., Stuart Pharmaceuticals/ICI, and The Upjohn Company.

Finally, I would like to dedicate this volume to the memory of Peter F. Curran, a close friend, teacher, and collaborator, on the fifteenth anniversary of his death. He died at the untimely age of 41, two years before the first Yale symposium on membrane transport was held in June 1976 in his honor. During his all too brief professional career, Pete directed his considerable energies and creative talents toward advancing our understanding of the mechanisms responsible for sodium and water absorption by epithelia and made a number of lasting contributions which form an essential part of the foundation on which this area of study is now based.

STANLEY G. SCHULTZ

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Chapter 1

Physical State of Cell Sodium

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I. INTRODUCTION

The state of Na^+ within the cell is central to three issues of concern to transport physiologists: the regulation of Na^+ flux across the plasma membranes, the relationship of the cytosolic Na^+ activity to the intracellular Na^+ and water contents (and thus to cell volume regulation), and the possible physiological roles of minor (or major) fractions of the total Na^+ contents. In examining these issues, we shall specifically address two questions: (1) Is the average Na^+ concentration (c^c_{Na}) of most biological cells a satisfactory index of the cytosolic Na^+ activity (a^c_{Na})? (2) If so, do some cells contain minor (or major) fractions of intracellular Na^+ having physiological significance?

In approaching these questions, we shall make use of three concepts: immobilization, compartmentalization, and heterogeneity of intracellular distribution. By *immobilization,* we refer to a marked prolongation of the

period during which the sodium nucleus and its microenvironment retain a fixed mutual orientation. This concept can be quantified by comparing the values of the correlation time (τ_c) in the cell and in simple aqueous solution (Shporer and Civan, 1972, 1977a). By *compartmentalization*, we refer to the existence of two or more intracellular populations of Na^+ whose rates of exchange are much slower than those of diffusional movement within the cell. By *heterogeneity of ionic distribution*, we refer to the possibility of a time-averaged nonuniformity of intracellular Na^+. Such heterogeneity can arise from immobilization or compartmentalization. However, even a single population of intracellular Na^+ could be nonuniformly distributed because of a nonuniform distribution of negative fixed charge sites within the cell.

II. RELATIONSHIP OF AVERAGE CONCENTRATION TO CYTOSOLIC ACTIVITY OF CELL SODIUM IONS

The chemical and electrochemical activities of Na^+ at the cytoplasmic membrane surface will affect the Na^+ flux rates by two mechanisms. First, the cytosolic activity of Na^+ will be a determinant of the fractional occupancy of transport sites, whether the transfer is electrogenic or electroneutral. Second, the differences in Na^+ chemical and electrochemical activity must contribute to the driving forces for electroneutral and electrogenic transfer, respectively, across the plasma membrane. In many epithelial preparations, it is possible to measure a^c_{Na} directly, by using either Na^+-sensitive microelectrodes and reference micropipettes or by measuring the reversal potential for Na^+ transfer through conductive channels. However, these approaches are not universally applicable, and many transport studies involve measurements either of total Na^+ content (n^c_{Na}) and total volume (v^c) or of radioactive tracer movement. Conclusions drawn from the latter measurements require the assumption, tacit or otherwise, that the average Na^+ concentration (calculated as n^c_{Na}/v^c), is proportional to a^c_{Na}. The validity of this assumption has periodically been challenged. Some experimental data [reviewed elsewhere (Shporer and Civan, 1977a)] have in fact suggested the alternative possibility that the bulk of the intracellular Na^+ and K^+ may be immobilized and/or compartmentalized.

A number of approaches have been taken in order to estimate the degree of intracellular immobilization. Of these, the most readily quantified has been the application of ^{23}Na nuclear magnetic resonance (NMR) spectroscopy, which will therefore be considered here in some detail.

A. Estimation of Fractional Intracellular Immobilization by ^{23}Na Nuclear Magnetic Resonance

1. Initial NMR Measurements

The first NMR measurements of intracellular ^{23}Na were conducted by Cope (1965, 1967), who studied samples of muscle, brain, and kidney before and after ashing the tissue. After ashing, the sample volumes were restored by adding solvent. The relative integrated intensity of the spectroscopic signal before ashing was characteristically only 30–40% of that obtained after destroying the tissue architecture. Under certain experimental conditions, the relative integrated intensity of the spectroscopic signal is proportional to the number of ^{23}Na nuclei contained within the sample. Cope assumed such a proportionality and concluded that two distinct populations of Na^+ were present in the samples studied. He suggested that 60–70% of the total intracellular Na^+ was immobilized and characterized by a signal broadened beyond the range of experimental detection. The remaining 30–40% was thought to constitute a separate pool of freely mobile Na^+, characterized by a narrower, easily detectable spectral signal. These early observations were soon confirmed by other investigators studying a variety of tissues. With the sole exception of human erythrocytes (Yeh *et al.*, 1973), measurements over the succeeding 8 years indicated that the bulk fraction of the expected ^{23}Na signal was not detectable by the NMR techniques available at that time. Averaging the 19 published estimates included in an earlier review (Shporer and Civan, 1977a), 58 ± 3% (mean ± SE) of the total expected NMR signal was undetectable by the NMR instrumentation available at that time.

The conclusions drawn from these early studies were strongly dependent on the assumption of proportionality between the area under the ^{23}Na signal and the number of Na^+ nuclei included in the NMR sample. Actually, under a number of conditions, the detected magnetization vector is not necessarily proportional to the number of ^{23}Na nuclei in a sample:

1. Heterogeneous populations of ^{23}Na nuclei
2. Heterogeneous energy transitions for all ^{23}Na nuclei
3. Instrumental effects (especially saturation) [discussed in detail by Gadian (1982)]

Of particular relevance in the present context is the concept (illustrated in Fig. 1) that the observed signal can reflect only part of the spectrum characterizing all of the Na^+ nuclei (Shporer and Civan, 1972). Nuclides such as ^{23}Na, characterized by a spin quantum number of 3/2, display four

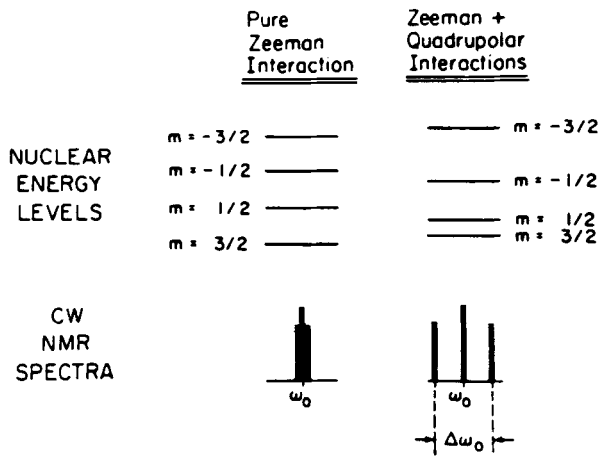

FIG. 1. Nuclear magnetic energy levels and spectral lines of nuclides (such as ^{23}Na and ^{39}K) with a spin number of $\frac{3}{2}$ (Civan, 1983). As illustrated on the left-hand side, in the absence of other energetic interactions, the four nuclear magnetic energy levels are equally spaced, producing superimposed narrow spectral lines. However, when the nucleus is subjected to a first-order nuclear quadrupolar interaction, the spectrum can be altered in two ways. Under certain conditions (displayed on the right-hand side), the two outer energy transitions (from $m = \frac{3}{2}$ to $m = \frac{1}{2}$, and from $m = -\frac{1}{2}$ to $m = -\frac{3}{2}$) are distorted, displacing the associated spectral signals. The conditions which lead to the appearance of satellite signals are (Rubinstein *et al.*, 1971; Baram *et al.*, 1973): $(e^2qQ)\tau \geqslant 1$ and $(\omega\tau_c) > 1$, where (e^2qQ) is the strength of the nuclear quadrupolar interaction, τ_c is the correlation time, and ω is the angular frequency of nuclear precession. (Reprinted by permission of John Wiley & Sons, Inc.)

nuclear magnetic energy states. Under the condition of a purely magnetic interaction with the external field, the four energy states are equally displaced, so that the energy transition (ΔE_i) between any two neighboring states is characterized by the same spectral frequency, ν_i; $\nu_i = (\Delta E_i)/h$, where h is Planck's constant. However, because of its relatively large nuclear quadrupolar coupling constant, ^{23}Na exchanges energy by electrical interaction with its microenvironment. In the presence of a first-order nuclear quadrupolar interaction, two of the three permitted energy transitions are distorted, resulting in a splitting of two satellite signals from the central undisplaced signal. Under certain conditions of molecular motion, the two satellites converge to form a single broad component. Under these circumstances, the nuclear quadrupolar interaction leads to the superposition of two signals (of different widths), both of which are emitted from all of the sodium nuclei (Fig. 2). In either case, whether the nuclear quadrupolar interaction produces splitting or superposition, the central fine line will present only 40% of the total anticipated ^{23}Na signal.

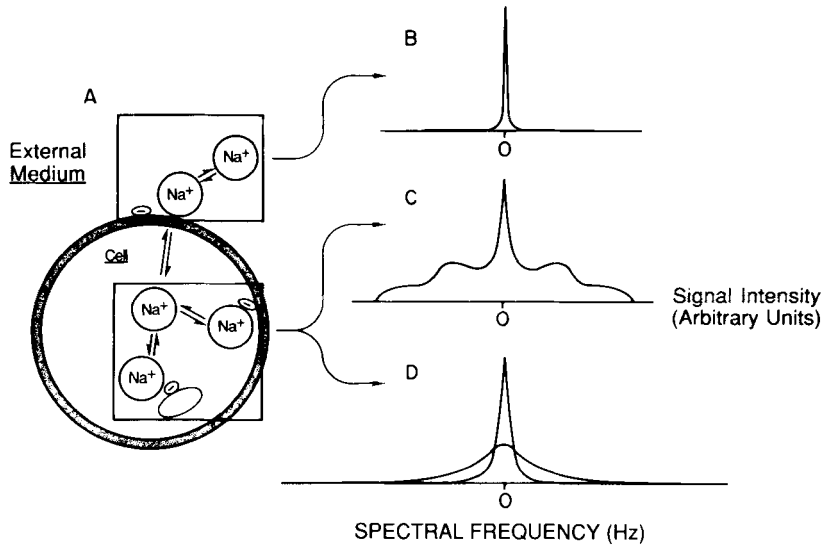

FIG. 2. Possible spectral changes produced by a first-order quadrupolar interaction with ^{23}Na nuclei. The extracellular Na^+ in (A) is considered to emit a narrow spectral line (B). On the other hand, a first-order quadrupolar interaction exerted on the intracellular Na^+ (A) may either produce satellite signals (C) or superposition of a relatively broad and relatively narrow signal (D). Superposition will occur when $(e^2qQ)\tau_c << 1$ and $(\omega\tau_c) \geq 1$. The satellite signals do not appear as discrete lines in (C), because of the random orientation of the ^{23}Na nuclei. In both (C) and (D), the relatively narrow central signal accounts for 40% of the total ^{23}Na spectrum.

Whether the total signal will be measured (by detecting either the satellite signals or superimposed broad spectral line) depends on the instrumentation available. The reduced dead time (improved response time) and enhanced sensitivity of instruments currently available very much improve the possibility of detecting broad ^{23}Na signals.

It should be appreciated that the same considerations apply to ^{39}K, the principal naturally occurring isotope of potassium. Like ^{23}Na, ^{39}K is characterized by a spin quantum number of 3/2 and a large nuclear quadrupolar coupling constant. Thus, when ^{23}Na undergoes a first-order nuclear quadupolar interaction, ^{39}K is likely to be subjected to a qualitatively similar effect in the same microenvironment. This consideration leads to the expectation that only 40% of the ^{39}K signal is also likely to be detectable in cells in which only 40% of the ^{23}Na signal is observed. This expectation has been clearly confirmed by Ogino *et al.* (1983), who used an external shift reagent to quantify the intracellular and extracellular ^{23}Na and ^{39}K

pools of yeast cells *(Saccharomyces cerevisiae)*. On the other hand, an exception has been reported by Fossel and Hoefeler (1986), who observed the full ^{23}Na signal, but only a small fraction of the ^{39}K spectrum of rat heart. Whether this unexpected observation reflects a larger quadrupolar interaction because of the increased number of electrons of the ^{39}K nuclide, compartmentalization, or some unidentified additional mechanism is unknown.

At least five lines of evidence support the concept (Shporer and Civan, 1972) that the undetected fractions of the total ^{23}Na spectrum reported for some cells reflect nuclear quadrupolar interactions. (1) This phenomenon can be reproduced by simple model systems (Shporer and Civan, 1972; Chen and Reeves, 1972). (2) One of the magnetic relaxation times characterizing ^{23}Na nuclei (T_1, the longitudinal relaxation time) has been found to have only a single component in muscle (Berendsen and Edzes, 1973; Shporer and Civan, 1974). The longitudinal relaxation time of ^{39}K has also been found to have only a single component in biological cells (Civan *et al.*, 1976; Shporer and Civan, 1977b). If cells contained free and immobilized populations of Na^+ and K^+, each of the populations would be expected to be characterized by a different value of T_1. (3) The T_1 for intracellular ^{23}Na displays little (if any) dependence on the nuclear magnetic resonance frequency (ω_o) (Shporer and Civan, 1974), as expected for freely mobile Na^+. T_1 should be directly proportional to ω_o^2 for immobilized sodium nuclei. (4) A more quantitative analysis has been conducted, based on measurements of the longitudinal and transverse relaxation times of ^{23}Na and ^{39}K. An upper limit of only a small percentage of ^{23}Na in frog striated muscle (Shporer and Civan, 1974: Monoi, 1976) and of ^{39}K in *Halobacterium halobium* (Shporer and Civan, 1977b) is likely to be immobilized. (5) The previously undetected ^{23}Na signal in renal tubular cells has now been observed with improved instrumentation (Gullans *et al.*, 1985; Rayson and Gupta, 1985).

2. Recent NMR Observations

On the basis of the percentage of detectability of ^{23}Na and ^{39}K signals, published NMR analyses fall into one of three categories:

1. In some tissues, $\cong$40% of the intracellular signals is NMR detectable (summarized in Shporer and Civan, 1977a; Goldberg and Gilboa, 1978; Ogino *et al.*, 1983). For the reasons discussed above, it is likely that this phenomenon reflects a first-order nuclear quadupolar interaction (Shporer and Civan, 1972).
2. In other tissues, most or all of the intracellular ^{23}Na and ^{39}K is likely

to be NMR detectable (Yeh *et al.*, 1973; Shporer and Civan, 1977b; Civan *et al.*, 1983; Pettegrew *et al.*, 1984; Ogino *et al.*, 1985; Gullans *et al.*, 1985; Rayson and Gupta, 1985; Wittenberg and Gupta, 1985). In general, these intracellular contents are also subject to a first-order quadrupolar interaction, as evidenced by the biexponentiality of the transverse relaxation. However, in contrast to the first set of reports, the signals reflecting the $\frac{3}{2}$ to $\frac{1}{2}$ and $-\frac{1}{2}$ to $-\frac{3}{2}$ transitions are not broadened beyond instrumental detection.

3. In a third group of tissues, $\leqslant$20% of the intracellular ^{23}Na and ^{39}K have been detected, even with modern instrumentation (Gupta *et al.*, 1985; Pike *et al.*, 1985; Fossel and Hoefeler, 1986).

In their initial manuscript (1972), Shporer and Civan emphasized that the NMR data do not exclude the possibility of minor populations of immobilized ^{23}Na. Some evidence for such minor pools has been reported (Adam *et al.*, 1987, 1988; Pettegrew *et al.*, 1984). However, the very low percentage of detectability reported for intracellular ^{39}K in cardiac muscle (Pike *et al.*, 1985; Fossel and Hoefeler, 1986) and for ^{23}Na in amphibian oocytes (Gupta *et al.*, 1985) cannot be ascribed to minor ion populations. These data could result from at least two mechanisms. First, the large NMR-undectectable signals could obviously reflect unusually substantial fractions ($\geqslant$20%) of ^{23}Na and ^{39}K which are truly immobilized. For reasons discussed later (p. 13), this interpretation may well be correct for amphibian oocytes. On the other hand, why this putative immobilization should appear in cardiac cells, as opposed to a great range of other biological cells, is obscure. It should be appreciated that there are some differences between the pulsed and continuous wave techniques used for generating NMR spectra. The two techniques are equivalent if the full spectral band width is excited in the pulse technique. However, if excitation is applied to only a fraction of the spectral band width, considerable differences in the signal characteristics can be expected.

Joseph and Summers (1987) have recently proposed that the flip-angle effect can be exploited to detect nuclear quadrupolar splitting. This effect may have even wider implications by possibly playing a role in having reduced the size of some of the reported ^{23}Na and ^{39}K signals. In examining this interpretation, it is necessary to consider how the spectral signal is generated.

A large, steady magnetic field (H_0) established along the z axis of the sample orients an excess of the ^{23}Na or ^{39}K magnetic dipoles parallel to the H_0 field. The resulting bulk magnetization vector (**M**) is not sensed by the receiving coil, whose vector is perpendicular to H_0. A signal is generated when a radiofrequency field (H_1) is briefly applied along the perpen-

dicular x axis, flipping the magnetization vector from its equilibrium position. A maximum value is detected when **M** is rotated 90° from the H_0 axis. This condition is met when $[\gamma H_1(\Delta t)]$ is $\pi/2$ radians; γ is the gyromagnetic ratio characteristic of the nuclide, and Δt is the duration of the small radiofrequency perturbation. When a broad band pulse sufficient to excite all three energy transitions (Fig. 1) is applied, the 90° radiofrequency pulse $[\gamma H_1(\Delta t_{90})]$ will not be very different in the biological sample and in a simple aqueous standard. Thus, the common practice of determining Δt_{90} by first analyzing the simple solution is entirely rational. However, with a relatively narrow band pulse, it is possible to excite solely the central energy transition, without affecting the other two transitions. As pointed out by Joseph and Summers, this is clearly the case for nuclear quadrupolar splitting, when the satellite signal frequencies lie outside the range of perturbing frequencies. It can also be the case for bioexponential superposition of ^{23}Na signals, when the frequency band of the perturbation is very much narrower than the broad spectral line (reflecting the two outer energy transitions).

Of importance in the present context is the fact that the effective gyromagnetic ratio exciting the central $\frac{1}{2}$ to $-\frac{1}{2}$ transition is twice that for exciting all three permitted energy transitions (Abragam, 1985; Schmidt, 1972; Fukushima and Roeder, 1981; Fenzke *et al.*, 1984). Thus, a radiofrequency pulse, $[\gamma H_1(\Delta t_{90})]$, which will produce a flip angle of 90° by exciting all nuclear magnetic energy transitions of ^{23}Na or ^{39}K in an aqueous calibrating solution, may rotate the magnetization vector for the central transition by 180° in a biological sample. No observable signal will be detected by such a 180° rotation. The implication of this theoretical analysis is that the detectability of even single homogeneous pools of ^{23}Na or ^{39}K (subjected to first-order nuclear quadrupolar interactions) may range from 0 to 100%, depending on how closely $[\gamma H_1(\Delta t_{90})]$ produces a true 90° rotation for the nuclides in the biological sample.

B. Application of Other Biophysical Techniques

The NMR studies summarized above strongly suggest that little intracellular Na^+ or K^+ is immobilized within biological cells. Clear examples of more than minor fractional immobilization are rare and have generally been incompletely documented by NMR analysis. This conclusion is also supported by measurements of (1) the intracellular electrophoretic mobility of K^+ (Hodgkin and Keynes, 1953), (2) intracellular coefficients of diffusion (Hodgkin and Keynes, 1953; Kushmerick and Podolsky, 1969), (3) cytoplasmic resistivity (Foster *et al.*, 1976), and (4) the capacity of hypothetical K^+ binding sites (Palmer and Gulati, 1976). On the other

hand, most biological cells contain a rich complement of intracellular organelles. The functional significance of the resulting compartmentalization depends upon the compositions of the fluids within the organelles and the rates of ion exchange with the surrounding cytosol.

One approach for quantifying compartmentalization is to compare the apparent intracellular activity coefficients for Na^+ (γ_{Na}^{app}) and for K^+ (γ_K^{app}) with the corresponding activity coefficients in simple aqueous solution. For each ion (i), γ_i^{app} is defined as the ratio of the intracellular activity (a_i^c) to the mean intracellular concentration (c_i^c):

$$\gamma_{Na}^{app} \equiv (a_{Na}^c)/(c_{Na}^c) \quad (1)$$

$$\gamma_K^{app} \equiv (a_K^c)/(c_K^c) \quad (2)$$

In the extracellular medium, the activity coefficient (γ_i°) is calculated as the ratio of activity to concentration measured for the same population of ions. Measurement of γ_i° therefore provides information concerning the interaction of the ion with the water molecules and other ions in its microenvironment. The apparent activity coefficients of Eqs. (1) and (2) are fundamentally different parameters. In the latter case, the activity is commonly measured with ion-selective microelectrodes, whose tips sense the ionic composition of the cytosol. On the other hand, the mean concentration is generally calculated as the ratio of the total intracellular ion to the total water content. For this reason, measurements of γ_{Na}^{app} and γ_K^{app} reflect the compartmentalization, as well as the physicochemical characteristics, of Na^+ and K^+ within the cell.

As discussed elsewhere (Civan, 1983), the intracellular Na^+ content is commonly measured either chemically or by electron-probe X-ray microanalysis (EPMA), although alternative techniques, such as ^{23}Na NMR and curve stripping analysis of radioactive fluxes, are also available. The water content can be determined either chemically or by measuring the fractional dry weight during EPMA. The ionic activities are usually determined with intracellular ion-selective microelectrodes and reference micropipettes or (under appropriate conditions) by measuring the reversal potential for Na^+ or K^+ through selective, conductive membrane channels. Alternative techniques for measuring a_{Na}^c, such as ^{19}F NMR analysis of Na^+-sensitive intracellular probes (Smith *et al.*, 1986), are also becoming available.

1. Possible Compartmentalization of Na^+ and K^+ in Model Epithelia

In general, measurement of γ_{Na}^{app} is rendered difficult by the characteristically low values of a_{Na}^c and c_{Na}^c in biological cells. Measurement of the apparent activity coefficients in epithelial tissue can be particularly com-

plicated by tissue heterogeneity (especially from subepithelial smooth muscle), cellular heterogeneity, and the difficulty of obtaining satisfactory intracellular electrophysiological records in some tissues. In large part, these problems can be circumvented by studying frog skin. Because of the technical advantages it presents, this model epithelium is particularly suitable for intracellular investigations of transepithelial Na^+ transport:

1. The intracellular ion contents and fractional water content can be measured by EPMA (Rick *et al.*, 1978b).
2. The epithelium can be removed from the underlying dermis without markedly altering its electrophysiological and transport properties (Fisher *et al.*, 1970), facilitating chemical analysis of the isolated epithelium.
3. With the exception of the outer layer of keratinized cells (the *stratum corneum*) and the mitochondria-rich cells, the epithelial cells form a syncytium (Farquhar and Palade, 1964; Ussing and Windhager, 1964; Nagel, 1976; Rick *et al.*, 1978b; DeLong and Civan, 1984), so that there is usually little variation in ionic and water contents among the principal cells of the epithelium. The total relative volume of the mitochondria-rich cells is so small that their water and ionic contents make a negligible contribution to the total measurements (Larsen *et al.*, 1988).
4. Stable intracellular electrical measurements can, under favorable conditions, be recorded over periods of 1 hour or more (Nagel, 1976; Helman and Fisher, 1977).
5. The favorable syncytial properties of the epithelium permit determination of intracellular ionic activities by recording the difference in membrane potential measured simultaneously with single-barreled ion-selective and reference microelectrodes (DeLong and Civan, 1983a).

Despite these substantial technical advantages, the presence of the *stratum corneum* does complicate certain experimental studies based on the preparation of membrane vesicles (Civan and Garty, 1989). For this reason, it is fruitful to complement studies of frog skin with studies of toad urinary bladder, a functionally analogous model epithelium.

The intracellular Na^+ and K^+ concentrations of frog skin have been analyzed under baseline conditions both by EPMA of whole skin (Rick *et al.*, 1978b, 1984) and by chemical analysis of isolated epithelium briefly rinsed to remove Na^+ from the *stratum corneum* and extracellular sites (Rotunno *et al.*, 1973; Aceves, 1977). Both techniques are open to uncertainties. The complexities of EPMA are discussed elsewhere (Civan,

1983), while even the brief rinsing used in the chemical analysis of isolated epithelia may lead to some depletion of cellular compartments. Averaging the published results obtained by both approaches, the mean values ± SEM for c^c_{Na} and c^c_K can be estimated to be 18 ± 3 and 144 ± 10 m*M*, respectively.

Intracellular Na^+ activity has been measured using ion-selective microelectrodes (Nagel *et al.*, 1981; Harvey and Kernan, 1984) and estimated from the reversal potential for Na^+ movement through the apical Na^+ channels (DeLong and Civan, 1984; Schoen and Erlij, 1985; Civan *et al.*, 1987). With the latter approach, γ°_{Na} is taken to be 0.76, as calculated from a limiting form of the Debye–Hückel equation. Averaging of the five series of published results shows that a^c_{Na} is calculated to be 13 ± 1 m*M* under baseline conditions.

A considerably wider range of values of intracellular K^+ activity has been reported for measurements using ion-selective microelectrodes (Nagel *et al.*, 1981; DeLong and Civan, 1983a; Harvey and Kernan, 1984; Garcia-Diaz *et al.*, 1985). Although double-barreled, ion-selective microelectrodes have been used (DeLong and Civan, 1983a; Harvey and Kernan, 1984), their tip diameters are necessarily larger than those of single-barreled microelectrodes, increasing the probability of leakage of cell K^+ through impalement-induced shunts. It seems likely that separate, simultaneous impalements of the epithelial syncytium with K^+-selective microelectrodes and reference micropipettes yield more accurate estimates of a^c_K. With this approach, a^c_K has been estimated to be 104 ± 3 (DeLong and Civan, 1983a) and 92 ± 8 m*M* (Garcia-Diaz *et al.*, 1985).

Inserting these published values into Eqs. (1) and (2), γ^{app}_{Na} can be estimated to be 0.70 ± 0.12 and γ^{app}_K to be 0.68 ± 0.08. These estimates are subject to the uncertainties inherent in comparing measurements obtained with different species in different laboratories under not necessarily identical conditions. However, the calculated means are very close to the activity coefficient of 0.76 characterizing Na^+ and K^+ in the external medium. This agreement strongly suggests that the great bulk of both intracellular Na^+ and K^+ is in ready exchange with cytosolic Na^+ and K^+ respectively.

Considerably less information is available concerning the kinetics of the responses of concentrations and activities (and thus of the apparent activity coefficients) for Na^+ and K^+ following experimental perturbations. However, reducing the serosal K^+ concentration can lower the intracellular K^+ activity to $\leq$13 m*M* (DeLong and Civan, 1983b), while ouabain has been reported to reduce c^c_K to $\cong$22 m*M* (Rick *et al.*, 1978b). EPMA has also confirmed that ouabain leads to the loss of the great bulk of K^+ from toad urinary bladder cells (Rick *et al.*, 1978a). In addition,

EPMA of frozen hydrated sections has shown that the combined addition of ouabain and removal of external K^+ reduces intracellular K^+ by ≅85% in toad bladder cells (Civan *et al.*, 1980). As far as intracellular Na^+ activity is concerned, measurements with ion-selective microelectrodes have suggested reductions in a^c_{Na} ranging from 40–50% (Nagel *et al.*, 1981) to ≅90% (Harvey and Kernan, 1984) after blocking apical Na^+ entry with amiloride. This range of reported reductions in a^c_{Na} is roughly comparable to the ≅60% fall in c^c_{Na} measured by EPMA after adding amiloride (together with vasopressin) (Rick *et al.*, 1984). These comparisons of perturbation-induced changes in a^c_i and c^c_i merit further examination. However, the data are thus far qualitatively consistent with the concept that both Na^+ and K^+ constitute functionally homogeneous pools within epithelial cells such as those of frog skin and toad bladder.

2. Possible Heterogeneity of Distribution of Intracellular Na^+ and K^+

The results of the preceding sections suggest that little intracellular Na^+ and K^+ is immobilized in most biological cells. Furthermore, little Na^+ and K^+ is likely to be compartmentalized in epithelia such as frog skin. By compartmentalization, we refer specifically to subpopulations of intracellular ions, exchanging at rates slow in comparison to those of diffusional movement within the cell. Defined in these restrictive terms, the absence of compartmentalization does not preclude a heterogeneous distribution of Na^+ or K^+ within the cell. For example, organelles whose electrical potential is more negative than that of the cytosol or which contain high densities of negatively charged sites would be expected to accumulate cations. A number of reports have suggested the presence of such heterogeneity of distribution and have raised possible physiological implications (summarized in Palmer and Civan, 1977; Civan, 1983).

Compartmentalization of Na^+ and K^+ is more commonly inferred (particularly from analysis of flux kinetics) than directly identified. To some extent, this may reflect both the technical difficulties of studying small organelles in whole-cell preparations and the potential problems of redistributing ions and water during the preparation of broken-cell samples. To the extent that this possibility has been directly quantified, heterogeneity of distribution of Na^+ and K^+ seems to be quantitatively modest in most cells. In an epithelial cell large enough for simultaneous nuclear and cytosolic recording with multiple microelectrodes, no gradient has been observed for either Na^+ or K^+ activity across the nuclear envelope (Palmer and Civan, 1977). EPMA of frog and toad skin cells and of toad urinary bladder cells has also indicated that c^c_{Na} and c^c_K have similar val-

ues in the nucleoplasm and in the cytosol. Taking into account the higher nuclear water content, calculations based on results obtained by EPMA of whole epithelia (Rick *et al.*, 1978a,b 1980, 1984; Civan *et al.*, 1980) suggest that the Na^+ and K^+ concentrations are only 5 ± 2 m*M* and 7 ± 2 m*M* lower, respectively, in the nucleus than in the cytosol.

EPMA of ultrathin sections has provided additional information concerning the distribution of Na^+ and K^+ between the cytosol and organelles smaller than the nucleus. Correcting for the relative water contents, there appears to be no significant difference in Na^+ or K^+ concentration between the mitochondria and the cytosol under baseline conditions in striated muscle (Somlyo *et al.*, 1977), smooth muscle (Somlyo *et al.*, 1979), or brain cells (Somlyo *et al.*, 1985). Analyses of rat liver parenchymal cells has suggested that the Na^+ and K^+ concentrations are probably similar in the rough endoplasmic reticulum as well as in the mitochondria, the nucleus, and the cytosol (Somlyo *et al.*, 1986). The major caveat in interpreting the results of the latter study is the uncertainty involved in assigning relative volume contents to the various organelles. In this study, the largest reported difference in measured Na^+ or K^+ content was the approximately twofold higher content of K^+ in the nucleus than in the mitochondria. However, this difference may primarily reflect the very different water contents of the two organelles. From data obtained by EPMA (Rick *et al.*, 1987a,b, 1980, 1984), the average nuclear water content for frog and toad skin cells and toad urinary bladder cells can be estimated to be 3.34 ± 0.09 kg water per kg dry weight. This value is twice as great as the value of mitochondrial water content of ≅1.72 kg water per kg dry weight, which can be estimated from the EPMA continuum counts reported by Somlyo *et al.* (1979). This suggests that the K^+ concentrations are similar in the mitochondria and in the nucleus.

C. Possible Special Conditions of Sodium Ions in Amphibian Oocytes

The discussions of the preceding sections suggest that, in striking contrast to Ca^{2+}, the state of Na^+ and K^+ in most cells is similar to that in the external bathing media. Immobilization, compartmentalization, and heterogeneity of intracellular distribution do not seem to exert substantial effects on most of the Na^+ and K^+ in most biological cells. In these cells, data analysis is likely to be more significantly affected by other factors, such as cellular heterogeneity, tissue heterogeneity, and tissue geometry [including unstirred compartments, particularly in the presence of recycling of Na^+ and K^+ across adjacent membranes (Civan 1981)]. On the other hand, data obtained with a broad range of techniques suggest that

the amphibian oocyte may constitute an exception to this general conclusion (Dick and McLaughlin, 1969; Horowitz and Fenichel, 1970; Century *et al.*, 1970; Century and Horowitz, 1974; Palmer *et al.*, 1978; Gupta *et al.*, 1985).

Palmer *et al.*, (1978) have reported measurements of a_i^c and c_i^c in both small immature and large mature frog oocytes, using intracellular ion-selective microelectrodes and electron-microprobe and chemical analyses. With development, c_{Na}^c became sixfold higher, while a_{Na}^c fell by $\cong$60%. The apparent activity coefficient for the intracellular Na^+ of the mature oocyte was calculated to be 0.08 $\pm$ 0.02, an order of magnitude lower than the activity coefficient for extracellular Na^+. These findings were in striking contrast to the observations that c_K^c fell slightly with development, while a_K^c increased by $\cong$70%. In the mature oocyte, γ_K^{app} ranged from 1.15 to 1.29. Given the large size of the mature oocyte and the ease of introducing multiple microelectrodes, the striking deviations of γ_{Na}^{app} and γ_K^{app} from the corresponding values of the extracellular activity coefficients could not be ascribed to technical artifacts. Rather, the data clearly established the existence of compartmentalization and/or immobilization of Na^+ within the oocyte. In addition, the cytosol is clearly richer in K^+ than are other compartments within the mature oocyte. The anatomic site of the putative organelle accumulating Na^+ and excluding K^+ was unknown. However, the development of the amphibian oocyte is associated with the appearance of yolk platelets, cytoplasmic vesicles, and pigment granules (Wishnitzer, 1966; Merriam, 1966; Dick *et al.*, 1970). Since the yolk platelets occupy one half of the volume of the mature amphibian oocyte, Palmer *et al.* (1978) proposed that these platelets would be the particularly likely site for compartmentalization.

Recent NMR studies have provided evidence supporting this concept that the yolk platelets provide at least part of the basis for the observed compartmentalization and/or immobilization of ^{23}Na in the amphibian oocyte. Gupta *et al.*, (1985) have reported that <7% of the total ^{23}Na contained within isolated yolk platelets was NMR detectable. Because of the possibility of loss of platelet Na^+ during the isolation procedure, it is difficult to quantify precisely how much of the NMR-invisible Na^+ fraction reflects the yolk platelet fraction of the intact oocyte. Gupta *et al.* have also presented evidence for two additional populations of ^{23}Na within the oocyte. The complexities of the NMR spectrum of this preparation cannot be ascribed to the flip-angle effect discussed in Section II,A,2, since the effect of varying the radiofrequency pulse width was carefully examined.

On the basis of the foregoing considerations, it seems likely that the

oocyte is one of the very few biological cells which significantly immobilize and compartmentalize intracellular Na^+. This phenomenon may have physiological importance. The *Rana* ovum is deposited in salt-poor ponds. However, Na^+ is needed as a substrate for the Na^+–K^+ exchange pump, which is ultimately responsible for establishing ionic and electrical gradients across the plasma membrane. These gradients are required for a wide range of cellular activities, including secondary active transport of nonelectrolytes and intercellular electrical coupling, and it is likely that they are essential for normal embryonic development following maturation and ovulation. The yolk platelets may serve as critical reservoirs of intracellular Na^+. This concept is supported by the observation that part of the Na^+ within the yolk platelets is released into the cytosol by ovulation of prophase-arrested, follicle-enclosed oocytes (Morrill *et al.*, 1971).

III. SUMMARY

Knowledge of the state of intracellular Na^+ is fundamental to a comprehensive study of transepithelial transport. We have examined this issue in three ways. One approach has been to quantify immobilization by ^{23}Na NMR spectroscopy. A second approach has been to quantify compartmentalization by comparing the apparent activity coefficient for intracellular Na^+ with the activity coefficient of Na^+ in simple aqueous solution. A third approach has been to examine the heterogeneity of distribution of intracellular Na^+ by the combined application of electrophysiological and electron microprobe techniques. In a wide variety of tissues, it seems unlikely that the great bulk of intracellular Na^+ is immobilized. Studies of one model epithelium, frog skin, suggest that little intracellular Na^+ appears to be either compartmentalized or heterogeneously distributed.

In at least one cell, the amphibian oocyte, immobilization, compartmentalization, and heterogeneity of distribution are likely to be substantial. The possible physiological relevance of this phenomenon is considered.

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Chapter 2

Intracellular Sodium Activities and Basolateral Membrane Potassium Conductances of Sodium-Absorbing Epithelial Cells

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I. INTRODUCTION

An early, natural outgrowth of the Koefoed-Johnsen–Ussing (KJU) (1958) double-membrane model for Na^+ absorption by isolated frog skin (Fig. 1) was the concept of the *Na^+ transport pool*, which is defined as the intracellular Na^+ originating from the outer, or mucosal, solution that is in transit or awaiting transport out of the cell across the basolateral (inner) membrane (Andersen and Zerahn, 1963; Frazier *et al.*, 1962). Further, it was not unreasonable to infer that changes in the rate of Na^+ entry across the apical membrane result in parallel changes in the size of this pool and, in turn, the turnover rate of the basolateral sodium pump. However, many early, and often ingenious, attempts to identify this active transport pool and to relate its size to the rate of transcellular Na^+

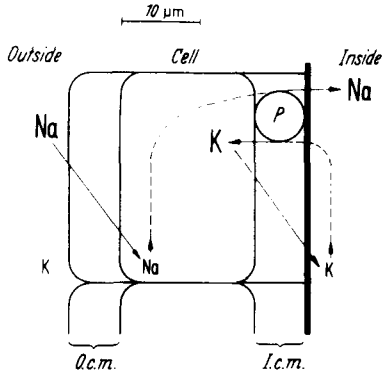

FIG. 1. The Koefoed-Johnsen–Ussing (1958) double-membrane model for Na^+ absorption by isolated frog skin. P, Pump; O.c.m., outer cell membrane; I.c.m., inner cell membrane.

transport, employing chemical techniques and/or analyses of the kinetics of isotope exchange, yielded equivocal, and sometimes conflicting, results [see Macknight and Leaf (1978) and Macknight *et al.* 1980) for reviews of this early literature]. Indeed, some investigators were unable to identify an intracellular active Na^+ transport pool and suggested that active transepithelial Na^+ transport may be the result of Na^+ skirting around the cells without entering the intracellular compartment (Zerahn, 1969; Cereijido and Rotunno, 1968). In view of the facts that all Na^+-absorbing epithelia are comprised of heterogeneous populations of cells, some of which are not involved in transcellular Na^+ transport, and that many, particularly frog skin, have very complex geometries, it is not surprising, in retrospect, that many of these early studies yielded equivocal, and even bizarre, results.

During the three decades that have elapsed since the introduction of the KJU model, three additional models of Na^+-absorbing epithelial cells have been firmly established. The principal differences among all of these models are the mechanisms responsible for Na^+ entry into the absorptive cells across their apical membranes. These mechanisms, illustrated in Fig. 2, are:

1. *Sodium entry by diffusion through highly selective channels that are blocked by micromolar concentrations of amiloride.* This mode of entry appears to be largely restricted to the apical membranes of tight epithelia, such as amphibian skin; amphibian and mammalian urinary bladders; amphibian, mammalian, and avian colons; and mammalian distal nephron. The properties of this channel have been recently reviewed by Garty and Benos (1988).

2. *Carrier-mediated Na^+ entry, coupled with the cotransport of organic solutes such as sugars and amino acids.* This entry mechanism has

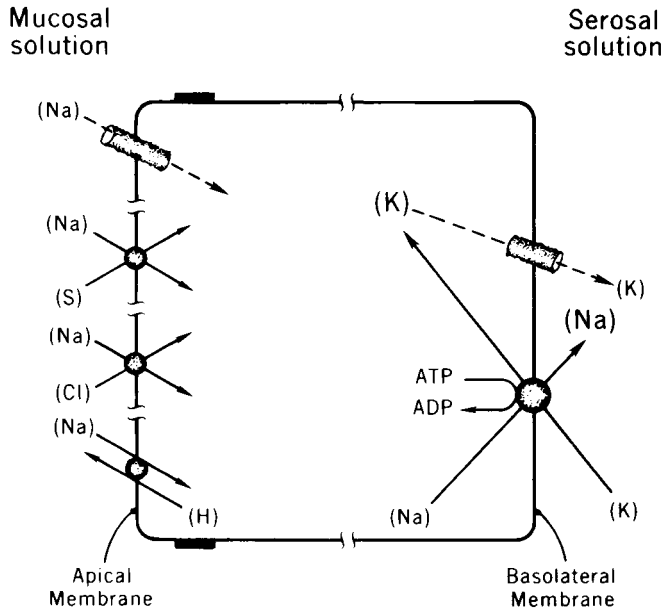

FIG. 2. A composite model illustrating the four well-established mechanisms that mediate Na^+ entry into Na^+-absorbing epithelial cells across their apical membranes and the pump–leak properties common to the basolateral membranes of all of these epithelia. ADP, Adenosine diphosphate; ATP, adenosine triphosphate.

been identified in the apical membranes of small intestine and renal proximal tubule in every animal studied throughout the phylogenetic scale.

3. *Carrier-mediated Na^+ entry, coupled with the cotransport of Cl^-.* This entry mechanism, which is inhibited by loop diuretics such as furosemide, appears to involve a neutral carrier complex consisting of one sodium ion, one potassium ion, and two chloride ions and has been clearly identified in small intestine, gallbladder, and the ascending limb of the loop of Henle.

4. *Carrier-mediated Na^+ entry, coupled with the countertransport of protons.* This entry mechanism is inhibited by millimolar concentrations of amiloride and has been identified in the apical membranes of gallbladder, small intestine, and renal proximal tubule.

A detailed discussion of these entry mechanisms can be found in a recent review by the author (Schultz, 1986).

In spite of the fact that different mechanisms are responsible for Na^+ entry into these different Na^+-absorbing cells, the basolateral membranes

of these cells all appear to share at least two common transport properties; namely, the ubiquitous Na^+–K^+ exchange pump (Na^+,K^+-ATPase) and a leak pathway for K^+, as postulated in the original KJU model.[1]

Further, during the past two decades, two approaches have been developed that permit the *direct* determination of intracellular Na^+ activities, $(Na)_c$, in *single* Na^+-absorptive cells: (1) the use of highly Na^+-sensitive (or selective) microelectrodes and (2) microelectrophysiological techniques that permit the determination of the current–voltage *(I–V)* relationships of the amiloride-inhibitable Na^+ channel in the apical membranes of tight epithelia. The intracellular Na^+ activity can then be calculated from the zero current or reversal potential of these *I–V* relationships, by employing the Nernst equation. In essence, this approach assumes that the amiloride-inhibitable Na^+ channel in the apical membrane simulates the behavior of a highly selective Na^+-sensitive electrode.

It should be noted that Na^+-selective microelectrodes provide a measure of $(Na)_c$ somewhere in the cytoplasm where the tip of the electrode happens to reside. On the other hand, the use of *I–V* relationships provides a measure of $(Na)_c$ at the interface between the inner surface of the apical membrane and the immediately adjacent cytoplasm. The results of recent studies by Turnheim *et al.* (1987) indicate that the values of $(Na)_c$ in rabbit colonic Na^+-absorptive cells, determined by employing these two different techniques, do not differ significantly.

In this chapter we will examine the relationship—or lack thereof—between $(Na)_c$, determined employing these two approaches, and the rates of transcellular Na^+ transport by several tight and leaky epithelia when these rates are stimulated either slowly or abruptly by physiological maneuvers that increase the rates of Na^+ entry across the apical membranes. We will also examine the effects of these maneuvers on the K^+ conductances of the basolateral membranes of these absorptive cells.

II. CHRONIC STIMULATION OF SODIUM ABSORPTION BY HYPERALDOSTERONISM

It is well established that chronic hyperaldosteronism, resulting directly from administration of mineralocorticoids or indirectly from dietary Na^+ deprivation or K^+ overloading, brings about a marked increase in the rates of transcellular Na^+ absorption by a number of epithelia in-

[1]As discussed by Schultz (1986), other transport mechanisms have been identified in the basolateral membranes of some epithelia, but the properties of *all* basolateral membranes of Na^+-absorbing cells appear to be dominated by the Na^+–K^+ pump and the K^+ leak.

cluding amphibian and mammalian colon and urinary bladder, and mammalian renal cortical collecting tubule. It is also generally accepted that the first step in this process is an increase in the conductance of the apical membranes to Na^+, due to an increase in the number of operant, amiloride-inhibitable Na^+ channels in that barrier (Garty, 1986).

The results of recent electrophysiological studies (Turnheim *et al.*, 1987) on segments of descending colon from rabbits maintained on a normal (high-sodium) diet and rabbits maintained on a sodium-deficient diet consisting of barley and distilled water for 7–10 days are summarized in Table I. As shown, the rate of Na^+ absorption, given by the amiloride-inhibitable short-circuit current (I_{Na}), was increased approximately threefold by Na^+ deprivation. However, at the same time, the electrical potential difference across the apical membrane (ψ^{mc}) and $(Na)_c$, determined using Na^+-selective microelectrodes, did not differ significantly between these two groups. Thus, inasmuch as the electrochemical potential difference for Na^+ diffusion across the apical membrane ($\Delta\bar{\mu}^m_{Na}$) was not significantly affected by chronic Na^+ deprivation, it follows that the chord conductance of the apical membrane under short-circuit conditions ($_0G^m_{Na}$) must have increased threefold, in parallel with the increase in I_{Na} (as shown in Table I). Nonetheless, the estimated ratios of the slope resistances of the apical membranes (r^m) to those of the basolateral membranes (r^s), i.e., (r^m/r^s), did not differ significantly between these two groups of tissues. Thus, the increase in the conductance of the apical membranes of tissues from Na^+-deprived animals appears to be, within

TABLE I
EFFECTS OF SODIUM DEPRIVATION ON ELECTROPHYSIOLOGICAL PROPERTIES AND INTRACELLULAR SODIUM ACTIVITIES IN DESCENDING RABBIT COLON[a]

Property	Control	Na^+-Deprived
I_{Na} ($\mu A/cm^2$)	29	99[b]
ψ^{mc} (mV)	−44	−39[c]
$(Na)_c$ (m*M*)	13	14[c]
$\Delta\bar{\mu}^m_{Na}$ (mV)	90	99[c]
$_0G^m_{Na}$ (mS)	0.29	1.1[b]
(r^m/r^s)	2.0	1.8[c]

[a]Data from Turnheim *et al.* (1987). All values were obtained under short-circuit conditions.
[b]Differs significantly from control ($p < 0.01$).
[c]Does not differ significantly from control ($p > 0.05$).

experimental error, matched by an increase in the conductance of their basolateral membranes.

These results are consistent with the findings of others that chronic hyperaldosteronism results in an increase in Na^+,K^+-ATPase activity in the basolateral membranes (Rossier *et al.*, 1984; O'Neil and Hayhurst, 1985) as well as a marked proliferation of basolateral membrane area (Kashgarian, 1980; Wade *et al.*, 1979); these observations will be discussed further by Rossier *et al.* (Chapter 9) and O'Neil (Chapter 11) in this volume. But what is perhaps most striking, in considering all of these observations, is the precision with which the increased pump–leak properties of the basolateral membranes parallel the increased rate of Na^+ entry across the apical membranes. Because of this precise parallelism, the cell is able to accommodate a large increase in transcellular Na^+ traffic with little or no pertubations of cell Na^+ and K^+ activities (Schultz, 1981; Wills and Lewis, 1980).

The findings reported in Table I are in complete agreement with the observations of Wills and Lewis (1980) that a twofold increase in the rate of Na^+ absorption by rabbit urinary bladders induced by chronic dietary Na^+ deprivation is not accompanied by significant changes in cell Na^+ and K^+ activities, determined by employing ion-selective microelectrodes. These findings are, however, difficult to reconcile with those of Eaton (1981), who reported that a twofold stimulation of active Na^+ absorption by rabbit urinary bladder induced by Na^+ deprivation was accompanied by a twofold increase in $(Na)_c$, also determined by using Na^+-selective microelectrodes.

Clauss *et al.* (1985) and Palmer and Speez (1986) have examined the effects of prolonged (18-hr to 3-day) treatment with mineralocorticoids on $(Na)_c$ in rabbit descending colon and toad urinary bladder, respectively. Both groups determined $(Na)_c$ from the reversal potential of the amiloride-inhibitable transepithelial current across tissues exposed to a sodium-free, high-potassium serosal solution. This technique, introduced by Fuchs *et al.* (1977), is designed to depolarize the electrical potential difference across the basolateral membranes and to diminish the resistances of those barriers so that the transcellular electrical properties of these preparations approach those of the apical membranes alone (Palmer, 1984); to the extent that these assumptions are correct, one need not resort to the use of microelectrodes to examine the properties of the apical membrane.

Both groups reported that the increase in I_{Na} induced by mineralocorticoids was accompanied by an increase in $(Na)_c$. Palmer and Speez (1986), however, also noted that the increase in $(Na)_c$ alone was not sufficient to account for the increase in I_{Na} and concluded "that aldosterone has independent stimulatory effects on Na entry across the apical membrane

and Na exit across the basolateral membrane but that the stimulation of the entry process is considerably greater."

At this point, it seems appropriate to voice some reservation regarding the use of K^+-depolarized epithelia in studies aimed at assessing the activity of the basolateral Na^+–K^+ pump. Evidence has been reported for the presence of a Na^+–Ca^{2+} countertransport mechanism in the basolateral membranes of several Na^+-absorbing epithelia, including toad urinary bladder (Chase, 1984; Schultz, 1986), which mediates the oppositely directed movements of Na^+ and Ca^{2+} across that barrier in response to the prevailing, combined electrochemical potential differences for these ions. Now, when the serosal solution is devoid of Na^+ and contains 1 m*M* Ca^{2+} (as in the studies of Clauss *et al.* and Palmer and Speez), the operation of this exchange mechanism would be expected to result in the extrusion of Na^+ from the cell across the depolarized basolateral membrane in exchange for Ca^{2+}.[2] This could result in an increase in cell Ca^{2+} activity, which, even in the micromolar range, has been shown to inhibit the Na^+–K^+ pump in human red cells (Yingst and Hoffman, 1984). Indeed, preliminary results mentioned by Thomas *et al.* (1983) indicate that increasing the activity of Na^+ in the mucosal solution, $(Na)_m$, results in an increase in $(Na)_c$ in *Necturus* urinary bladder when the tissue is bathed by a high-potassium, sodium-free serosal solution; as will be discussed below, this increase in $(Na)_c$ is not observed when the serosal surface of the tissue is bathed by the normal amphibian saline solution.

Thus, it is possible that the failure of pump activity to keep pace with the mineralocorticoid-induced increase in the rate of Na^+ entry across the apical membranes, shown in the studies of Clauss *et al.* (1985) and Palmer and Speez (1986), is a consequence of suppression of pump activity by the experimental technique employed in these studies. A definitive resolution of this issue, however, awaits further study.

III. RAPID STIMULATION OF TRANSCELLULAR SODIUM ABSORPTION

A. Studies on *Necturus* Urinary Bladder

Thomas *et al.* (1983) have examined the effects on $(Na)_c$ of increasing the rate of Na^+ entry across the amiloride-inhibitable channels in the api-

[2]Inasmuch as this countertransport process involves the exchange of three sodium ions for each calcium ion, it is extremely sensitive to changes in the chemical potential difference for Na^+ across the basolateral membrane as well as the electrical potential difference across that barrier. Depolarization of this electrical potential difference would enhance the rate of Na^+ extrusion in exchange for Ca^{2+}.

cal membranes of *Necturus* urinary bladder and, in turn, the rate of transcellular Na^+ transport by simply exposing this membrane to mucosal solutions containing 5 m*M*, 15 m*M*, and 45 m*M* Na^+ in random order. $(Na)_c$ was determined from the reversal potentials of the *I–V* relationships of the amiloride-inhibitable Na^+ current across the apical membrane (I^m_{Na}) and the electrical potential difference across that barrier (ψ^{mc}), by employing the Nernst equation. Typical examples of the *I–V* relationships, determined from the impalement of a single cell exposed to these three mucosal Na^+ activities, are shown in Fig. 3; clearly, there is an excellent fit of the experimental data (points) to the predictions of the Goldman–Hodgkin–Katz (constant field) equation (Goldman, 1943; Hodgkin and Katz, 1949) over a wide range of values of ψ^{mc} and, particularly, over the range encountered under most physiological conditions.

The effects of increasing $(Na)_m$ on the rate of transcellular Na^+ transport, I_{sc}, and $(Na)_c$ are shown in Fig. 4. Increasing $(Na)_m$ from 3.8 to 34.2 m*M* was accompanied by the expected hyperbolic increase in I_{sc} but was not accompanied by significant increases in $(Na)_c$; indeed, analyses of the results of the individual studies (Fig. 5) indicate that there is no significant relationship between $(Na)_c$ and $(Na)_m$ or between $(Na)_c$ and the value of I_{sc} determined when $(Na)_m = 34.2$ m*M*.

Finally, Fig. 6 illustrates the relationship between the steady-state slope conductance of the basolateral membranes under short-circuit conditions, ${}_0g^s_\infty$, corrected for capacitative transients as described by Schultz *et al.* (1985), as a function of the I_{sc}. Clearly, ${}_0g^s$ increases with increasing I_{sc}. Demarest and Finn (1987) have demonstrated that the electrical properties of this barrier are dominated by its highly selective conductance to K^+. Thus, it is not unreasonable to infer that the direct relationship shown in Fig. 6 is attributable, at least in part, to a parallelism between pump rate and K^+ leak at the basolateral membrane.

B. Studies on *Necturus* Small Intestine

The rates of Na^+ entry across the apical membranes of small intestine and renal proximal tubule can be markedly stimulated by simply adding sugars or amino acids to the mucosal solution (Schultz, 1986). The electrophysiological responses of *Necturus* small intestine villus cells to the addition of alanine to the mucosal solution are illustrated in Fig. 7. Immediately after the addition of this amino acid, there is a marked depolarization of ψ^{mc} and a decrease in (r^m/r^s); both of these effects can be readily attributed, at least in part, to the activation of rheogenic and conductive carrier mechanisms in the apical membrane that mediate Na^+-coupled amino acid entry into the cells. This initial response is followed by a spon-

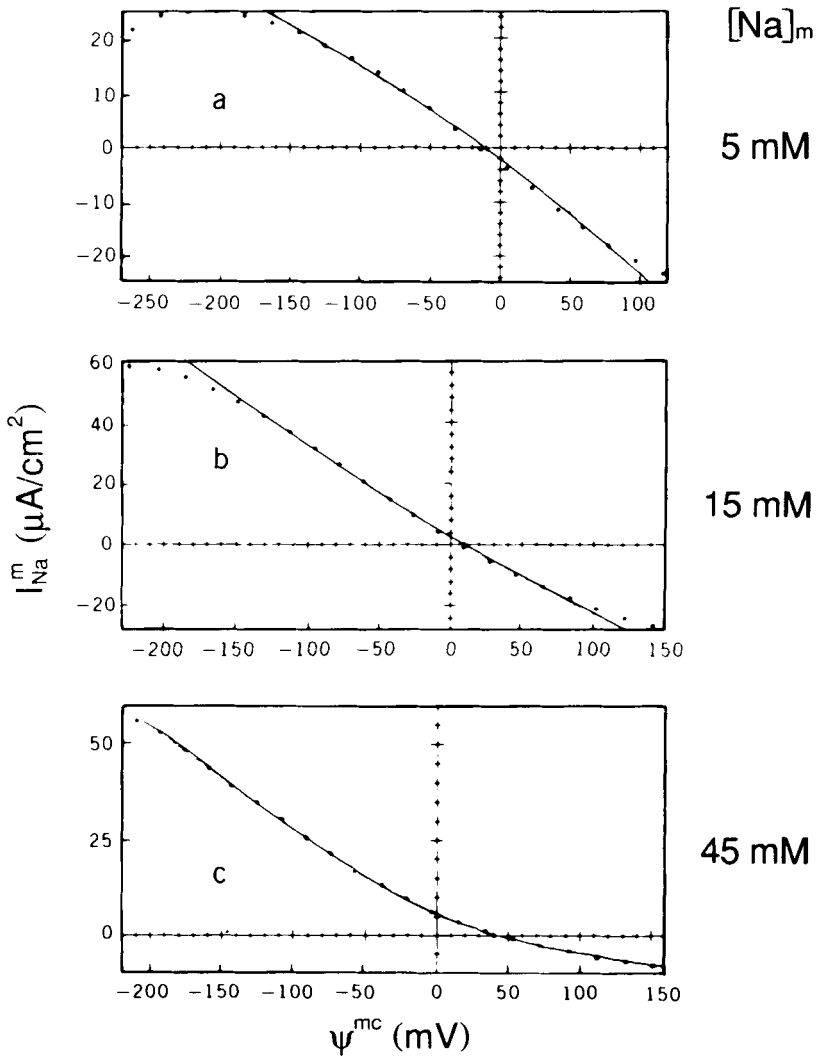

FIG. 3. Relationships between the Na^+ current across the apical membrane (I^m_{Na}) of *Necturus* urinary bladder and the electrical potential difference across that barrier (ψ^{mc}) when the Na^+ activity, $(Na)_m$, in the mucosal solution is 3.8 m*M*, 11.4 m*M*, or 34.2 m*M* [corresponding to Na^+ concentrations, $[Na]_m$, of (a) 5, (b) 15, and (c) 45 m*M*, respectively]. The points are the experimental data and the solid curves show the least-squares fit of these data to the Goldman–Hodgkin–Katz constant-field equation. (From Thomas *et al.*, 1983. Reprinted by permission of Springer-Verlag.)

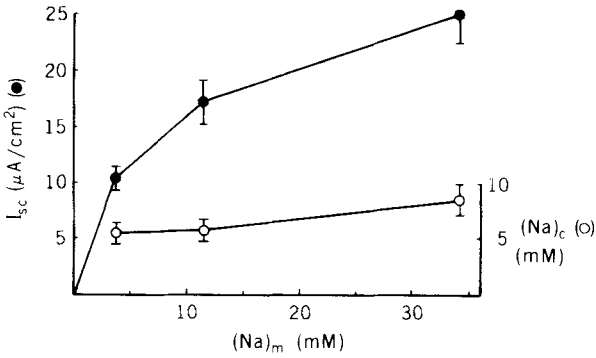

FIG. 4. Relationships among $(Na)_m$, I_{sc}, and $(Na)_c$ in *Necturus* urinary bladder. (From Thomas *et al.*, 1983. Reprinted by permission of Springer-Verlag.)

taneous repolarization of ψ^{mc} and an increase in (r^m/r^s) which can be attributed, at least partially, to an increase in the conductance of the basolateral membranes to K^+ (Gunter-Smith *et al.*, 1982; Grasset *et al.*, 1983; Lau *et al.*, 1984; Lapointe *et al.*, 1986). Similar responses are elicited by the addition of sugars, such as galactose, to the mucosal solution.

The results of studies (Hudson and Schultz, 1984) designed to determine the effect of a galactose-induced stimulation of the rate of Na^+ entry across the apical membrane on $(Na)_c$, by employing Na^+-selective micro-

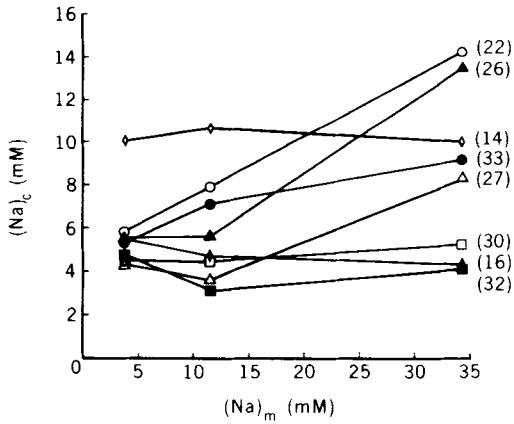

FIG. 5. Relationships between $(Na)_m$ and $(Na)_c$ in the studies reported by Thomas *et al.* (1983). The values in parentheses are those of the I_{sc} when $(Na)_m$ = 34.2 m*M*. (Reprinted by permission of Springer-Verlag.)

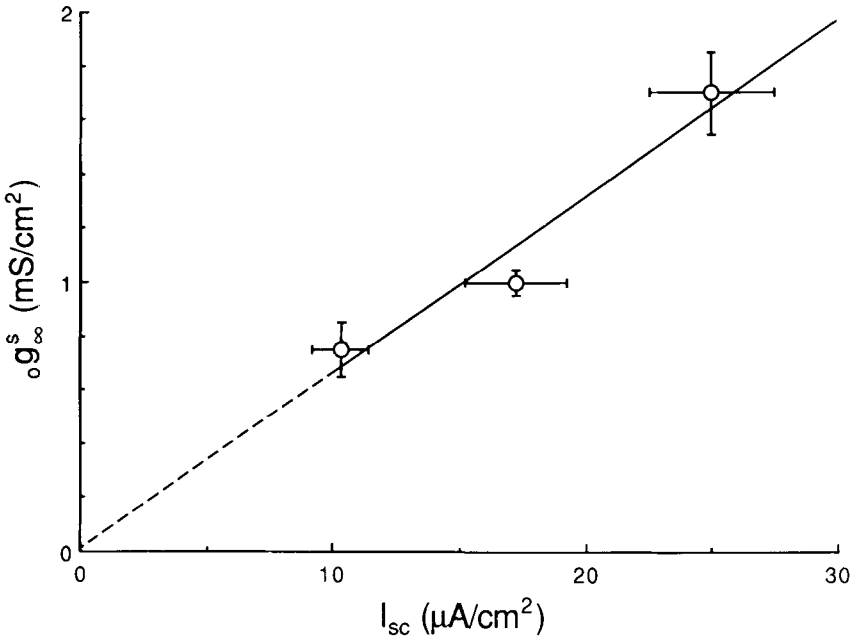

FIG. 6. Relationship between the steady-state slope conductance of the basolateral membrane of *Necturus* urinary bladder when the tissue is short-circuited ($_0g_\infty^S$) and the short-circuit current, I_{sc}. The solid line is the least-squares fit of the data points. The dashed extrapolation should be viewed as suggestive but unestablished.

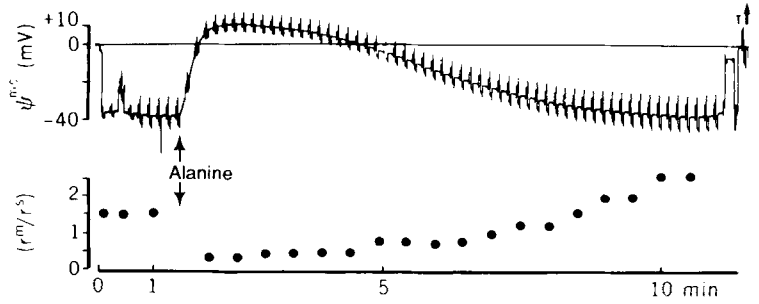

FIG. 7. Effects of the addition of alanine to the solution bathing the apical membrane of the *Necturus* small intestine on the electrical potential difference across that membrane (ψ^{mc}) and the ratios of the slope resistances of that barrier to those of the basolateral membrane (r^m/r^s). (From Gunter-Smith *et al.*, 1982.)

electrodes, are given in Table II. By 1–2 min following the introduction of the sugar into the mucosal solution, at a time corresponding to the peak of the depolarization of ψ^{mc} and the maximal decrease in (r^m/r^s), $(Na)_c$ increased to a value almost twice that observed in the absence of the sugar. This finding is not surprising and adds to the already ample body of evidence supporting this cotransport mechanism. The large, rapid increase in $(Na)_c$ also supports the notion that the intracellular Na^+ compartment may be quite small compared to the rapid rates at which Na^+ may enter this compartment across the apical membrane (Schultz, 1981). After reaching its peak value, $(Na)_c$ slowly declined in parallel with the repolarization of ψ^{mc} and the increase in (r^m/r^s); and, by 12–15 min, when the rate of transcellular Na^+ transport reached a steady-state value approximately fourfold greater than that in the absence of the sugar, $(Na)_c$ did not differ significantly from the control value.

Lee and Armstrong (1972) have also reported that the sugar-induced stimulation of transcellular Na^+ absorption across bullfrog small intestine is not accompanied by a significant increase in $(Na)_c$.

C. Studies on Rabbit Gallbladder

Diamond (1964) demonstrated that the rate of fluid absorption by rabbit gallbladder, secondary to neutral NaCl absorption, is doubled by the addition of HCO_3^- (plus CO_2) to the mucosal and serosal bathing solutions. This effect has been confirmed in our laboratories (Moran *et al.*, 1986) by

TABLE II
INTRACELLULAR SODIUM ACTIVITIES IN *Necturus* SMALL INTESTINE BEFORE AND AFTER STIMULATION OF TRANSCELLULAR SODIUM TRANSPORT BY ADDITION OF GALACTOSE TO MUCOSAL SOLUTION[a]

State	$(Na)_c$ (m*M*)	I_{sc} ($\mu A/cm^2$)
Control	12	13
1–2 min after introducing 10 m*M* galactose to the mucosal solution	21[b]	18[b]
10 min after introducing galactose to the mucosal solution	14[c]	51[b]

[a]From Hudson and Schultz (1984).
[b]Differs significantly from control.
[c]Does not differ significantly from control.

direct measurements of bidirectional and net fluxes of Na^+ across this epithelium (Table III).

The effects of HCO_3^- (plus CO_2) on ψ^{mc}—(r^m/r^s) estimated from the voltage–divider ratio, $f = (\Delta\psi^{mc}/\Delta\psi^{cs})$, and $(Na)_c$ in rabbit gallbladder—are illustrated in Fig. 8. We see that $(Na)_c$ initially increases and, within 1–2 min, reaches a value that is almost twice that observed under control conditions; thereafter, it decreases and, by 12–15 min, it does not differ significantly from the control value. Further, after an initial decrease in f and a small depolarization of ψ^{mc}, both values increase and, by 12–15 min, ψ^{mc} closely approximates the equilibrium potential for K^+ across the basolateral (and apical) membranes (Gunter-Smith and Schultz, 1982).

As discussed by Boulpaep and Sackin (1980), the voltage–divider ratio may not provide an accurate estimate of (r^m/r^s), particularly in leaky epithelia, because of complications arising from distributed resistances along the lateral membranes bounding the intercellular spaces. For this reason, we examined the effects of serosal $Ba,^{2+}$ a well-established blocker of K^+ channels in a wide variety of biological membranes, on ψ^{mc} and f before and after the addition of HCO_3^- (plus CO^2). As shown in Table IV, (1) the addition of 5 m*M* Ba^{2+} to the serosal solution, in the absence of HCO_3^-, resulted in a significant depolarization of ψ^{mc} and a marked decrease in f; (2) the subsequent introduction of HCO_3^- (plus CO_2) to both bathing solutions (with 5 m*M* Ba^{2+} still present in the serosal solution) elicited minimal changes in ψ^{mc} and f; but (3) the subsequent removal of Ba^{2+} from the serosal perfusate resulted in a marked increase in f, and ψ^{mc} hyperpolarized to a value that approaches the expected equilibrium potential for K^+. These findings are certainly consistent with the notion that, in spite of the uncertainties inherent in quantitatively interpreting

TABLE III
EFFECT OF BICARBONATE ON Na^+ ABSORPTION BY RABBIT GALLBLADDER[a]

Condition	J_{Na}^{ms}	J_{Na}^{sm}	J_{Na}
Bicarbonate-free	28	20	8
20 m*M* bicarbonate	33[b]	19	14[b]

[a] J_{Na}^{ms}, J_{Na}^{sm}, *and* J_{Na} are the unidirectional mucosa-to-serosa and serosa-to-mucosa fluxes and the net flux of Na^+, respectively, expressed in $\mu Eq/cm^2$ hr.

[b] Significantly different from bicarbonate-free condition ($p < 0.01$).

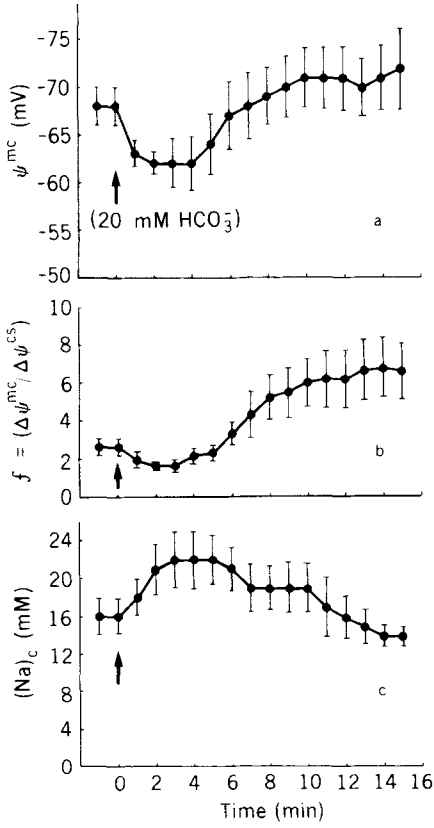

FIG. 8. Effects of the addition of HCO_3^- (plus CO_2) to the solutions bathing the mucosal and serosal surfaces of rabbit gallbladder on (a) ψ^{mc}, (b) f, and (c) $(Na)_c$. The bars reflect the variance among tissues, but the same patterns of responses were observed in all of the tissues studied. (Data from Moran *et al.*, 1986.)

voltage–divider ratios in leaky epithelia, the stimulation of transcellular Na^+ transport by HCO_3^- (plus CO_2) is accompanied by an increase in the conductance of the basolateral membrane to K^+. Similar findings have been reported for *Necturus* gallbladder by Petersen and Reuss (1985).

As discussed previously (Schultz, 1986), the precise mechanism(s) responsible for Na^+ entry across the apical membranes of gallbladder absorptive cells have not been firmly established and remain a matter of some controversy; evidence has been presented favoring a Na^+–H^+ countertransport process and a K^+–Na^+–$2Cl^-$ cotransport process, as well as the coexistence of both of these carrier-mediated processes. In spite of this uncertainty, the finding that the stimulation of transcellular Na^+ absorption by HCO_3^- was accompanied by a rapid increase in $(Na)_c$ indicates that the initial effect of this maneuver is stimulation of Na^+ en-

TABLE IV
EFFECTS OF BICARBONATE ON ELECTROPHYSIOLOGICAL PROPERTIES OF RABBIT GALLBLADDER[a]

Mucosal solution	Serosal solution	ψ^{mc} (mV)	$f = (\Delta\psi^{mc}/\Delta\psi^{cs})$
HCO_3^--free	HCO_3^--free	−66	3.8
HCO_3^--free	HCO_3^--free + Ba^{2+}	−51	0.7
20 m*M* HCO_3^-	20 m*M* HCO_3^- + Ba^{2+}	−54	2.6
20 m*M* HCO_3^-	20 m*M* HCO_3^-	−78	8.5

[a]Average results of five experiments in which, after a single cell was successfully impaled, the compositions of the mucosal and serosal perfusates were changed in the order given.

try across the apical membrane. The subsequent decline in $(Na)_c$ mimics the pattern observed in *Necturus* small intestine following stimulation of Na^+ entry by activating apical membrane Na^+-coupled sugar or amino acid cotransport processes.

Finally, Reuss and Petersen (1985) and Reuss (1987) have noted that inhibition of transcellular Na^+ absorption in *Necturus* gallbladder by adenosine 3′,5′-cyclic monophosphate is not accompanied by a significant decrease in $(Na)_c$. Thus, it appears that pump rates at the basolateral membranes of gallbladder epithelial cells parallel the rates of Na^+ entry across their apical membranes when Na^+ entry is stimulated as well as when it is inhibited.

IV. CONCLUSIONS AND SPECULATIONS

The findings summarized in this chapter indicate that the pump–leak properties of the basolateral membranes of Na^+-absorbing epithelial cells are "plastic" and tend to parallel the rates of Na^+ entry across the apical membranes of these cells, regardless of the mechanisms that are responsible for these entry processes. Among other things, this parallelism prevents inordinate increases in cell Na^+ and K^+ contents, and thus volume, in response to a large increase in the rate of Na^+ entry and Na^+–K^+ pump activity (Schultz, 1981).

For the case of slow (chronic) stimulation of transcellular Na^+ transport by mineralocorticoids, there is compelling evidence for increased mRNA-directed synthesis of the Na^+,K^+-ATPase (pump units) and the expression of these pumps in the basolateral membranes (Geering *et al.*,

1982, 1983; Petty *et al.*, Rossier *et al.*, 1984; O'Neil and Hayhurst, 1985). There is also compelling evidence for a marked proliferation of the basolateral membranes (Kashgarian, 1980), which undoubtedly contain the channels responsible for the K^+ conductance of that barrier. Thus, the increase in pump–leak properties of the basolateral membrane under these conditions is readily explicable. But, as noted above, it is quite remarkable that these increases in overall pump and leak activities appear to precisely parallel each other, within experimental error.

However, the pump–leak responses to abrupt increases in Na^+ entry and, in turn, transcellular Na^+ transport occur within minutes and certainly cannot involve *de novo* synthesis of either new pump units or plasma membrane.

As for the increase in basolateral membrane K^+ conductance, there is a growing body of inferential evidence that this may be part of a volume regulatory response to cell swelling. Increases in the permeabilities of the plasma membranes of several nonepithelial cells (Hoffmann, 1987; Grinstein *et al.*, 1984; Siebens, 1985; Howard and Wondergem, 1987) and the basolateral membranes of several epithelial cells (Dellasega and Grantham, 1973; Grantham *et al.*, 1977; Welling *et al.*, 1985; Davis and Finn, 1985; Foskett and Spring, 1985; Germann *et al.*, 1986; Dawson *et al.*, 1988; Larson and Spring, 1987; Lau *et al.*, 1984, 1986; Lewis *et al.*, 1985; Ussing, 1982; Richards and Dawson, 1986; Roy and Sauve, 1987) to K^+ and/or Cl^- have been observed following exposure of these cells to hypotonic solutions and play an essential role in the volume regulatory decrease (VRD) exhibited by these cells under these conditions. Further, the results of some of these studies suggest that these increases in K^+ and/or Cl^- permeabilities are mediated by an increase in $(Ca)_c$. While many of these suggestions are based on indirect evidence, Wong and Chase (1986) have reported that (1) exposure of isolated toad urinary bladder cells to a hypotonic solution results in rapid swelling followed by a spontaneous restoration of the original volume (i.e., VRD); (2) cell swelling was accompanied by an increase in membrane permeability to Ca^{2+} and an increase in $(Ca)_c$; and (3) preventing the increase in $(Ca)_c$, either by reducing the concentration of Ca^{2+} in the suspension medium or by loading the cells with excess quin 2, inhibited cell volume regulation.

Finally, Hudson and Schultz (1988) have demonstrated that swelling of Ehrlich ascites tumor cells resulting from the Na^+-dependent uptake and intracellular accumulation of glycine is accompanied by a marked stimulation of membrane Cl^-, and perhaps K^+, channels and that Cl^- channels having identical properties are also activated by swelling induced by ex-

posure of these cells to a hypotonic medium.[3] In short, the effects of gradual swelling under isotonic conditions due to the accumulation of intracellular osmolytes are indistinguishable from the effects of rapid swelling under hypotonic conditions. Thus, the common denominators are cell swelling and, presumably, stress generated in the plasma membrane and/or membrane-attached cytoskeletal elements.

How are these stresses sensed? This question is not entirely resolved, but the recent discovery of membrane channels that are activated by stretch (SA channels) may be the clue. Stretch-activated channels were first described by Guharay and Sachs (1984) in membranes of tissue-cultured chick pectoral muscle, where they appear to subserve the physiological function of stretch sensors. Since then, SA channels have been described in a large variety of cells, many of which do not appear to have obvious physiological functions as mechanotransducers. In recent reviews of this subject, Sachs (1986a,b) has speculated that some of these SA channels may be involved in osmoregulation.

Stretch-activated K^+ channels have been identified in the epithelial cells lining the frog lens (Cooper *et al.*, 1986) and in the basolateral membrane of the *Necturus* proximal tubule (Sackin, 1987). Further, Christensen (1987) has reported the presence of SA Ca^{2+} channels in the basolateral membrane of the *Necturus* choroid plexus, which, when activated, result in the stimulation of nearby Ca^{2+}-activated K^+ channels that are normally closed. Calcium-activated K^+ channels have also been identified in the basolateral membranes of rabbit (Sepulveda and Mason, 1985) and rat (Morris *et al.*, 1986) enterocytes.

It should be noted that these volume-regulatory responses are very sensitive and can be activated by very small increases in cell volume. For example, an increase in the K^+ conductance of the basolateral membranes of *Necturus* enterocytes can be elicited by exposing these cells to a solution that is only 6% hypotonic with respect to control (Lau *et al.*, 1984), and a 3–6% increase in cell volume is sufficient to activate Cl^-, and perhaps K^+, channels in the plasma membranes of Ehrlich ascites tumor cells (Hudson and Schultz, 1988). Likewise, SA channels appear to be exquisitely sensitive. For example, (1) the SA K^+ channel in the basolateral membrane of *Necturus* proximal tubule cells can be activated by membrane tensions that could be generated by as little as a 1% increase in cell volume (Sackin, 1987); (2) the cation-selective SA channel

[3]Cahalan and Lewis (1987) have reported evidence derived from whole-cell patch recordings that exposure of T lymphocytes to a hypotonic medium results in activation of Cl^- channels and a secondary activation of voltage-dependent K^+ channels.

identified by Cooper *et al.* (1986) is activated by the application of only 30–40 mmHg suction to the patch pipette, which corresponds to the osmotic pressure generated by a difference of only a few milliosmoles; and (3) Martinac *et al.* (1987) have identified a pressure-sensitive ion channel in the giant spheroplasts of *Escherichia coli* that can be activated by an osmotic pressure difference across the membrane corresponding to a difference of only a few milliosmoles.[4] Thus, it is quite possible that the increases in $(Na)_c$ observed within 1–2 min after stimulating Na^+ entry across the apical membranes of the *Necturus* small intestine (Table II) and rabbit gallbladder (Fig. 8)—together with the fact that Na^+ entry is coupled with, in the first instance, the entry of galactose and, in the second instance, directly or indirectly, with the entry of Cl^-—could result in an increase in cell osmolyte content and swelling sufficient to trigger the activation of SA channels and, in turn, VRD responses.

These findings suggest the following hypothesis for the parallelism between basolateral membrane K^+ conductance and the rate of transcellular Na^+ transport observed in the studies described above, as well as in a wide variety of other Na^+ absorbing epithelia (Schultz, 1986; Lang *et al.*, 1986): (1) An increase in the rate of Na^+ entry across the apical membrane results in an increase in intracellular osmolyte content—this is doubly true for Na^+ entry processes coupled to the entry of other solutes such as sugars, amino acids, and Cl^-; (2) this results in cell swelling, which activates SA channels in the basolateral membranes; (3) these SA channels may be K^+ and/or Cl^- channels; and/or Ca^{2+} channels that result in an increase in $(Ca)_c$ and the indirect activation of Ca^{2+}-dependent K^+ and/or Cl^- channels. Alternatively, as suggested by Wong and Chase (1986), an increase in $(Ca)_c$ could promote exocytotic processes that bring about the insertion of K^+ and/or Cl^- channels from cytoplasmic sources.

Another, but by no means mutually exclusive, explanation for the pump–leak parallelism stems from the recent findings that the K^+ leak pathways in the basolateral membranes of frog (Lang *et al.*, 1986) and *Necturus* renal proximal tubules (Sackin and Palmer, 1987; Kawahara *et al.*, 1987), *Amphiuma* renal collecting tubule (Horisberger and Giebisch, 1988) and *Necturus* small intestine (Costantin *et al.*, 1989) exhibit voltage-dependent properties consistent with inward (or anomalous) rectification; that is, the K^+ conductance decreases when the basolateral mem-

[4]If these notions are correct, the sensitivity of SA channels approaches the accuracy with which one can prepare and measure the osmolarity of experimental solutions. However, because many cell membranes are highly invaginated, it is not clear how much additional volume the cell can accommodate before significant tension is developed in the membrane or cytoskeletal elements.

brane is depolarized and progressively increases with hyperpolarization of that barrier. Inasmuch as the Na^+–K^+ pump is rheogenic (or electrogenic), an increase in pump rate will bring about a hyperpolarization of the electrical potential difference across the basolateral membrane, which, in turn, would promote an increase in the K^+ conductance of that barrier. An increase in the K^+ conductance of that barrier, mediated by a separate set of K^+ channels activated by an increase in cell volume, would likewise bring about an increase in the conductance of this inward rectifying system.

Finally, let us briefly consider the matter of the Na^+ transport pool and Na^+ pump activity. While there is no doubt that maneuvers that bring about an increase in $(Na)_c$ will result in an increase in basolateral membrane pump activity, unless the latter is inhibited or saturated, the results summarized above indicate that there is no necessary causal relationship between these two parameters; physiological maneuvers that markedly stimulate the rate of Na^+ entry across the apical membranes and, in turn, pump activity often are not associated with a discernible increase in $(Na)_c$.

There are at least three possible explanations for these findings that are not mutually exclusive. The first, and the simplest, explanation is that the turnover rates of a fixed number of operant pumps already present in the basolateral membrane are increased by some intracellular signal other than an increase in $(Na)_c$. As discussed by Hoffman (1986), pump rate can be influenced by many factors other than $(Na)_c$.

A second possibility is that the membrane contains quiescent sodium pump units which are activated by some intracellular signal other than an increase in $(Na)_c$.[5]

A third possibility is the insertion (recruitment) of additional pump units into the basolateral membrane from cytoplasmic sources. This could result from a marked decrease in the transit time of newly synthesized pump units between the Golgi stack and that barrier or from promoting the recycling of endosomes containing "old," recently endocytosed pump units (Pollack *et al.*, 1981). Possible signals for such recruitment might include a transient increase in $(Na)_c$, an increase in cell volume, and/or an increase in $(CA)_c$.[5]

[5]Proverbio *et al.* (1988) have recently presented evidence that swelling of rat renal cortical cells results in the activation of a Na^+-ATPase (pump) that is not dependent on the presence of K^+, is insensitive to ouabain, and is inhibited by ethacrynic acid (see also Chapter 6 of this volume). Siebens (1985), in a review of cell volume control, has cited the results of a number of studies suggesting that Na^+–K^+ pump activity is increased by cell swelling resulting from exposure of flounder, duck, and *Amphiuma* erythrocytes and human lymphocytes to hypotonic media.

V. SUMMARY

The results of studies on a variety of Na^+-absorbing epithelial cells indicate that the pump–leak properties of the basolateral membranes are not fixed but, instead, are "plastic" and respond in a parallel fashion to increases in the rate of Na^+ entry across the apical membranes in response to physiological stimuli. The precise mechanisms responsible for this concerted behavior are far from clear, but there is evidence that they may be related to the mechanisms that are involved in volume preservation by these cells. Some promising clues to the underlying mechanisms have surfaced in recent years from the application of new approaches, including the patch-clamp and reconstitution techniques, for the study of the regulation of ion channels; the development of probes for the determination of cell Ca^{2+} activities, pH, etc.; and molecular biological techniques for the elucidation of the properties and cytokinetics of the ubiquitous Na^+–K^+ pump. It is not unreasonable to expect that within the next decade the application of these techniques, and techniques yet to be evolved, will provide us with a comprehensive understanding of how Na^+-absorbing epithelial cells work at the cellular and molecular levels.

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Chapter 3

Feedback Regulation of Epithelial Sodium Channels

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I. INTRODUCTION

Sodium reabsorption by tight epithelia involves two basic membrane transport processes: Na^+-selective channels in the apical membrane, which allow Na^+ to move into the cell from the mucosal fluid, and an ATP-dependent sodium pump, which drives Na^+ out of the cell against an electrochemical activity gradient. This view, first put forward by Koefoed-Johnsen and Ussing (1958) to describe transepithelial Na^+ transport in the frog skin, has been confirmed and elaborated on by many laboratories since then, and has been extended to many other high-resistance Na^+-reabsorbing epithelia, such as the urinary bladders of amphibians, reptiles, and some mammals; the colons of amphibians and mammals; and the mammalian renal collecting tubule.

In the simplest form of this model, the parameter which ties together

the two processes is the intracellular Na^+ concentration. Thus, an increase (or a decrease) in the rate of Na^+ entry through the channels will lead to an increase (or a decrease) in intracellular Na^+ concentration, which will stimulate the sodium pump due to the increased (or decreased) availability of a rate-limiting substrate (Na^+) for the pump reaction. On the other hand, an increase (or a decrease) in the activity of the pump will lead to a decrease (or a increase) in cell Na^+, which will increase (or decrease) the driving force for Na^+ entry. The overall rate of transport can, in principle, be modified by changes of either the entry or exit steps, with the cell Na^+ concentration serving as a signal from one side of the cell to the other to speed up or slow down.

Shortly after the publication of the Koefoed-Johnsen–Ussing model, another paper from the same laboratory suggested the presence of an additional control mechanism for coupling the two membranes and regulating intracellular Na^+ (MacRobbie and Ussing, 1961). This was the idea that increases in intracellular Na^+—caused, for example, by inhibition of the sodium pump—could diminish not only the driving force for Na^+ entry but also the Na^+ permeability of the apical membrane. This idea is put into schematic form in Fig. 1, which illustrates the Na^+ entry and exit steps across the apical and basolateral membranes, respectively. Intracellular Na^+ can be increased by an increase in the concentration of Na^+ outside the apical membrane, by a primary increase in Na^+ permeability (P_{Na}), or by inhibition of the Na^+–K^+ pump. The feedback pathway, in which increased cell Na^+ leads to a decrease in P_{Na}, can, in principle, involve either a direct effect of cell Na^+ on the Na^+ channels or an indirect effect, as will be discussed below.

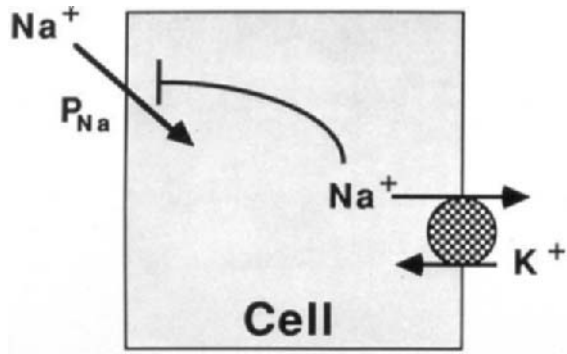

FIG. 1. Schematic representation of an epithelial cell showing the basic model of Koefoed-Johnsen and Ussing together with a putative negative feedback interaction through which intracellular Na^+ reduces apical membrane Na^+ permeability. This effect could, in principle, involve either direct or indirect effects of cellular Na^+.

The potential role of this pathway is to limit changes in cell Na^+, and possibly also in cell volume, under two conditions. One is during stimulation of apical Na^+ influx, as is expected to occur during hormonal stimulation of apical Na^+ permeability or during increases in mucosal Na^+ concentration. Another condition could involve inhibition of the sodium pump, as might occur during hypoxia or as the result of circulating inhibitors of the Na^+,K^+-ATPase. In this chapter we discuss the existence, mediators, and significance of such a negative feedback control mechanism.

II. OBSERVATIONS OF FEEDBACK CONTROL

A. Inhibition of Sodium Pumping

As mentioned above, the earliest observation of this type of negative control mechanism was made by MacRobbie and Ussing (1961). These authors measured changes in the thickness of the frog skin epithelium and made the simple observation that the application of strophanthidin, an inhibitor of the Na^+–K^+ pump, to the serosal medium abolished transepithelial transport as measured by the short-circuit current, but did not induce cell swelling. They concluded that the apical membrane could sense that the pump was being shut off, responding by diminishing its permeability to Na^+ and hence the rate of Na^+ entry into the cell. Since the basolateral membrane is relatively impermeable to Na^+ under all conditions, this would make Na^+ virtually impermeant to the cell and would prevent swelling.

This protocol has been repeated many times, using a variety of techniques to evaluate changes in apical Na^+ entry. Ouabain has been shown to decrease the unidirectional Na^+ movement from mucosa to cell (Biber, 1971; Erlij and Smith, 1973; Turnheim *et al.*, 1978; Chase and Al-Awqati, 1981), the electrical conductance of whole epithelia (Hviid-Larsen, 1973; Kirk and Dawson, 1985), and the conductance of the apical cell membrane (Helman *et al.*, 1979; Schoen and Erlij, 1985) in a variety of epithelia.

Despite the conclusion that severe inhibition of the sodium pump does result in a decrease in apical Na^+ permeability, the role of such a mechanism in the regulation of Na^+ reabsorption under physiological conditions depends on the extent to which intracellular Na^+ must rise in order to trigger the response. One approach to this question has been to measure the effect of ouabain at different levels of mucosal Na^+. Since the basolateral membrane is thought to have a very low Na^+ permeability, the de-

gree to which intracellular Na^+ rises after inhibition of the pump should depend on the concentration in the mucosal fluid.

Results have been somewhat variable. In the frog skin, Erlij and Smith (1973) found a decrease in the rate of uptake of Na^+ from the mucosal medium after ouabain treatment with 10 m*M* mucosal Na^+. Biber (1971) found a similar effect with 6 m*M* Na^+. Thompson and Dawson (1978), studying the turtle colon, could not detect an effect of ouabain on Na^+ uptake with 16 m*M* mucosal Na^+, but did see inhibition with 112 m*M*. Chase and Al-Awqati (1981) reported effects in the toad urinary bladder at 12.5 m*M* but not 2.5 m*M*. Thus, except for the case of the turtle colon, it appears that rather modest increases in cell Na^+ could trigger a Na^+ permeability decrease.

There are two difficulties with the interpretation of these results. First, the quantitative relationship between the mucosal Na^+ concentration and the extent of the ouabain-induced rise in intracellular Na^+ concentration is unknown. Second, ouabain could produce a depolarization of the apical membrane, particularly when the mucosal Na^+ concentration is high. This decrease in the electrical driving force for Na^+ entry could account, at least in part, for the subsequent inhibition of Na^+ entry.

A second way to analyze this problem is to measure intracellular Na^+ activities and changes in apical Na^+ permeability in the same preparation. This type of experiment has not been extensively pursued. Eaton (1981) measured intracellular Na^+ in the rabbit urinary bladder by using ion-sensitive microelectrodes. Apical Na^+ permeability was also measured electrically. P_{Na} was found to be essentially constant over a wide range of intracellular Na^+ activities, from 10 to 30 m*M*, after application of ouabain. Eaton concluded that, at least in this tissue, intracellular Na^+ activity itself does not control P_{Na} under physiological conditions. It is not clear whether the lack of change of P_{Na} in this preparation is due to differences in the way in which Na^+ channels are regulated in the rabbit bladder, or to the relatively small changes in intracellular Na^+ activity that were observed.

In experiments with K^+-depolarized toad bladder, in which the intracellular Na^+ activity was measured from the reversal potential for amiloride-sensitive Na^+ current, Palmer (1985) found little change in P_{Na}, with increases in cell Na^+ activity up to 50 m*M*. In this preparation, however, Na^+–Ca^{2+} exchange at the basolateral membrane, which may be an important factor in the feedback response (see below) was altered due to the high K^+-,Na^+-free serosal solution. Thus, it is not appropriate to use these results in this context. Clearly, more extensive measurements using this type of protocol on different epithelial preparations would be very useful.

B. Stimulation of Sodium Influx

Another protocol that has been used to demonstrate feedback control is to provide the epithelium with a rapid increase in the mucosal Na^+ concentration and to observe the time course of the rate of transport, usually measured as the short-circuit current. Such an experiment is shown in Fig. 2. The tissue employed was the urinary bladder of an aquatic salamander *(Ambystoma tigrinum)*.

When Na^+ is introduced into the mucosal medium, the short-circuit current increases rapidly, reaching a peak value as quickly as the solution can be exchanged (in this case, a few seconds). It then decays, with a time course lasting several minutes. During this time, there is an increase in the transepithelial electrical resistance. Similar results were obtained by Turnheim *et al.* (1978) using rabbit colon. We have also seen this be-

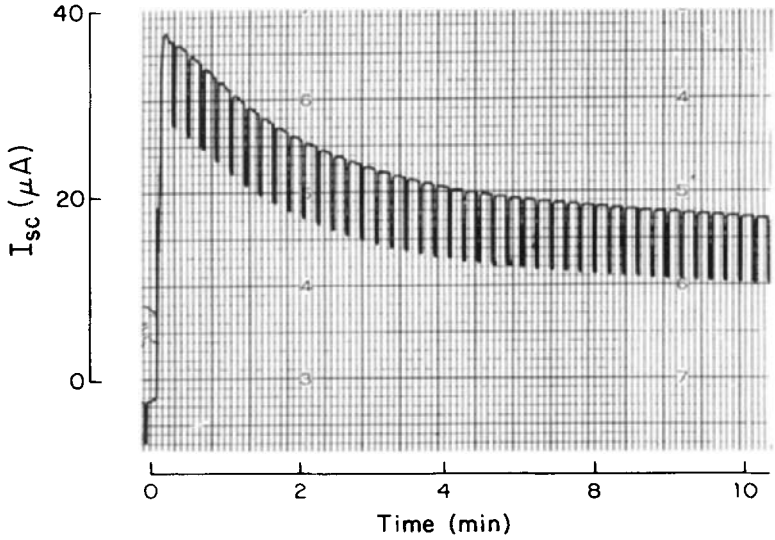

FIG. 2. Time-dependent decline in short-circuit current after introduction of mucosal Na^+ in the urinary bladder of *Ambystoma tigrinum*. The bladder was initially bathed with NaCl–Ringer's solution on the serosal side and KCl solution on the mucosal side. The short-circuit current under these conditions is slightly negative, implying net movement of positive charge from serosa to mucosa, and the transepithelial resistance is large. After changing the mucosal solution to a NaCl-containing solution (time 0), the short-circuit current reached a peak value of 37 μA for a nominal tissue area of 1 cm^2 and then relaxed to a lower steady-state level after about 10 min. The transepithelial conductance is proportional to the size of the downward current deflections arising from a change in the clamping voltage of 10 mV. The conductance increases upon addition of Na^+ and then gradually declines as the steady state is approached.

havior in the toad urinary bladder, although in this tissue the time course of changes in short-circuit current are often more complex.

There are several different mechanisms which could be involved in this response. These phenomena will affect Na^+ transport to different extents, and over different time domains.

First, there is, at least in frog skin, a rapid relaxation component that occurs over the span of a few seconds. This phenomenon was observed and studied in detail by Lindemann and colleagues (Fuchs *et al.*, 1977; Lindemann and Van Driessche, 1978). It is thought to involve a self-inhibition of the apical Na^+ permeability by *external* Na^+, possibly resulting from the interaction of Na^+ with an external modifier site. This mechanism might also underlie the observation that the number of conducting channels in the frog skin apical membrane, as measured by noise analysis, is decreased as the mucosal Na^+ concentration increases (Van Driessche and Lindemann, 1979).

A second possible effect that would occur over this time domain involves depolarization of the apical membrane, which results from the increase in the flow of positively charged Na^+ ions into the cell. This depolarization will have the immediate effect of diminishing the electrical driving force for Na^+ entry. In addition, however, studies of single channel behavior in the rat cortical collecting tubule (CCT) have indicated that the probability of a channel's being open is decreased by depolarization of the apical membrane (Palmer and Frindt, 1988). This effect will be realized by the channels over a time period comparable to the time constants for spontaneous channel opening and closing, which are both in the range of seconds. Such an effect probably did not contribute to the relaxations observed by Fuchs *et al.* (1977) in the frog skin, since in those experiments the epithelia were K^+ depolarized and voltage clamped, minimizing changes in the apical membrane voltage. In any case, these effects would all be too rapid to be detected with the limited time resolution of the protocol illustrated in Fig. 2.

A third component of relaxation involves the loading of the cells with, and the subsequent decrease in, the chemical driving force for Na^+ entry. We have analyzed the contribution of such an effect to the observed relaxations using a numerical model of the toad urinary bladder (Strieter *et al.*, 1987). The results of a simulated experiment, similar to that of Fig. 2, using an epithelium with a constant apical Na^+ permeability is shown in Fig. 3. When the mucosal Na^+ concentration is rapidly increased, cell Na^+ increases gradually, approaching a steady state with a half-life of about 2 min. This time course is reasonably close to that observed for the decrease in current shown in Fig. 2. However, there is virtually no relaxation of the short-circuit current in the simulated experiment. Thus,

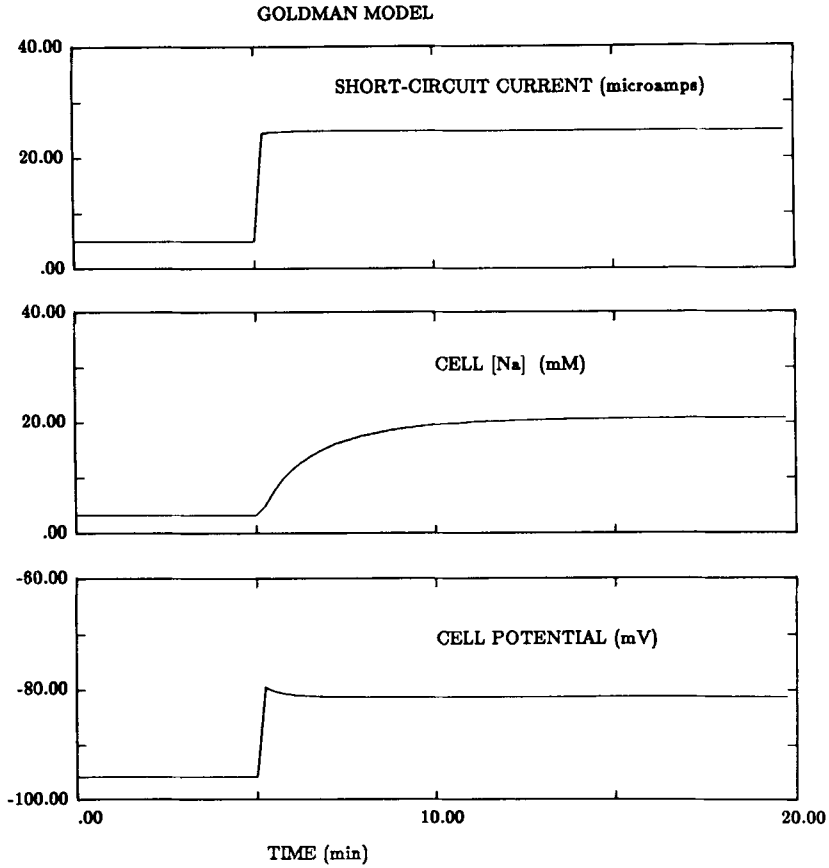

FIG. 3. Time dependence of short-circuit current in a mathematical model of an amphibian epithelium (Strieter *et al.*, 1987). In the model, passive ion fluxes across apical and basal-lateral membranes and tight junctions were modeled using the constant-field (Goldman) equation with constant (time- and concentration-independent) permeability coefficients. The Na^+ permeability coefficient was 0.65×10^{-7} cm/sec. The sodium pump at the basolateral membrane was assumed to be a Na^+–K^+ exchanger with fixed 3:2 stoichiometry. It was modeled kinetically with three identical, noninteracting sites for intracellular Na^+. A step increase in the mucosal Na^+ concentration, with Na^+ replacing K^+, resulted in a rapid increase in the short-circuit current which declined only slightly with time. The time course of changes in intracellular Na^+ concentration and cell potential are also shown.

it seems unlikely that the changes in the ionic composition of the cell per se can account for a significant part of the decrease in current shown in Fig. 2 and studied by Turnheim *et al.* (1978). Rather, it appears that changes in membrane permeability must be involved.

The fourth component of relaxation occurs with a time course of minutes and is the one which is prominent in Fig. 2. It involves an increase in transcellular resistance which is consistent with either a time-dependent decrease in apical Na^+ permeability or a depolarization of the cell due to a decrease in basolateral K^+ conductance. These effects are reversible; if Na^+ is removed and the cells are allowed to rest for a sufficient time, the whole relaxation curve can be reproduced. If Na^+ permeability is increased independently, such as by the addition of antidiuretic hormone or cyclic adenosine monophosphate, the magnitude of both the peak and relaxation components are increased.

This phenomenon may reflect, at least in part, a decrease in apical membrane Na^+ permeability which is secondary to the increase in Na^+ influx into the cell. Alternatively, changes in basolateral membrane transport properties, particularly a decrease in K^+ permeability, could depolarize the cell and diminish the electrical driving force for Na^+ entry. To the extent that the apical P_{Na} is affected, it seems likely that the fundamental mechanisms involved when apical Na^+ entry is increased are similar to those which mediate the effects of inhibition of sodium pump activity on apical Na^+ influx (discussed above). There are a number of changes in the intracellular milieu that might occur under these circumstances and that account for a decrease in Na^+ channel activity subsequent to either inhibition of Na^+ efflux or stimulation of Na^+ influx. These factors are considered in detail below.

III. POSSIBLE MEDIATORS OF FEEDBACK CONTROL

A. Intracellular Sodium

Perhaps the simplest mechanism for accounting for this type of feedback control would be a direct effect of an increase in intracellular Na^+ on the apical membrane Na^+ channels. This possibility is particularly attractive in light of the suggestion of down-regulation of Na^+ permeability by external Na^+, perhaps mediated by an allosteric modifier site (Section II,B). Invoking a similar site on the inside of the membrane at least has a pleasant element of symmetry.

However, there is no strong evidence that intracellular Na^+ per se modifies the apical Na^+ permeability. Palmer *et al.* (1980) found, after

application of ouabain to the serosal side of the K^+-depolarized toad urinary bladder, only small changes in Na^+ permeability, as the intracellular Na^+ increased from 2 to 12 m*M*. In a subsequent study using a similar technique, intracellular Na^+ was allowed to vary over an even wider range—6 to 50 m*M* in activity—with no significant change in P_{Na} (Palmer, 1985). As discussed above, Eaton (1981) found in rabbit urinary bladder that P_{Na} was not affected by ouabain-induced changes of cell Na^+ over the range of ~10–30 m*M*. These changes in intracellular Na^+ are large compared with those normally expected and measured under physiological conditions. Finally, in excised, inside-out patches from the apical membrane of the rat CCT, no effect on Na^+ channel activity was detected even when the solution bathing the cytoplasmic side of the patch was changed from Na^+-free Ringer's solution to one containing 140 m*M* Na^+ (Fig. 4A).

A plausible interpretation of these findings is that down-regulation of apical Na^+ permeability results from an indirect consequence of high intracellular Na^+, rather than from the Na^+ itself acting directly on the channels. Three possible mediators are discussed below.

B. Intracellular Calcium

The possibilities that intracellular Ca^{2+} might increase secondary to an increase in cell Na^+ and that Ca^{2+} might be a more direct effector of Na^+ permeability have been proposed by several groups (Grinstein and Erlij, 1978; Taylor and Windhager, 1979; Chase and Al-Awqati, 1981). The rationale behind these proposals is that cytoplasmic Ca^{2+} levels are dependent on the activity of a Na^+–Ca^{2+} exchanger on the basolateral membrane. Increases in cell Na^+ will slow or reverse the flow of Ca^{2+} out of the cell via this mechanism (Taylor and Windhager, 1979).

There is good evidence that such an exchanger does exist, at least in some epithelia. Lee *et al.* (1980) demonstrated that removal of Na^+ from the serosal side of the proximal tubule of *Necturus* resulted in an increase in the cytoplasmic Ca^{2+} activity, presumably by reducing the driving force for Ca^{2+} extrusion coupled to Na^+ entry through the Na^+–Ca^{2+} exchanger. Chase and Al-Awqati (1981) measured Na^+-dependent Ca^{2+} transport in basolateral membrane vesicles from the toad bladder. Lorenzen *et al.* (1984) showed that application of ouabain to block the sodium pump and increase intracellular Na^+ in the *Necturus* proximal tubule also results in substantial increases in intracellular Ca^{2+} activity.

There is also considerable support for the notion that cytoplasmic Ca^{2+} activity can control Na^+ permeability in tight epithelia. There are several lines of indirect evidence for this from studies of intact epithelia. First, re-

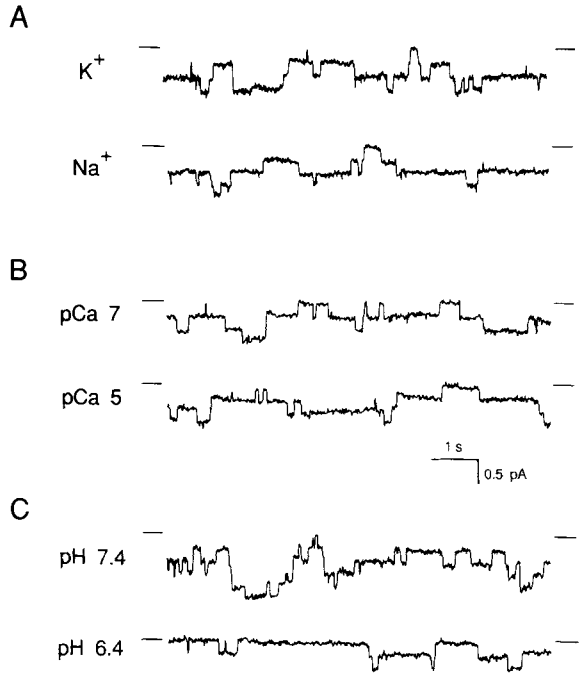

FIG. 4. Direct effects of putative ionic mediators of feedback control on Na^+ channels. Current traces were obtained using the patch-clamp technique from an excised, inside-out patch of the luminal membrane of the rat cortical collecting tubule. The pipette solution contained 140 m*M* NaCl and the pipette potential was held constant at 60 mV, positive with respect to the bath. The current transitions represent openings and closings of individual Na^+ channels, with the current being carried by Na^+ moving from pipette to bath. A downward current deflection corresponds to a channel opening. The standard bath solution contained 140 m*M* KCl at pH 6.9 (buffered with hydroxyethylpiperazine sulfonic acid [HEPES]) and pCa 7 (buffered with ethylene glycol tetraacetic acid [EGTA]). The tracings were obtained before and after replacing KCl in the bath with NaCl (A), changing the pCa of the bath from 7 to 5 (B) and changing the pH of the bath from 7.4 to 6.4 (C). The effects of the various substitutions were evaluated by calculating the mean number of open channels (NP_o) from tracings much longer than those shown. Values of NP_o were: (A) 1.74 in KCl and 1.74 in NaCl; (B) 2.08 with pCa 5 and 2.38 with pCa 7; (C) 3.11 at pH 7.4 and 0.81 at pH 6.4.

moval of Na^+ from the serosal solution, despite increasing the driving force for Na^+ reabsorption, causes a decrease in the transepithelial transport rate in the frog skin (Grinstein and Erlij, 1978) and the toad bladder (Taylor and Windhager, 1979; Chase and Al-Awqati, 1981), consistent with a decrease in apical Na^+ permeability that could arise from an increase in cellular Ca^{2+}. Second, the application of calcium ionophores de-

creases Na^+ transport in the toad bladder (Ludens, 1978; Wiesmann *et al.*, 1978), although increases were observed in the frog skin (Balaban and Mandel, 1979), which may be related to the stimulation of prostaglandin synthesis (Erlij *et al.*, 1986). Third, in K^+-depolarized toad bladders, in which the serosal medium was Na^+-free, raising the serosal Ca^{2+} concentration was found to decrease the amiloride-sensitive Na^+ conductance under conditions in which the intracellular Na^+ concentration was high (Garty and Lindemann, 1984; Palmer, 1985). The interpretation of this finding was that, under these conditions, raising the extracellular Ca^{2+} could increase the rate of Ca^{2+} entry into the cell via Na^+–Ca^{2+} exchange.

Finally, there are a number of direct observations of the effects of cytoplasmic Ca^{2+} on epithelial Na^+ channels. Chase and Al-Awqati (1983) and Garty and colleagues (Garty, 1984; Garty *et al.*, 1987) have demonstrated in toad bladder membrane vesicles that Ca^{2+} can inhibit amiloride-sensitive, conductive Na^+ fluxes. The concentrations of Ca^{2+} necessary to produce these effects were in the physiological range of 1 μM or less. The high affinity of amiloride for blocking these Na^+ fluxes and the demonstration that the fluxes were conductive (Garty, 1984; Garty *et al.*, 1987) imply that they are Na^+ channel mediated. On the other hand, Palmer and Frindt (1987) found no effect of cytoplasmic-side Ca^{2+} on Na^+ channels in excised membrane patches from rat CCT. This insensitivity is illustrated for the range of 10^{-7} to 10^{-5} M in Fig. 4B.

A second effect of Ca^{2+} was reported by Garty and Asher (1985, 1986). They reported that preincubation of permeabilized toad bladder cells with solutions of different Ca^{2+} concentrations could affect the Na^+ fluxes measured in vesicles subsequently isolated from these cells. This effect was temperature dependent and very sensitive to the Ca^{2+} concentration in the range of 10^{-7} M. Another apparently indirect effect of intracellular Ca^{2+} on Na^+ channels was observed in cell-attached patches of rat CCT after exposure to the calcium ionophore ionomycin (Palmer and Frindt, 1987). The mean number of open channels in such patches diminished 5–10 min after addition of the ionophore. This effect was dependent on the presence of extracellular Ca^{2+}. In both of these lines of investigation, the mechanism through which Ca^{2+} was producing these effects remained obscure.

C. Intracellular pH

Another solute which is transported by a Na^+-dependent mechanism in many cells is the proton. Although the regulation of cell pH is poorly understood in most tight epithelia, a Na^+–H^+ exchange process has been observed in rabbit CCT by Chaillet *et al.* (1985). If such a mechanism

were operative under normal or ouabain-inhibited conditions, changes in cell Na^+ could lead to parallel changes in the H^+ concentration in the same way as was proposed for Ca^{2+} (see Section III,B).

There is indirect evidence from intact tissue studies to suggest that Na^+ channels may be sensitive to pH. In the frog skin, Ussing and Zerahn (1951) found that an acute application of CO_2 to the external medium caused a rapid, profound decrease in the rate of Na^+ transport. This effect is likely to involve an acidification of the cytoplasm, since CO_2 will diffuse into the cell rapidly and become hydrated to form HCO_3^- and H^+. It was not determined whether this maneuver affected transport at the apical or basolateral membrane. However, in studies of K^+-depolarized toad bladder, Palmer (1985) reported that a similar application of CO_2 reversibly inhibited the apical Na^+ permeability.

Studies of the possible direct effects of pH on Na^+ channels have generated conflicting results. In toad bladder vesicles, Garty *et al.* (1985) found that when the internal Ca^{2+} concentration was kept low, changes in internal pH over the range of 7–8 did not affect Na^+ fluxes, while acidification below pH 7 was inhibitory. However, when the concentration of Ca^{2+} inside the vesicles was raised, alkalinization of the internal compartment decreased amiloride-sensitive Na^+ fluxes, apparently by making the channels more sensitive to Ca^{2+} (Garty *et al.*, 1987). On the other hand, in inside-out patches from rat CCT, Palmer and Frindt (1987) observed that alkalinization of the solution bathing the cytoplasmic side of the patch over the range of 6.4–7.4 strongly increased channel activity, as measured by the patch-clamp technique. An example of this sensitivity is shown in Fig. 4C.

These data lead to the prediction that Na^+ channels in the toad bladder and rat CCT could respond in opposite ways to alkalinization of the cytoplasm, at least under some conditions. In the toad bladder the Na^+ permeability should *decrease* or show no change, depending on the cytoplasmic Ca^{2+} activity. In rat CCT, in which the pH dependence seems to be shifted toward higher pH values and in which there does not seem to be a direct effect of cell Ca^{2+} on the channels, apical Na^+ permeability should *increase* with cell alkalinization.

D. Cell Metabolism

An interaction of cell metabolism with apical Na^+ permeability was suggested by the studies by Hong and Essig (1976), who found that the transcellular, amiloride-sensitive conductance of the toad bladder was diminished by 2-deoxyglucose (2-DG), an inhibitor of glycolysis. This finding was confirmed in the K^+-depolarized toad urinary bladder by Palmer

et al. (1980), who reported that 2-DG decreased apical Na^+ permeability. These findings were extended by Garty *et al.* (1983), who showed that the effect of 2-DG could be reversed by the addition of pyruvate, an exogenous Krebs cycle intermediate that can bypass the glycolytic pathway. Hviid-Larsen (1973) also found evidence that metabolic inhibition by CN^- blocked apical Na^+ conductance in the frog skin.

It is possible that the metabolic stress arising from an increase in the rate of transepithelial Na^+ transport could produce a similar inhibition of P_{Na}. Such a mechanism could account for part of the slow fall in the transport rate after Na^+ entry is increased (Fig. 2). It presumably does not play a role in the experiments in which Na^+ exit is decreased. Under these conditions, the metabolic load of the cell should decrease, since energy use by the Na pump is slowed.

The intracellular mediators of this effect are completely unknown. It is possible that intracellular Ca^{2+} could increase under conditions of metabolic stress, due to a decrease in the availability of energy for the active extrusion of Ca^{2+}. This has not been demonstrated, however, and interruption of a major metabolic pathway will probably produce myriad changes in the concentrations of cellular metabolites, any of which could potentially affect Na^+ channels.

IV. CONCLUSIONS

Some of the events that might be associated with an increase in Na^+ influx into the cell, as with an increase in the Na^+ concentration of the mucosal fluids, are shown diagrammatically in Fig. 5. Although this scheme may be oversimplified, it is also possible that it is overcomplicated. It is not clear whether all (or any) of these potential feedback loops are important during the normal operation of the cell or even during the kinds of *in vitro* manipulations that have been performed.

A number of questions regarding the effects of the putative mediators of feedback control must be addressed. Why are direct effects of cytoplasmic Ca^{2+} on Na^+ channels seen in toad bladder vesicles but not in excised patches from rat CCT? What are the mediators of the indirect effects of Ca^{2+} on both of these systems? What accounts for the apparent difference in pH sensitivity? Are there cellular metabolites (e.g., adenosine diphosphate or triphosphate) that directly affect Na^+ channel activity?

Another important goal is to define the conditions under which cell Ca^{2+}, cell pH, and cell metabolites might change, and the extent of these changes. For example, it is not known whether a simple maneuver, such

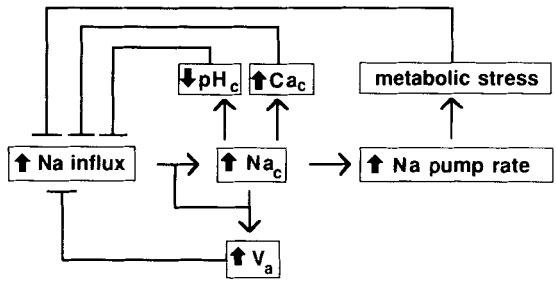

FIG. 5. Schematic representation of possible negative feedback influences on Na^+ transport across the apical membrane of a tight epithelium. An increase in Na^+ influx into the cell will lead directly to a depolarization of the apical membrane voltage (V_a) and to an increase in cell Na^+ (Na_c). The change in V_a, which could also become more positive as cell Na^+ increases and cell K^+ decreases in compensation, will decrease Na^+ influx directly by decreasing the driving force for Na^+ entry. The increase in (Na_c) will lead to secondary changes in cell Ca^{2+} (Ca_c) and cell pH (pH_c), which could decrease apical membrane Na^+ permeability by interactions with Na^+ channels. Finally, the ensuing increase in transepithelial Na^+ transport will create changes in cell metabolism. The metabolic stress could be sufficient to down-regulate apical Na^+ permeability.

as increasing the Na^+ delivery to the mucosal surface of a tight epithelium (e.g., Fig. 2), produces any significant changes in cell Ca^{2+} or cell pH, or whether this constitutes a metabolic stress large enough to substantially alter the concentrations of key metabolic intermediates. In this regard, the development of techniques for measuring these parameters using electrodes, fluorescent dyes, and NMR should be particularly useful.

It will also be interesting to ask whether any of the parameters considered here can act as messengers for hormones which control Na^+ transport in these epithelia. Can hormones such as aldosterone, antidiuretic hormone, and insulin affect cell Ca^{2+}or cell pH, and would such changes account for part of their natriferic effects?

Finally, to complete our picture of the way these tissues work, it will be necessary to understand how ion transport at the basolateral membrane is regulated by these and other factors (see Lewis and Donaldson, Chapter 5, this volume). Transporters on this membrane not only serve to catalyze Na^+ exit from the cell to complete the pathway for reabsorption, but also help to determine the cell potential and hence the electrical driving force for Na^+ entry. A comprehensive view of how both of these membranes function and interact will be necessary in order to understand the regulation of epithelial Na^+ transport.

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Chapter 4

Electron Microprobe Analysis of Cell Sodium in Epithelia

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I. ELECTRON MICROPROBE ANALYSIS

Originally designed for nonbiological materials (Castaing, 1951), during recent years electron microprobe analysis has become widely used also for biological specimens. Compared to other techniques for chemical analysis of intracellular ion concentrations, this method excels by allowing the simultaneous analysis of a large number of elements and by affording a very high spatial resolving power.

Application of the technique to biological soft tissues is, however, not without problems. For the analysis of readily diffusible ions such as Na^+ and K^+, perhaps the most important consideration is whether the specimen preparation preserves their original distribution (Rick *et al.*, 1982). Conventional preparation techniques used for electron microscopy are certainly not appropriate. It is now generally accepted that the only method which satisfactorily maintains the original distribution of ions is

fast-freezing, followed by cryosectioning and analysis in the frozen-hydrated state or, after additional freeze-drying, analysis in the frozen-dried state.

The studies reported here were performed on thin, freeze-dried cryosections in a scanning transmission electron microscope using an energy-dispersive X-ray detection system. Details of the experimental protocol, specimen preparation, and X-ray microanalysis have been published earlier (Bauer and Rick, 1978; Dörge *et al.*, 1978; Rick *et al.*, 1982).

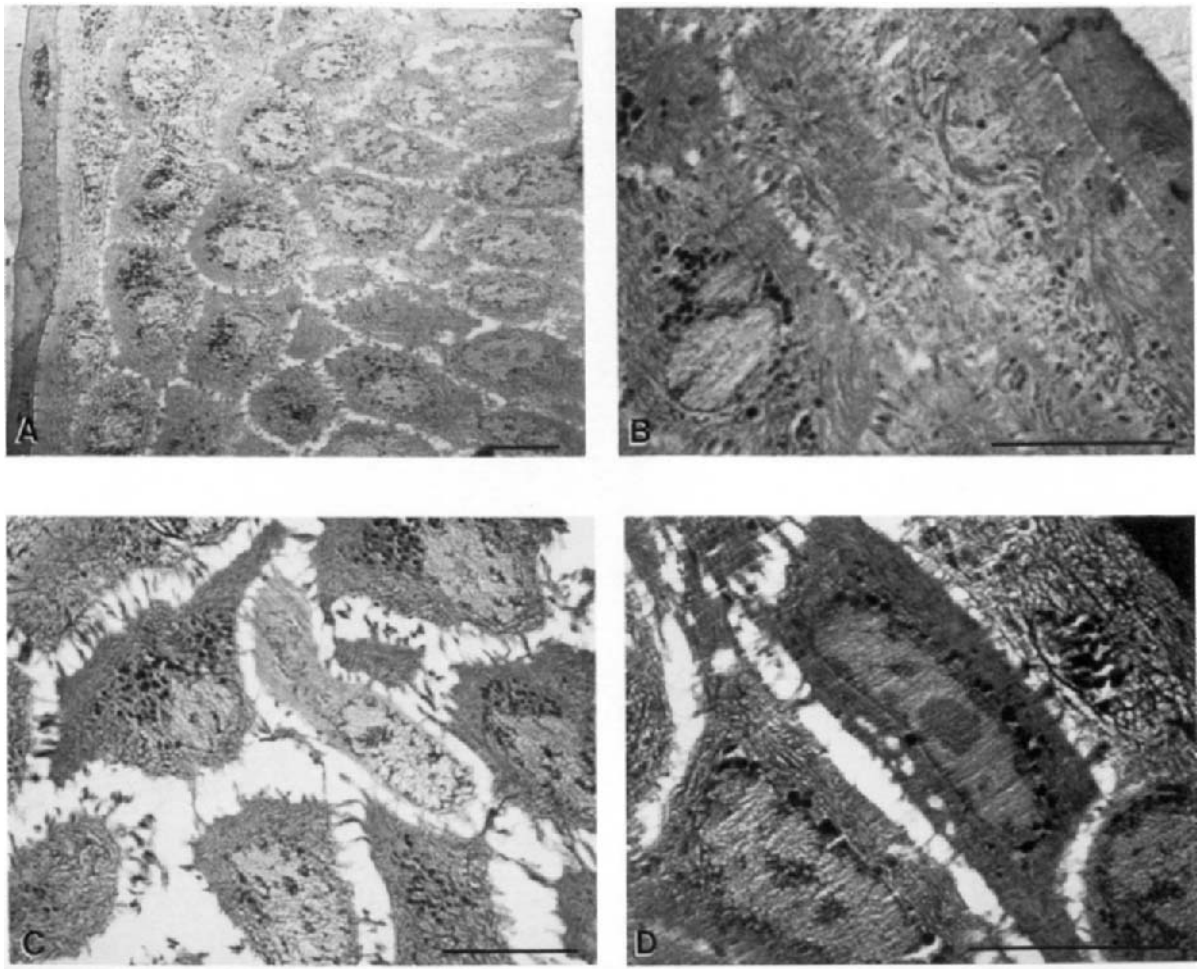
A. Epithelial Morphology in Freeze-Dried Cryosection

Visualization of a thin cryosection from fresh, unstained, and unfixed tissue is inherently difficult, mainly because of the lack of electron optical contrast. In a freeze-dried section, virtually the only contrast provided is that of slight differences in the local dry matter content. An additional problem is the formation of ice crystals during freezing of the tissue, which inevitably leads to some distortion of the cellular fine structure and to a slight redistribution of water-soluble elements.

Figure 1A shows a scanning electron transmission image of a freeze-dried, 0.5-μm-thick cryosection obtained from a split frog skin epithelium *(Rana pipiens)*. Clearly, the preparation looks different from conventionally fixed, stained, and plastic-embedded sections. Nevertheless, the different epithelial cell layers, spaces between cells, and nucleus and cytoplasm can be easily differentiated. At higher magnification (Fig. 1B), it is evident that the cytosol is highly structured, filled with an abundance of intermediate filaments and granules. The filaments seem to extend throughout the cell; in rare instances, filaments can be traced extending from desmosome to desmosome.

A cell type that is almost completely lacking intermediate filaments and granules is the mitochondria-rich cell (Fig. 1C). The cytoplasm of this cell has a lighter, more homogeneous appearance. The tubular network present in the apical cytoplasm corresponds to mitochondria. In the cells comprising the different epithelial layers of the epithelium (principal cells), occasionally large intracellular vacuoles can be recognized. Typically, this organelle is located in the basal cytoplasm, below the nucleus (Fig. 1D).

FIG. 1. Histology of the frog skin epithelium in a thin, freeze-dried cryosection obtained from fresh, shock-frozen tissue. All four scanning electron transmission images were obtained under routine analytical conditions (20 kV acceleration voltage, 0.5 nA probe current). Scale marker, 5μm. For further details, see text.


A
B
C
D

B. Spatial Resolving Power of Analysis

The spatial resolving power attainable in electron microprobe analysis is a function of the size of the specimen volume excited by electrons which, in turn, will depend on the section thickness, electron beam size, and acceleration voltage. With available instrumentation, a lateral resolution of only a few nanometers is feasible.

Figure 2 demonstrates spatial resolution obtained in a 0.5-μm-thick cryosection at an acceleration voltage of 20 kV. The line profiles reflect the Na^+ and K^+ distribution as measured by slowly moving a 50-nm-diameter electron beam across the basal cell membrane of a frog skin epithelial cell. Inside the cell, the K^+ concentration is high, whereas outside, the K^+ concentration is very low. From the S-shaped curve, a lateral resolution of ~200 nm can be calculated. A mirrorlike profile is obtained for Na^+. Surprisingly, the resolution is significantly lower than that for K^+, only ~300 nm. Also, the sum of Na^+ and K^+ at the cell boundary is slightly higher than in the cytosol or in the extracellular space, a finding which points to some binding of Na^+ at the cell membrane.

II. STATE AND DISTRIBUTION OF INTRACELLULAR IONS

Electron microprobe analysis measures the total chemical concentration of an element regardless of its binding state. Thus, the method provides no direct information on exchangeablity or activity of ions. Nevertheless, by combining electron microprobe analysis with ion-selective electrodes, some insights into the state of intracellular ions can be gained. As shown in Table I, there is generally a good agreement between chemical concentrations and ion activities, indicating that the activity coefficient inside the cell is not much different from that of a watery solution of similar ionic composition. Moreover, most intracellular ions appear to be osmotically active (Rick and DiBona, 1987).

By ionic substitution experiments, it can be shown that small, monovalent ions are not irreversibly bound to intracellular structures. Na^+ is readily exchanged by K^+ or Li^+, K^+ by Rb^+ or Na^+, and Cl^- by Br^- (R. Rick, unpublished observations). On the other hand, divalent or polyvalent ions often are not fully exchangeable. Frog skin epithelial cells, for example, maintain a Mg^{2+} concentration of ~5–7 m*M*, despite the fact that they are incubated for hours in Mg^{2+}-free solutions.

Figure 3 illustrates the subcellular distribution of Na^+, K^+, and Cl^- for a typical frog skin epithelial cell. Compared to the nucleus, cytoplasmic measurements performed in the raster mode always show somewhat

TABLE I
COMPARISON OF CHEMICAL CONCENTRATIONS AND ION ACTIVITIES IN EPITHELIAL CELLS

Specimen	Na^+	K^+	Cl^-	$Na^+ + K^+$	Ref.
Frog cornea					
Chemical concentration (mmol/liter)	10.1	149.3	23.4	159.4	Rick *et al.* (1985)
Ion activity (mmol/liter)	14.0	106.0	22.0	120.0	Reuss *et al* (1983)
Activity coefficient	1.39	0.71	0.94	0.75	
Frog skin					
Chemical concentration (mmol/liter)	11.5	151.2	41.7	162.7	Rick *et al.* (1978a)
Ion activity (mmol/liter)	14.0	132.0	18.0	146.0	Nagel *et al.* (1981)
Activity coefficient	1.22	0.87	0.43	0.89	
Rat proximal tubule					
Chemical concentration (mmol/liter)	24.2	190.7	25.3	214.9	Beck *et al.* (1982)
Ion activity (mmol/liter)	13.1				Yoshitomi and Frömter (1985)
Activity coefficient	0.54				

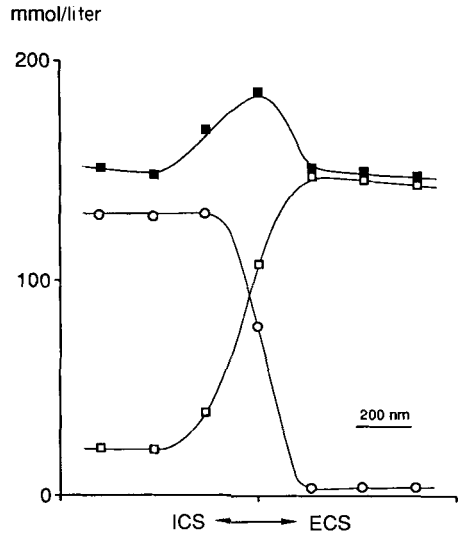


FIG. 2. Spatial resolving power of X-ray microanalysis (20 kV, 0.5 nA). ■, $Na^+ + K^+$; □, Na^+; ○, K^+. For further details, see text.

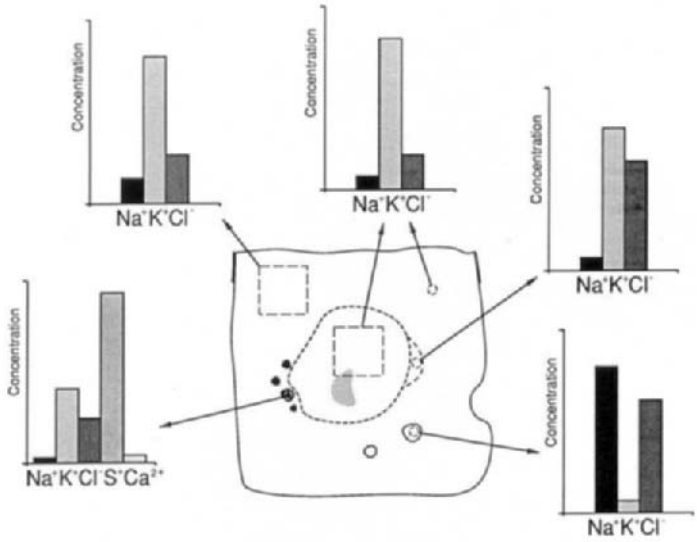


FIG. 3. Subcellular distribution of Na^+, K^+, and Cl^-. The heights of the bars reflect the relative concentrations in different subcellular compartments. For further details, see text.

higher Na^+ and Cl^- concentrations. This is probably due to the inclusion of intracytoplasmic organelles with elevated Na^+ and Cl^- concentrations. Indeed, spot analyses reveal an inhomogeneous distribution of Na^+ and Cl^- in the cytoplasm. In some cytoplasmic areas, however, the ion-concentration profile is virtually identical to that of the nucleus, suggesting that true cytosolic concentrations are identical to the nuclear values.

Larger, easily identifiable organelles have either a normal cellular distribution of Na^+ and K^+, as sometimes observed for swollen nuclear cisternae, or a completely reversed distribution of these ions, as typically seen in vacuoles.[1] Common to both structures is a high Cl^- concentration. Granules appear to have low Na^+ and K^+ concentrations; however, when expressed per liter of water space, the Na^+ and K^+ concentrations are similar to the cytosol. Again, the Cl^- concentration of granules is higher than in organelle-free cytoplasm. Both in frog skin and toad urinary bladder, granules show elevated Ca^{2+} concentrations.

Contamination by subcellular compartments probably explains why in most epithelia the cytoplasmic concentrations of Na^+, Ca^{2+}, and Cl^- are

[1]Quantitative interpretation of measurements in watery compartments containing no macromolecular matrix is complicated by the fact that, after freeze-drying, the electrolytes are no longer uniformly distributed. Nevertheless, it is possible to extract relative ion-concentration profiles, as depicted in Fig. 3.

TABLE II
NUCLEAR AND CYTOPLASMIC ION CONCENTRATIONS OF EPITHELIAL CELLS[a]

Specimen	Na^+	K^+	Cl^-	P	Ca^{2+}	d.w.	Ref.
Frog cornea							
Nucleus	5.8	124.4	17.5	117.2	0.4	20.8	Rick *et al.* (1985)
Cytoplasm	6.7	119.0	16.7	92.2	0.6	21.1	Rick *et al.* (1985)
Frog skin							
Nucleus	5.1	115.0	32.9	144.4	0.3	21.8	Rick *et al.* (1978a)
Cytoplasm	7.4	111.8	36.5	98.8	1.1	25.4	Rick *et al.* (1978a)
Rat proximal tubule							
Nucleus	19.2	149.0	19.2	151.4	0.2	21.1	Beck *et al.* (1982)
Cytoplasm	18.0	142.3	33.7	193.0	1.2[b]	30.4	Beck *et al.* (1982)

[a]Element data are expressed as mmol/kg wet weight, dry weight (d.w.) is given as g/dl.
[b]From F.X. Beck (personal communication).

slightly higher than the nuclear values. Table II compares nuclear and cytoplasmic concentration values obtained in the frog cornea, frog skin, and the rat proximal tubule. While in the frog skin and tubular cells the Cl^- concentration in the cytoplasm significantly exceeds that of the nucleus, this is not the case for the frog cornea, an epithelium exhibiting only a small number of intracytoplasmic organelles. Similarly, in frog skin and in the rat proximal tubule, the Ca^{2+} concentration in the cytoplasm is significantly higher than in the nucleus; however, this is not the case in the frog cornea. Experimental conditions that produced significant changes in ion concentrations always had an identical effect on both cellular compartments, consistent with the now generally held tenet that the nuclear membrane is freely permeable to small ions.

III. ANALYSIS OF EPITHELIAL ION TRANSPORT

In the past, we utilized this technique to investigate the functional organization of epithelia and the relationship between intracellular Na^+ and transepithelial Na^+ transport (Rick *et al.*, 1987a,b, 1984, 1988). For the purpose of the following discussion, the cellular space will be considered only one distributional space and the cellular cencentration, therefore, will be expressed as mean intracellular concentrations, based generally on separate measurements in nucleus and organelle-free cytoplasm.

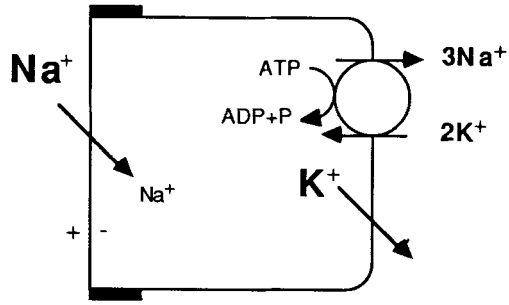


FIG. 4. Two-barrier model of transepithelial Na^+ transport. ADP, Adenosine diphosphate; ATP, adenosine triphosphate. (After Koefoed-Johnsen and Ussing, 1958.)

A. Identity of Sodium Transport Compartment

In transepithelail transport studies, the epithelium often is considered as a black box. Perhaps the most famous of these boxes is the one proposed by Koefoed-Johnsen and Ussing (1958) for active Na^+ reabsorption in tight epithelia. According to this model, transepithelial Na^+ transport can be explained by two different steps (see Fig. 4): Na^+ passively enters the epithelial cell, as we know now, by way of a Na^+-selective channel (Lindemann and Van Driessche, 1977) and is then actively extruded by the Na^+–K^+ pump. K^+, which has been taken up by the cell in exchange for Na^+, recirculates across the inner cell membrane via a K^+ channel.

Originally developed for the frog skin epithelium, Ussing assumed that it is only the basal cell layer which represents the Na^+ transport compartment. Later, he and others concluded that the whole epithelium is engaged in transepithelial Na^+ transport (Ussing and Windhager, 1964), or that only the outermost living cell layer, the stratum granulosum, is involved (Voûte and Ussing, 1968). A further possibility is the mitochondria-rich cell, which has independent access to the apical bath and certainly offers all of the cellular energy machinery necessary to support an active transport mechanism.

To answer the question as to which epithelial cell type or layer constitutes the Na^+ transport compartment, we performed the experiment depicted in Fig. 5. Under control conditions, the Na^+ concentrations in all epithelial layers are low and the K^+ concentrations are high. The only exception is the outer cornified cell layer, which always showed an ion

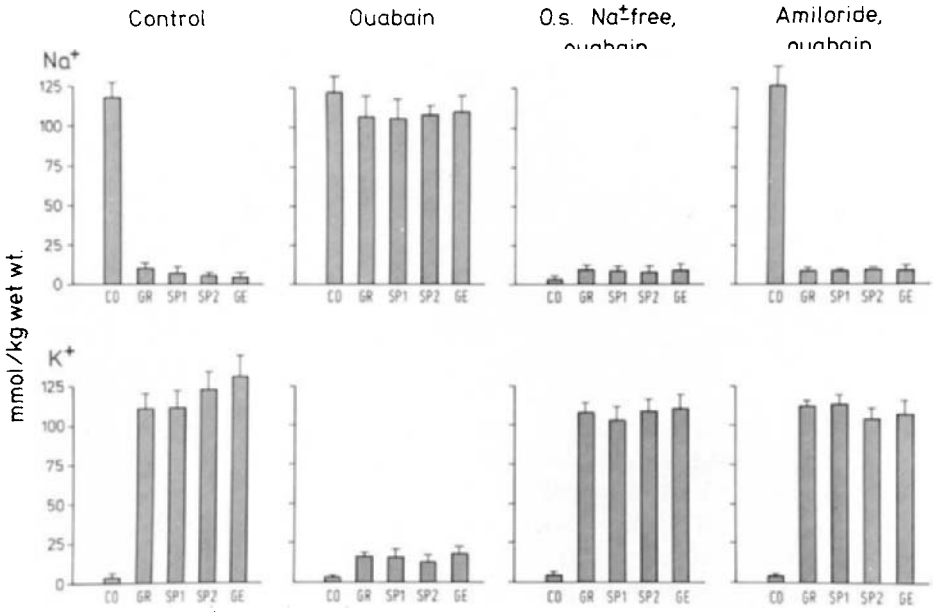


FIG. 5. Na^+ and K^+ concentrations in the different epithelial layers of frog skin *(Rana temporaria)* under control conditions, after the addition of ouabain (100 μM, 90 min), and when, simultaneous with the application of ouabain, either the outside (0.s.) was incubated with Na^+-free choline Ringer's solution or amiloride (100 μM) was added. The different epithelial layers are the stratum corneum (CO), granulosum (GR), outer and inner spinosum (SP1 and SP2, respectively), and germinativum (GE). Means ± 2SEM. (From Rick *et al.*, 1978a. Reprinted with permission.)

composition similar to that of the outer bath. After inhibition of the Na^+–K^+ pump by ouabain, an almost complete exchange of K^+ for Na^+ could be observed in all cell layers.

While the Na^+ concentration increase after ouabain is consistent with the expected behavior of a Na^+ transport compartment, it certainly does not prove that all epithelial layers are engaged in transepithelial Na^+ transport, since nonpolarized cells may also experience a Na^+ increase under this condition. What needs to be demonstrated, in addition, is that the Na^+ accumulating after ouabain is in a transcellular transport pathway. To test this, in two additional skin pieces, simultaneous with ouabain, we blocked the influx of Na^+ from the apical side either by omitting Na^+ from the outer bath or by selectively blocking the apical Na^+ channel with amiloride. Under these conditions, the effect of ouabain is completely abolished in all layers.

The results not only prove that all epithelial layers participate in transepithelial Na^+ transport, but also demonstrate that the epithelium forms a functional syncytium. Since the deeper epithelial layers have no direct access to the outer bath, the abolishment of the Na^+ increase in the deeper layers by amiloride or Na^+ removal can only be explained if there are direct cell–cell communication pathways between the layers. Thus, the pathway of transepithelial Na^+ transport in the frog skin epithelium can be described as shown in Fig. 6. Na^+, almost unimpeded by a highly permeable stratum corneum, enters the outermost living cell layer in an amiloride-sensitive step. From there, it diffuses via low-resistance cell–cell pathways into all of the deeper epithelial layers and is transported in a ouabain-sensitive step into the basolateral spaces of the epithelium.

The syncytial Na^+ transport compartment, however, does not comprise all epithelial cell types. An obvious exception are the cornified cells, which appear to be functionally dead cells, freely exchanging with the outer bath. Moreover, the various cell types of the epithelial glands do not participate in transepithelial Na^+ transport. Instead, they seem to be engaged in Cl^- secretion of the epithelium, which normally is dormant, but can be elicited by norepinephrine or isoproterenol (Mills *et al.*, 1985).

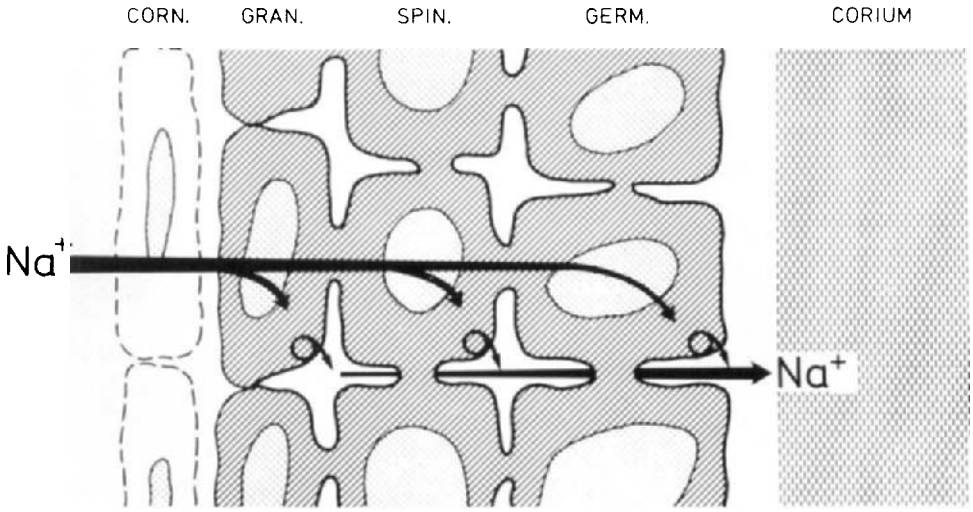


FIG. 6. Pathway of transepithelial Na^+ transport in the frog skin epithelium. CORN., Stratum corneum; GRAN., granulosum; SPIN., spinosum; GERM., germinativum. (From Rick *et al.*, 1980. Reprinted with permission.)

B. Possible Role of Mitochondria-Rich Cells

An additional, and perhaps the most interesting, exception is the mitochondria-rich cell. After the addition of ouabain, the Na^+ concentration in these cells is also detectably increased; however, the increase is only about one third of that observed in neighboring cells of the transport syncytium. Moreover, the Cl^- concentration in mitochondria-rich cells is significantly lower than in principal cells. Taken together, these findings rule out direct cell–cell coupling between mitochondria-rich cells and principal cells (Rick *et al.*, 1978a).

In principle, it is possible that the mitochondria-rich cell constitutes a parallel transepithelial transport pathway for Na^+, as this cell type has independent access to the inner and outer bath. Indeed, the Na^+ increase after the addition of ouabain can be blocked partially by removing Na^+ from the outer bath (Rick *et al.*, 1978a). However, the Na^+ increase was not inhibitable by amiloride, excluding the possibility that the mitochondria-rich cell is responsible for the bulk of transepithelial Na^+ transport.

Stimulated by a recent report of Larsen *et al.* (1987), which showed a swelling of mitochondria-rich cells in the toad skin epithelium after the addition of millimolar concentrations of ouabain, we recently reinvestigated the ouabain sensitivity of mitochondria-rich cells (R. Rick, unpublished observations). The experiments were performed in split skins of *Rana pipiens,* using a ouabain concentration of only 0.1–1 μM, sufficient to inhibit the rate of Na^+ reabsorption by ~80%.

Figure 7 depicts the results of a typical experiment. Again, after the addition of ouabain, the Na^+ concentration in all epithelial cell layers is markedly increased, although not in mitochondria-rich cells. In most skins, a decrease in the Cl^- concentration was also detectable. The decrease in the Cl^- concentration of syncytium cells was accompanied by a decrease in the cellular water fraction, which is consistent with cellular shrinkage. This observation differs from previous results in *Rana temporaria* and *Rana esculenta,* which, when supramaximal concentrations of ouabain were used, provided no evidence for changes in cell volume or Cl^- concentration (Rick *et al.*, 1978a).

Figure 8 shows the same data in a different graphic representation, displaying the Na^+ concentration as a function of the Cl^- concentration. In the control, the mitochondria-rich cell can be easily differentiated from principal cells by the much lower Cl^- concentration, but not by the Na^+ concentration. After the addition of ouabain, both Na^+ and Cl^- concentrations are different.

These results suggest that the mitochondria-rich cells are not sensitive

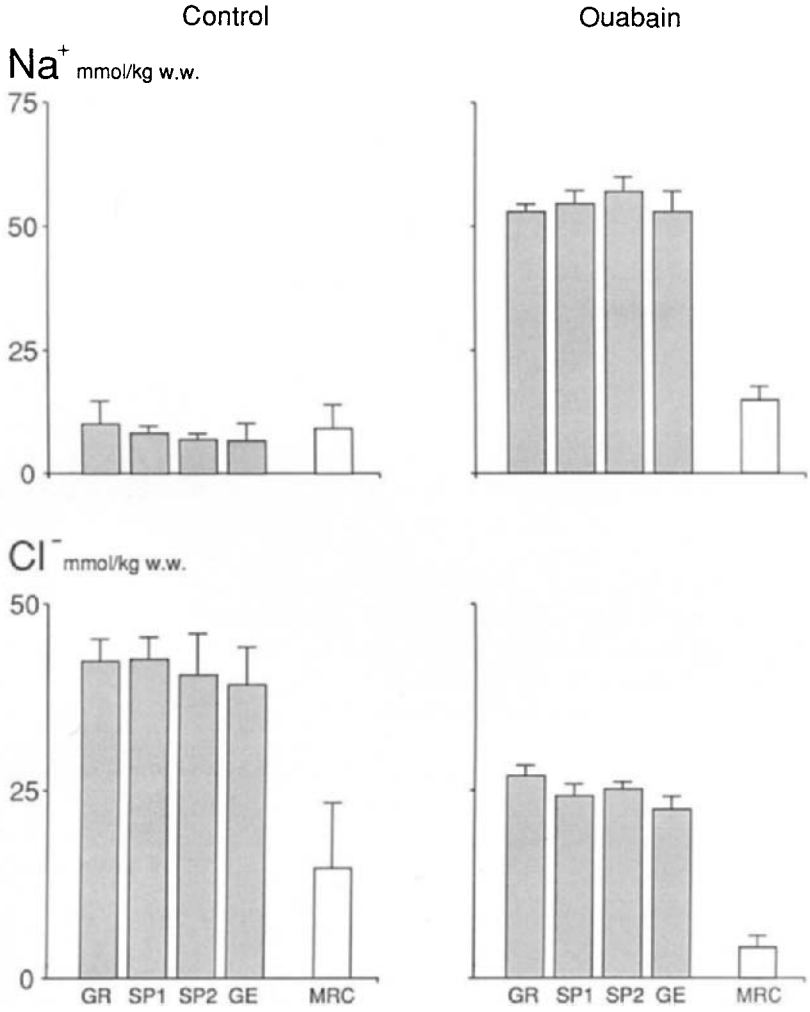


FIG. 7. Na^+ and Cl^- concentrations in the different epithelial layers and mitochondria-rich cells (MRC) of frog skin *(Rana pipiens)* under control conditions and after the addition of ouabain (1μM, 90 min). Abbreviations are as in Fig. 5. For further details, see Fig. 5.

to low concentrations of ouabain. However, this is not true for all mitochondria-rich cells. In ~50% of all experiments, a subpopulation of cells was detectable that was even more sensitive to ouabain than principal cells. This is evident in Fig. 9, which summarizes the data from three individual experiments. Under control conditions, with the exception of a single cell, all mitochondria-rich cells show a low Na^+ concentration,

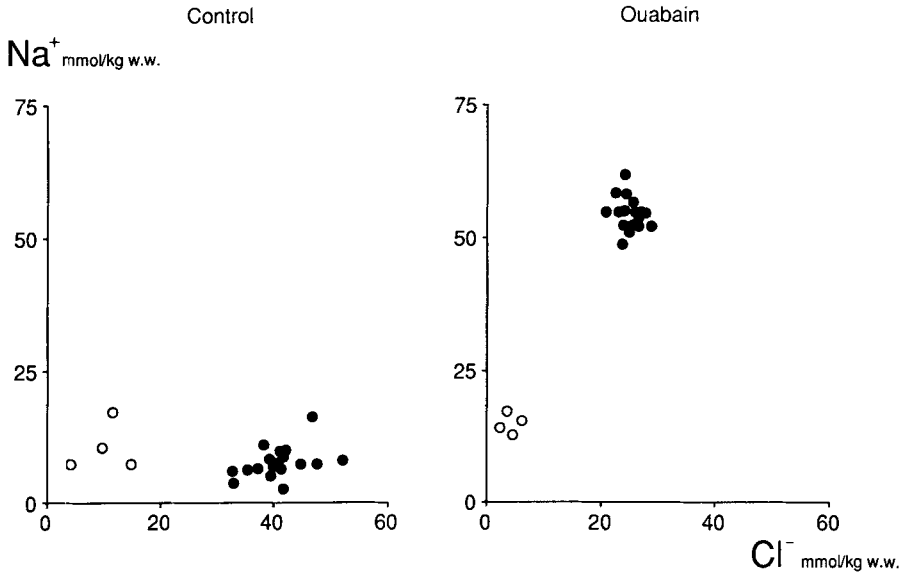


FIG. 8. Na^+ and Cl^- concentrations in syncytium cells (●) and mitochondria-rich cells (○) under control conditions and after the addition of ouabain. Same experiment as in Fig. 7.

whereas after the addition of ouabain, almost one half of the cells display a Na^+ concentration higher than that of syncytium cells. The increase in Na^+ concentration in mitochondria-rich cells was invariably accompanied by an increase in cell volume, as judged by an increased water fraction. Swelling was especially pronounced in cells that also experienced a large Cl^- concentration increase.

Mitochondria-rich cells also differ from principal or syncytium cells with regard to the exchangeability of cellular Cl^- (Rick *et al.*, 1986). While in some mitochondria-rich cells, as in principal cells, Cl^- is only slowly exchangeable and even then only from the inner bath, in other cells Cl^- is readily exchangeable from the outer or inner bathing medium (Dörge *et al.*, 1989). Thus, a subtype of mitochondria-rich cells might have transport characteristics consistent with the role of a transcellular Cl^- shunt pathway, as previously suggested by Voûte and Meier (1978).

C. Regulation of Transepithelial Sodium Transport

Conceivably, Na^+ transport can be modulated by effects on the apical membrane, stimulating or inhibiting the apical Na^+ influx, or by effects on the basolateral membrane, regulating the sodium pump activity or K^+

permeability. Evidently, stimulators and inhibitors of transepithelial Na^+ transport will have different effects on the intracellular Na^+ concentration, depending on whether they act on the Na^+ influx or on the sodium pump. An agent that primarily stimulates the Na^+ influx should increase the intracellular Na^+ concentration, while an agent that primarily stimulates the sodium pump should lower the intracellular Na^+ concentration.

The following discussion will deal with the effects of two groups of hormones that have a stimulatory effect on Na^+ reabsorption in tight epithelia.

1. Antidiuretic Hormone and Isoproterenol

In many tight epithelia, the antidiuretic hormone arginine vasopressin (AVP), in addition to stimulating the water permeability, also stimulates Na^+ reabsorption. In frog skin, AVP leads to a doubling of the transport rate within only a few minutes. The stimulation of Na^+ transport is fully inhibitable by the addition of amiloride.

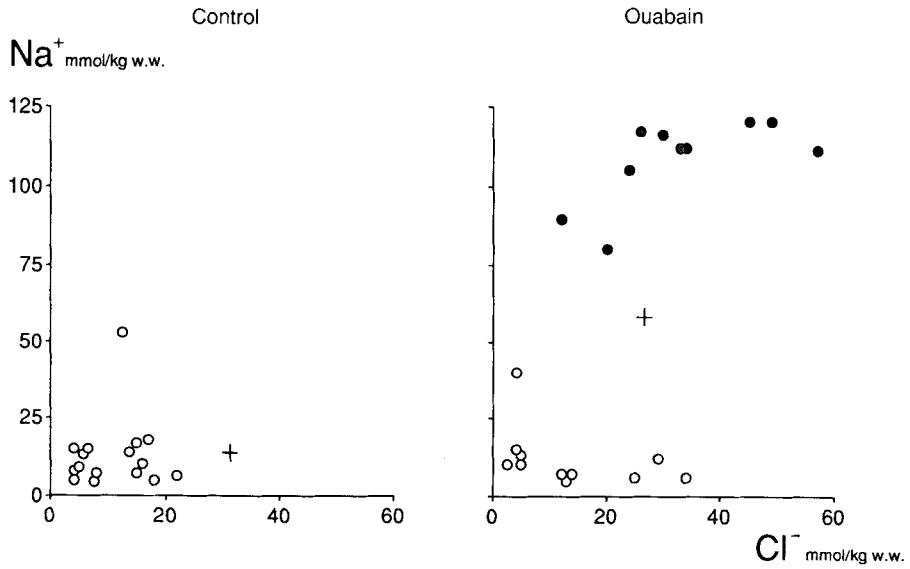


Fig. 9. Na^+ and Cl^- concentrations in mitochondria-rich cells under control conditions and after the addition of ouabain. Data are from three individual experiments. Filled and hatched circles indicate swollen and slightly swollen cells, respectively; open circles represent unchanged cells; crosses represent mean values of principal cells. For further details, see text.

Figure 10 shows the typical changes in the intracellular Na^+ and K^+ concentrations in an experiment performed in *Rana temporaria*. Compared to the control, after the addition of AVP the Na^+ concentration in all living epithelial cell layers is significantly increased, from ~10 m*M* to ~30 m*M*. The increase is completely abolished by the subsequent addition of amiloride. In fact, the Na^+ values are even slightly lower than under control conditions. No significant changes were detectable in mitochondria-rich cells. The results demonstrate that AVP stimulates transepithelial Na^+ transport by primarily increasing the apical, amiloride-sensitive Na^+ influx. The hormone obviously has no effect on mitochondria-rich cells.

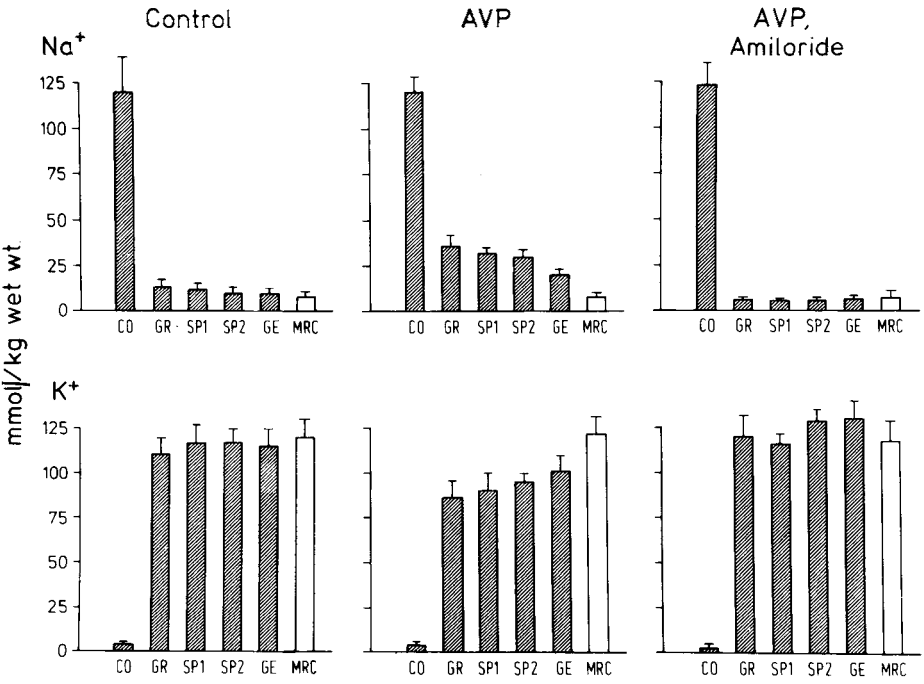


FIG. 10. Na^+ and Cl^- concentrations in the different epithelial layers and mitochondria-rich cells of frog skin *(Rana temporaria)* under control conditions, after the addition of AVP (150 mU/ml, 60 min), and when, simultaneous with AVP, amiloride (100 μ*M*) was added. Abbreviations are as in Fig. 5. For further details, see Fig. 5. [From R. Rick, A. Dörge, and K. Thurau. Electron microprobe analysis of frog skin epithelium: Pathway of transepithelial sodium transport. *In* "Ion Transport by Epithelia" (S.G. Schultz, ed.), Society of General Physiologists Series, Vol. 36, pp. 197–208. Raven, New York, 1981. Reprinted with permission.]

Figure 10 not only shows an increase in the mean intracellular Na^+ concentration after the addition of AVP, but also an increased Na^+ concentration gradient between outer and inner epithelial cell layers. In contrast, after the addition of amiloride, no concentration gradient is detectable. Similar increased Na^+ gradients were observed under all conditions in which the rate of transepithelial Na^+ transport was increased. This finding agrees well with the notion that Na^+ diffuses from outer to inner cell layers. However, increased Na^+ concentration gradients were also detectable under conditions leading to an uncoupling of the functional syncytium, such as high carbon dioxide tensions, Na^+-free inner bathing medium, or passing of large depolarizing (inward) currents (R. Rick, unpublished observations).

Another hormone which leads to short-term, fast stimulation of transepithelial Na^+ transport is epinephrine. In frog skin, the natriferic action of the hormone appears to be mediated by a β-receptor. Isoproterenol leads to a doubling of the Na^+ transport rate and an increase in the Na^+ concentration in all epithelial layers (Rick *et al.*, 1980), suggesting that, like AVP, isoproterenol primarily enhances the apical Na^+ influx. The effects are amiloride sensitive and can be prevented by prior incubation with propranolol.

In addition, we tested the effect of prostaglandins and cyclic adenosine monophosphate (cAMP), which provided essentially the same results (R. Rick, unpublished observations). Since prostaglandins, β-stimulators, and AVP are all known to increase the intracellular levels of cAMP, it is most likely that the natriferic effect of these agents is mediated by cAMP. Of course, the demonstration that the primary effect is on the apical membrane does not reveal by which mechanism cAMP stimulates the apical Na^+ permeability. From electical shot-noise analysis in the toad urinary bladder, it seems likely, however, that cAMP increases the number of apical Na^+ channels, either by insertion of new channels or by activation of preexisting channels (Li *et al.*, 1982).

2. ALDOSTERONE

For the mineralocorticoid aldosterone, the same mechanism of action has been suggested (Palmer *et al.*, 1982). On the other hand, the effect of aldosterone is markedly different from that of AVP or epinephrine. It acts much more slowly, requiring the synthesis of intracellular proteins, and it is dependent on the availability of metabolic substrates (for review see Garty, 1986). Furthermore, aldosterone potentiates the effect of AVP, indicating that the ways by which the two hormones act are not completely identical.

Since aldosterone (in addition to its stimulatory effect on Na^+ transport) induces molting of the frog skin epithelium, we investigated the effect of aldosterone in the toad urinary bladder (Rick *et al.*, 1989). Figure 11 shows a freeze-dried cryosection of the toad urinary bladder epithelium. Unfortunately, the preservation of the cellular fine structure is not nearly as good as in the split frog skin epithelium, mainly due to the fact that the folding of the bladder surface and the presence of an apical mucous layer prevent optimal freezing of the tissue. Nevertheless, different epithelial cell types and some subcellular structures such as subapical granules of granular cells can be easily differentiated.

The experiments were performed on animals that were adapted to different salinities in order to stimulate or reduce their endogenous levels of aldosterone. Accordingly, two different response patterns were observed. In bladders from salt-adapted toads, the initial short-circuit current was low and showed a marked increase upon addition of aldosterone. Conversely, in water-adapted toad bladders, the initial current was high, but showed no further increase after the addition of aldosterone. Instead,

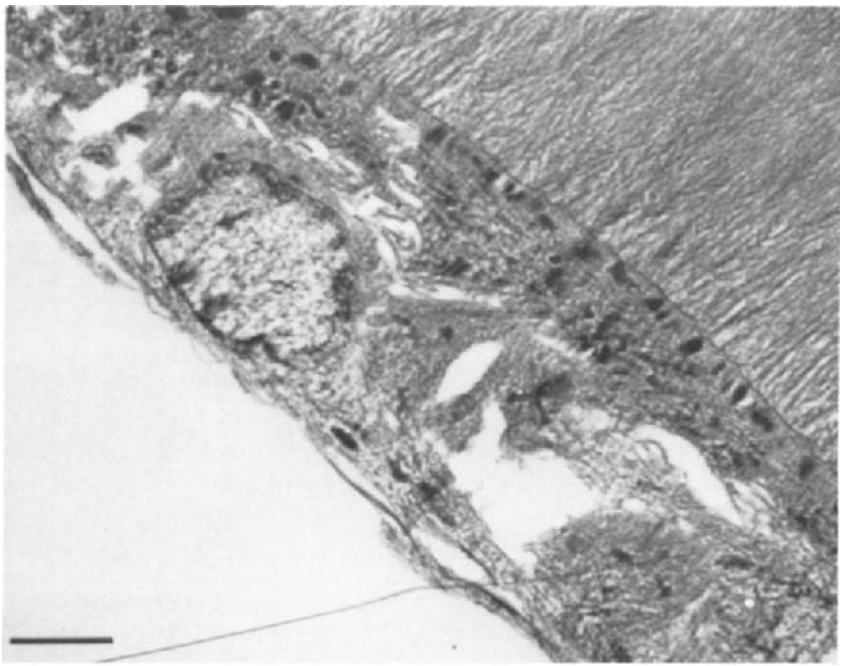


FIG. 11. Scanning electron transmission image of a freeze-dried cryosection of toad urinary bladder (*Bufo marinus*). Scale marker, 2μm. For further details, see text.

a large decrease in the short-circuit current was apparent in the untreated hemibladder.

Table III lists the values of the short-circuit current observed at the time of freezing for both experimental protocols. In addition, Table III contains two further experimental series: one using a tenfold lower concentration of aldosterone, and one in which Na^+ transport was stimulated by AVP. It is evident that the relative stimulation of transport rate was similar in all four experimental groups.

Table IV summarizes the effects of aldosterone and AVP on the intracellular electrolyte concentrations of granular cells. No significant differences are apparent among the three experimental protocols employed in the aldosterone experiments. Compared to AVP, the increase in intracellular Na^+ concentration is greatly attenuated, in particular in experiments with 50 n*M* aldosterone.

The differential effects of the two hormones are obvious in Fig. 12, which plots the Na^+ concentration increase in granular cells as a function of the relative I_{sc} increase. It is evident that, regardless of the degree of transport stimulation, aldosterone always produced a much smaller increase in the Na^+ concentration than AVP. In fact, in the majority of the experiments performed with a lower concentration of aldosterone, intracellular Na^+ was not significantly increased. Notably, no correlation is apparent between intracellular Na^+ increase and the degree of transport stimulation.

This result suggests that the mechanisms of action of AVP and aldosterone are not exactly alike. Obviously, aldosterone effects a parallel stimu-

TABLE III

EFFECT OF ALDOSTERONE AND ARGININE VASOPRESSIN ON Na^+ TRANSPORT RATE IN URINARY BLADDERS OF WATER- AND SALT-ADAPTED TOADS[a]

Condition	I_{sc} (control)	I_{sc} (experimental)	I_{sc}/I_{sc}
Aldosterone			
Water-adapted (500 n*M*)	14.0 ± 2.6	39.3 ± 16.2	2.8 ± 0.6
Salt-adapted (500 n*M*)	9.9 ± 4.9	28.3 ± 10.4	3.1 ± 0.6
Salt-adapted (50 n*M*)	19.7 ± 16.5	33.9 ± 20.7	3.0 ± 1.5
Arginine vasopressin			
Water-adapted (150 mU/ml)	16.2 ± 4.5	34.6 ± 10.2	2.0 ± 0.6

[a]Means ± SD ($\mu A/cm^2$). Numbers of toads were 3, 3, 7, and 5, respectively. Readings were taken at the end of incubation, immediately prior to freezing. Relative stimulation of the short-circuit current (I_{sc}/I_{sc}) was calculated considering the spontaneous variations in the I_{sc} as monitored in the control hemibladder.

TABLE IV

EFFECT OF ALDOSTERONE AND ARGININE VASOPRESSIN ON Na^+, K^+, AND Cl^- CONCENTRATIONS OF GRANULAR CELLS IN TOAD URINARY BLADDER[a]

Condition	Na^+	K^+	Cl^-
Water-adapted toads (500 n*M* aldosterone)			
Control (n = 74)	9.0 ± 6.1	131.5 ± 21.8	39.9 ± 10.7
Experimental (*n* = 81)	14.0 ± 9.4[b]	125.4 ± 20.3[b]	36.3 ± 12.1[b]
Salt-adapted toads (500 n*M* aldosterone)			
Control (*n* = 93)	8.5 ± 4.6	118.4 ± 17.6	46.9 ± 12.5
Experimental (*n* = 121)	12.7 ± 6.5[b]	115.6 ± 14.9	40.7 ± 10.3[b]
Salt-adapted toads (50 n*M* aldosterone)			
Control (*n* = 214)	8.5 ± 2.2	121.4 ± 11.2	34.0 ± 6.6
Experimental (*n* = 262)	10.9 ± 2.6	117.9 ± 15.0	27.3 ± 5.9[b]
Water-adapted toads (150 mU/ml AVP)			
Control (*n* = 112)	11.9 ± 4.9	124.7 ± 16.9	38.6 ± 5.9
Experimental (*n* = 131)	31.4 ± 12.3[b]	102.9 ± 17.4[b]	39.3 ± 6.5

[a]Means ± SD of *n* cells (expressed as mmol/kg wet weight). Numbers of toads were 3, 3, 7, and 5, respectively.

[b]Changes significant at the $2p < 0.05$ level (paired analysis).

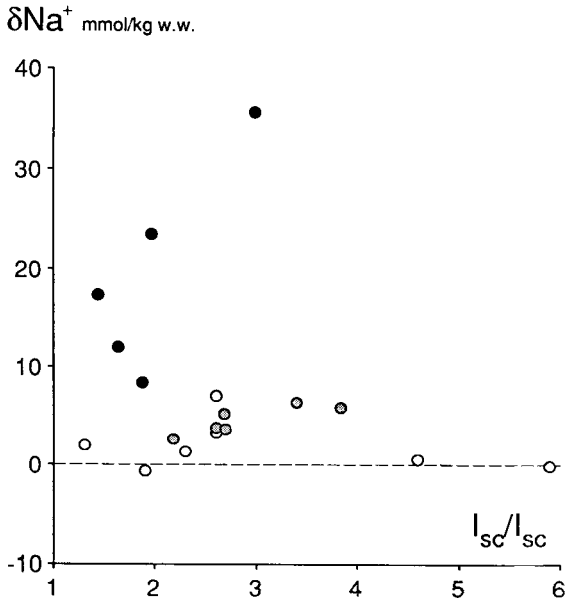


FIG. 12. Na^+ concentration increase (δNa^+) plotted as a function of the relative stimulation of the short-circuit current (I_{sc}/I_{sc}). Hatched circles and open circles indicate experiments with aldosterone (500 n*M* and 50 n*M*, respectively); filled circles represent experiments with arginine vasopressin (150 mU/ml). For further details, see text.

lation of the sodium pump and Na^+ influx so that the intracellular Na^+ concentration is only minimally changed. The advantage of such a dual mechanism may be that it presents a smaller challenge to the homeostasis of intracellular ion concentrations.

After aldosterone, but not after AVP, a decline in the Cl^- concentration of granular cells was detectable (Rick *et al.*, 1988). The fall in the cellular Cl^- concentration can be explained by an aldosterone-induced intracellular alkalinization (Oberleithner *et al.*, 1987). In fact, an alkalinization could also be responsible for the stimulation of the pump, as the pump rate is pH dependent (Eaton *et al.*, 1984). Alternatively, the stimulation of the pump process may be explained by an aldosterone-induced synthesis and expression of new pump units (Geering *et al.*, 1982; Rossier *et al.*, Chapter 9, this volume). We may also speculate that the long-term increase in the intracellular Na^+ concentration by itself contributes to the stimulation of the pump mechanism.

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Chapter 5

Sodium Dependence of Cation Permeabilities and Transport

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I. INTRODUCTION

Unlike excitable membranes, which contain a mixture of Na^+ and K^+ channels and Na^+,K^+-ATPase (sodium pump) within the same membrane, epithelia have an asymmetric distribution of channels and transporters, with specific channel and transporter populations confined to either the apical or basal pole of the cell. This asymmetric distribution is a consequence of the tight junction, a continuous loop of lipoprotein, which binds individual cells together to form an epithelial sheet. The tight junction acts as the demarcation line between apical and basolateral membranes and serves as a barrier which prohibits the intermixing of the api-

cal and basolateral domains. It is this localization of specific channels and transporters to specific membrane domains which produces the vectorial transport of electrolytes and nonelectrolytes from one compartment (e.g., the one bathing the apical membrane) to the opposing compartment (the one bathing the basolateral membrane).

The classic example is the vectorial transport across the frog skin. The original model, proposed by Koefoed-Johnsen and Ussing (1958), localized a Na^+ conductance to the apical (pond-facing) membrane and a K^+ conductance and a Na^+–K^+ pump to the basolateral (blood-facing) membrane (Fig. 1A). Transepithelial Na^+ transport occurred by the inward movement of Na^+ (from pond to cell cytoplasm) down a net electrochemical gradient and by extrusion across the basolateral membrane in exchange for K^+. K^+ then diffused (down a net electrochemical gradient) from cell to blood through a K^+ conductance found in the basolateral membrane.

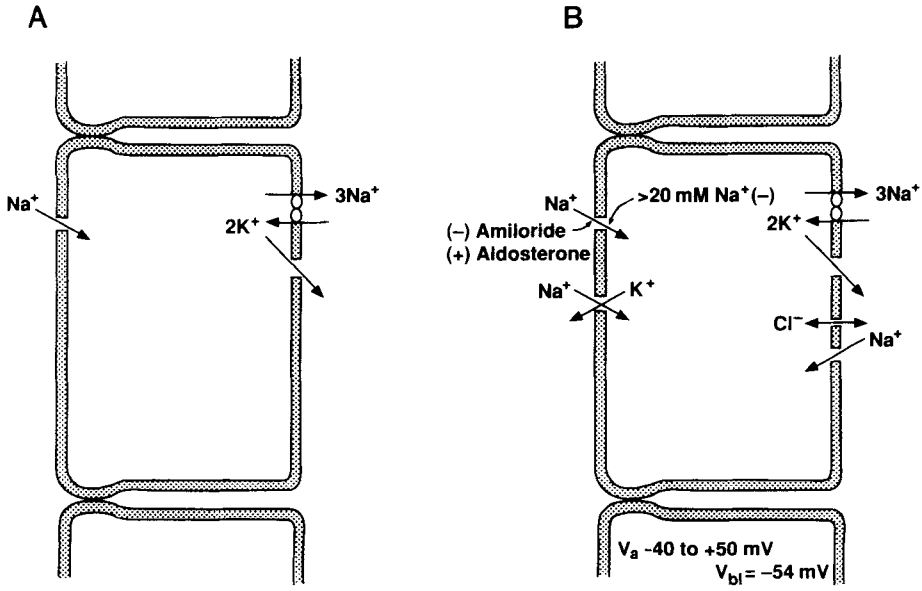

FIG. 1. Static models of Na^+ transport in tight epithelia. (A) The original double-membrane model as proposed by Koefoed-Johnsen and Ussing (1958); (B) double-membrane model of Na^+ transport in the rabbit urinary bladder obtained from a modification of the model shown in (A). In this model, apical Na^+ permeability is decreased by amiloride, luminal protease activity, and intracellular Na^+ activity and increased by aldosterone. In addition to the K^+ conductance of the original model, Cl^- and Na^+ conductance has been incorporated into the basolateral membrane.

II. EVIDENCE FOR MEMBRANE TRANSPORT REGULATION

This model is static in nature and has no built-in provisions for the alteration of net Na^+ transport. It is now well known that not only the apical membrane Na^+ permeability (the number of open Na^+ channels) but also the basolateral membrane K^+ permeability is modulated in a number of tight (high-resistance) epithelial tissues. In particular, the hormone aldosterone increases apical and basolateral membrane permeability, while the diuretic amiloride decreases the permeability of these membranes (Fig. 1B).

From the above static model, one would predict the following observations if one increases transepithelial Na^+ transport by simply increasing the rate of Na^+ entry across the apical membrane.

1. *Cell Na^+ activity should increase.* For the steady state, an increase in apical Na^+ entry must be equaled by an increased rate of Na^+ extrusion across the basolateral membrane. At constant pump density (and kinetics), an increased extrusion can only occur by an increase in substrate, i.e., cell Na^+ activity (Lewis and Wills, 1983). However, experimentally, no change in steady-state intracellular Na^+ activity has been determined (Wills and Lewis, 1980; Hudson and Schultz, 1984; Moran *et al.*, 1986).

2. *Cell K^+ activity might increase.* This occurs since an increase in cell Na^+ extrusion will result in increased K^+ uptake. At constant basolateral membrane K^+ permeability (in the absence of a change in basolateral membrane potential), an increase in K^+ flow from cell to plasma can only occur by increasing the chemical driving force for outward K^+ movement i.e., increase K^+ Nernst potential. Again, the experimental finding differed from the expected, and intracellular K^+ activity was found to be constant and independent of the rate of apical membrane Na^+ entry (Wills and Lewis, 1980; Nagel *et al.*, 1981).

3. *Basolateral membrane potential will decrease.* An outwardly directed K^+ gradient results in a basolateral membrane K^+ diffusion potential attenuated by a small but finite basolateral membrane Na^+ permeability. However, an inwardly directed Na^+ gradient results in a Na^+ diffusion potential at the apical membrane. Since the tight junctions do not have infinite resistance (Frömter and Diamond, 1972), circular current flow occurs such that an increase in apical membrane Na^+ permeability will depolarize the basolateral membrane voltage. The extent of this depolarization is a function of the apical and basolateral membrane resistances and the tight junctional resistances [see Lewis *et al.*, (1978) for equations which describe the above relationship]. Thus, in the ab-

sence of an increase in cell K^+ activity, the basolateral membrane voltage must depolarize. Here, the actual experimental result was basolateral membrane potential that was constant and independent of the rate of Na^+ entry across the apical membrane (Lewis *et al.*, 1976; Helman and Fisher; 1977; Higgins *et al.*, 1977; Schultz *et al.*, 1977).

Obviously, from the comparison of theoretical predictions from the model with experimental results, it is necessary to modify the original model so that it is more interactive, i.e., changes in the properties of one membrane must be communicated to the opposing membrane so that appropriate modifications of its transport properties can be made. Such communications have been labeled "homocellular regulation" (Schultz, 1981) and "cross-talked" (Diamond, 1982).

III. EXAMPLES OF MEMBRANE TRANSPORT REGULATION: CROSS-TALK

To date, four examples of such regulation have been described. The first example was described in detail above. Either increasing or decreasing apical membrane Na^+ permeability causes parallel increases or decreases (respectively) in the basolateral membrane K^+ conductance and pump density of kinetics. The end result is that basolateral membrane voltage, cell K^+ activity, and cell Na^+ activity remain unaltered at steady state. This constancy of membrane voltages and all ion activites also occurs in intestinal epithelia, where increased Na^+ entry is coupled with sugars and amino acids (see Chapter 2 by Schultz, this volume).

The second (and perhaps first-described) example is that inhibition of Na^+,K^+-ATPase (localized at the basolateral membrane) with ouabain causes an inhibition of the apical membrane Na^+ permeability (Lewis and Diamond, 1976; Frömter and Gebler, 1977; Helman *et al.*, 1979) and perhaps the basolateral K^+ permeability (MacRobbie and Ussing, 1961).

The third example is that exposure of a tight epithelium to hyperosmotic solution results in a decrease in apical membrane Na^+ entry rate and a decrease in basolateral membrane conductance. Conversely, exposure of a tight epithelium to a hyposmotic solution results in an increase in apical membrane Na^+ entry rate and an increase in basolateral membrane conductance (Finn and Reuss, 1975; Lopes and Guggino, 1987).

The last example of such regulation was unexpected and first demonstrated on the toad urinary bladder by Singer and Civan (1971) and in more detail by Lewis *et al.* (1985). In brief, these authors found that transepithelial Na^+ transport is dependent on the anion species bathing the

basolateral membrane. The replacement of chloride with a large (and hopefully impermeant or poorly permeant) anion, such as gluconate, caused a decrease in both apical and basolateral membrane conductance (Lewis *et al.*, 1985; Wills *et al.*, 1988).

IV. POSSIBLE SIGNAL FOR REGULATION OF MEMBRANE PROPERTIES

What is abundantly clear from the above examples is that changing the transport properties at one membrane (over times ranging from seconds to hours) causes a parallel change in the properties of the opposing (series) membrane. What is not known is the cellular signal that provides (by some unknown pathway) the modulation of these different membrane conductances. Moreover, how are these different signals generated, sensed, transduced, and translated? Is it by a common (single) receptor/sensor and effector mechanism, or are there a multitude of signal sensors and effectors?

A number of strategies can be used to address such a monumental question. The most straightforward approach is to first determine (from the available data or by experimental manipulations) whether there is a common signal which then mediates the coordinated changes in membrane transport properties. Next, one can attempt to uncouple selectively the membrane response from the signal using pharmacological manipulations of known regulatory pathways. Last, one can attempt to isolate the individual transporters to determine what possible physical or chemical processes might be involved in their regulation.

Three of the four examples of transport regulation suggest that alterations in cell volume might be the initial signal for the observed changes in both apical membrane Na^+ permeability and basolateral membrane K^+ permeability.

1. Addition of a hyperosmotic solution, which will cause cell shrinkage, decreases both Na^+ and K^+ permeability (Finn and Reuss, 1975). The extent of the decrease will depend on whether the cells possess volume-regulatory mechanisms.

2. Replacement of a permeant anion with one that is impermeant will result in a loss of the permeant anion and a counterion (K^+) with a proportionate amount of water (volume loss) from the cell (Lewis *et al.*, 1985; Costa *et al.*, 1987). Such an anion replacement causes a decrease in both Na^+ and K^+ permeability (Lewis *et al.*, 1985; Costa *et al.*, 1987; Chen and Lewis, 1987; Wills *et al.*, 1988).

3. Inhibition of apical Na^+ entry using amiloride will result in a decrease in cell K^+ uptake (via Na^+,K^+-ATPase). The efflux of K^+ and a counterion (Cl^- or HCO_3^-) with a proportionate amount of water will result in a small volume decrease, which will then reduce the K^+ permeability (Davis and Finn, 1982). The final result is small loss of volume and decreased basolateral K^+ permeability.

Conversely, the following evidence suggests that an increase in cell volume increases basolateral membrane K^+ permeability and apical membrane Na^+ permeability.

1. Dilution of the serosal bathing solution (by decreasing NaCl) results in an increase in apical membrane Na^+ permeability and basolateral membrane K^+ permeability (Finn and Reuss, 1975). Since such a hyposmotic solution will result in cell swelling, the increase in K^+ permeability is most probably a volume-regulatory response, in which K^+ and a counterion exit the cell, thus reducing the cellular osmotic pressure.
2. Replacement of Cl^- with a weak acid (e.g., acetate) will result in cell swelling (Cooke and Macknight, 1984; Lewis *et al.*, 1985) due to nonionic diffusion of undissociated acetic acid into the cell, the subsequent cellular dissociation of acetic acid to a H^+ and acetate, the exchange of the H^+ for Na^+, and the exchange of Na^+ for K^+ (Na^+,K^+-ATPase). Since acetate is less permeant than Cl^-, the cell swells because of potassium acetate accumulation and a subsequent influx of water. Such a replacement of Cl^- with acetate results in an increase in apical membrane Na^+ permeability and basolateral membrane K^+ permeability, as demonstrated by Lewis *et al.* (1985) in toad bladder.
3. Increasing Na^+ transport, e.g., removing amiloride, adding serosal aldosterone, or increasing mucosal Na^+ concentration, results in an increase in basolateral membrane K^+ conductance (Schultz and Hudson, 1986). Thus, an increased influx of Na^+ will stimulate via Na^+,K^+-ATPase, an increased influx of K^+ (in exchange for Na^+) and a counterion (Cl^- or HCO_3^-), with a proportionate amount of water. The end result is a small increase in volume, which will then increase the K^+ permeability.

V. TRANSPORT AND CELL VOLUME

The above-mentioned nonphysiological pertubations (osmotic challenge and anion substitutions) have suggested that cell volume might be the initial signal that sets into action a chain of events which ultimately result in the modification of membrane permeability properties. We have stated that inhibition of transport will result in the cellular loss of K^+, a

counterion, and water, and the stimulation of transport will result in the gain of K^+, a counterion, and water.

What is the identity of this counterion? Above, we suggested two possibilities, i.e., Cl^- and HCO_3^-; other possibilities include phosphates and metabolic end products (in addition to CO_2/HCO_3^-), such as lactate (lactic acid).

We will investigate each of these candidate counterions (anions) in turn.

1. *Chloride.* This anion is important for the maintenance of normal cell volume, since its replacement with an impermeant anion (e.g., gluconate) results in cell shrinkage and loss of Na^+ and K^+ permeability. Returning Cl^- to the solution results in return of permeability and transport. Under these conditions, Cl^- is passively distributed (in equilibrium with the membrane potential) across the basolateral membrane (Lewis *et al.*, 1978). In order for Cl^- to act as the sole intracellular counterion during transport stimulation, its intracellular concentration must increase. Such increases have not been measured. It is doubtful that Cl^- plays a primary role in cell volume alterations during transport changes. However, a permissive role cannot be excluded (see below).

2. *Bicarbonate.* Normal aerobic metabolism via glycolysis and oxidative phosphorylation produces 38 mol of ATP and 6 mol of CO_2 for each mole of glucose. Some of the CO_2 generated in this manner diffuses out of the cell, while the remainder is converted to H^+ and HCO_3^- either spontaneously or by catalysis with carbonic anhydrase. Thus, during increased Na^+ transport, there is increased CO_2 production and perhaps an increased level of cell HCO_3^-. As a consequence, aerobic metabolism will cause an increase in cell K^+ and HCO_3^-. This will lead to an increase in cell osmotic pressure, which will be dissipated by an influx of water, i.e., cell swelling. An interesting feature of this system is that during cell volume increase, cell Cl^- is diluted and the cell then has a (K^+) Cl^- influx. This influx continues until Cl^- is in electrochemical equilibrium and the cell is in an isosmotic steady state. Although Cl^- concentration is not increasing, cell Cl^- content is and thus Cl^- plays a passive (permissive) role in isosmotic volume increase. A new steady state is achieved when the rate of HCO_3^- production is equal to its loss via diffusion or electroneutral exchange.

3. *Lactate.* Glycolysis in the presence of adequate O_2 results in the production of pyruvate, which is then used in oxidative phosphorylation to produce adenosine triphosphate (ATP) (see item 2), with only a small amount of pyruvate being converted to lactate (lactic acid) by lactate dehydrogenase. Studies by Lynch and Balaban (1987) demonstrated in

Madin–Darby canine kidney (MDCK) cells that, of the ATP utilized by the Na^+,K^+-ATPase, some 40% was produced by oxidative phosphorylation, with the remaining 60% coming from glycolysis. Therefore, during Na^+ transport, a significant quantity of lactic acid is generated. The interesting feature of this study is that cell ATP concentration was unaffected whether the source of ATP was only from oxidative phosphorylation (using glutamine as a substrate) or from aerobic glycolysis (glucose). This constancy of ATP suggested to the authors a direct coupling between ATP generated by glycolysis (glucose $\rightarrow$ 2 lactate + 2 ATP) and Na^+,K^+-ATPase, a model previously suggested for the red blood cell Na^+,K^+-ATPase. In the absence of aerobic glycolysis, ATP produced by oxidative phosphorylation has a restricted access (diffusion limited) to the Na^+,K^+-ATPase.

If the production of lactic acid is directly linked to Na^+ transport, then lactic acid production (the anion lactate) can be a significant osmotic/anionic participant in cell volume increase during increased Na^+ transport. As in the case of HCO_3^- (see item 2, above), lactic acid and passive Cl^- redistribution will result in a cell volume increase.

In this section, we see that cell volume and metabolism (Na^+ transport) can be intimately linked. The advantage of this system should be obvious: Thus, increased Na^+ entry will lead to increased Na^+ exit (via the Na^+,K^+-ATPase). This increased pump activity increases both CO_2 (HCO_3^-) production through oxidative phosphorylation and lactic acid (lactate + H^+) production through aerobic glycolysis. The cellular production and increase of these anions will cause an accumulation of K^+ and Cl^-. This accumulation will result in an increased cell osmolarity, which will be dissipated by an influx of water and consequent cell swelling.

VI. REGULATORY MECHANISMS OF CHANNELS AND TRANSPORTERS

Although cell volume might be the initial signal for alterations in membrane permeabilities, we must next ask what the transducing mechanism is by which a volume shift causes channel regulation. Before addressing this question, we should perhaps ask a more general question: What is known about both intrinsic (e.g., voltage sensitivity) and extrinsic (e.g., Ca^{2+}) channel regulation? We will consider three different types of channels, two of which (already commented on in Section III) are apical membrane Na^+ conductance and basolateral membrane K^+ conductance. The

third is an integral component of our previous discussions and is basolateral membrane Cl^- conductance.

Starting with the basolateral membrane, we will summarize what is known about both K^+ and Cl^- conductance in that membrane.

A. Potassium Ion Channels

Using the patch-clamp techinque, a number of different types of K^+ channels have been localized to the basolateral membrane of urinary tract epithelium, intestinal epithelium, and secretory epithelia, such as pancreatic acinar cells, the lacrimal gland, the trachea, and the choroid plexus.

The K^+ channels in secretory cells (in general) seem to be stimulated by increases in cell Ca^{2+} concentration. Thus, hormone- or neurotransmitter-induced secretion by these epithelia is a consequence of activation of a basolateral membrane NaCl influx mechanism, activation of an apical membrane Cl^- conductance (through which Cl^- exits the cells), an increase in Na^+ extrusion and K^+ uptake via Na^+,K^+-ATPase in the basolateral membrane, and activation (by increased cell Ca^{2+} concentration) of a K^+ channel to compensate for the increased K^+ influx via Na^+,K^+-ATPase. This increase in cell Ca^{2+} is thought to involve both receptor-mediated release of cell Ca^{2+} and extracellular Ca^{2+} influx through Ca^{2+} channels (see, e.g., Marty *et al.*, 1984; Maruyama *et al.*, 1983). Whether the influx of extracellular Ca^{2+} is a result of channel regulation by a second messenger (e.g., cyclic AMP) is not clear. However, recent patch-clamp studies by Christensen (1987) on the secretory epithelium choroid plexus (which produces cerebral spinal fluid by a mechanism similar to that outlined above) has uncovered a nonselective cation channel (Ca^{2+}-permeable) whose open probability is increased by stretch, i.e., a stretch-activated "calcium" channel. Thus, during a stimulation of transport, the cells will swell slightly; this swelling increases the probability of the cation channel being open (P_o) and consequently increases the Ca^{2+} influx through this stretch-activated channel. This will increase cell Ca^{2+} concentration, which in turn will activate a Ca^{2+}-sensitive, voltage-gated K^+ channel that is also present in the same membrane (Christensen and Zeuthen, 1987). Similarly, a cell volume increase by exposure of the cells to a hyposmotic solution will increase the P_o of the stretch-activated channel, increase Ca^{2+} influx and activate the voltage-sensitive maxi-K^+ channel, which will then allow the cells to lose K^+ and Cl^- (the latter through a Cl^- conductance) and water, the end result being cell shrinkage.

In renal proximal tubules, a more direct linkage between volume changes and K^+ channel activation has recently been reported. Sackin and Palmer (1987) have found two types of K^+ channels in the basolateral

membrane of the *Necturus* proximal tubule. They differ in three respects. First, they differ kinetically; one has a long mean open time $\tau_o > 20$ msec, the other a short mean open time $\tau_o < 1$ msec. Both types have $K^+ : Na^+$ selectivities in the range of 12–14 : 1. In cell-attached patches with 112 m*M* KCl in the pipette, the long open-time channel has a single-channel conductance of 47 pS (picosiemens) and the short open-time channel a conductance of 30 pS. The voltage sensitivity of the long open-time channel was not reported, but the short open-time channel had a P_o which decreased as the membrane potential depolarized. The most striking property of the short open-time channel was that in cell-attached or cell-excised inside-out patches, application of a negative pressure to the pipette increased the open probability of this K^+ conductance (Sackin, 1987). Thus, in a similar, but more direct, manner than the choroid plexus, cell swelling will result in an increase in membrane tension, which causes an opening of a K^+-selective channel. From considerations of membrane compliance and cell geometry, Sackin (1987) estimated that a 1% increase in cell volume was required for a significant increase in the open probability of the K^+ channel. It is interesting to point out that the probability of the channel opening with increasing membrane stretch was greater for intact patches than for excised patches, suggesting loss of a secondary regulatory process.

In the turtle colon, two types of basolateral membrane K^+ conductances have been identified (Germann *et al.*, 1986). The first type is Ba^{2+} blockable, equally selective for K^+ and Rb^+, and not blocked by lidocaine or quinidine. The second conductance is only observed in swollen cells, is blocked by Ba^{2+}, lidocaine, and quinidine, and is more selective for K^+ than for Rb^+ (6 : 1). Using the patch-clamp technique on the basolateral membrane of single isolated cells from the turtle colon, Richards and Dawson (1986) found a K^+-selective channel (the selectivity was not quantitated) which had a 47 pS conductance (KCl in pipette) in the cell-attached configuration and was not voltage gated, but was blocked by lidocaine and quinidine. These observations (particularly the blocker sensitivity) suggest that this channel might represent the swelling-induced K^+ conductance previously reported from transepithelial experiments. One reason these authors were unable to quantitate the K^+ selectivity of this channel was that, upon excising the patch (inside-out configuration), the spontaneous channel activity rapidly disappeared. Such inactivation was not reversed by changing bath Ca^{2+}, and indicates the loss of a cofactor (e.g., a phosphorylated site or G-binding protein) necessary for continued channel activity.

The basolateral membrane of the rabbit urinary bladder is highly conductive to K^+ (Lewis *et al.*, 1978). This K^+ conductance has been shown

to decrease when the bladder cells are shrunken using either a hyperosmotic challenge or replacement of a permeant anion with an impermeant anion (Donaldson and Lewis, 1988). Using the patch-clamp technique on the basolateral membrane of freshly dissociated rabbit bladder cells, Lewis and Hanrahan (1985) found a K^+-selective channel. In symmetric 150 m*M* KCl, this channel had a conductance of 220 pS, a linear current–voltage relationship, P_o that increased as the membrane was depolarized, a complex kinetic scheme, an infrequently occurring subconductance state, a tendency to occur in clusters of three channels, and the ability to be unaffected by changes in cytoplasmic Ca^{2+} (10^{-8} to 10^{-3} *M*). Whether these channels are altered by stretch is unknown. What is known is that, in some instances, K^+ channel activity became apparent only after excising the membrane patch. Since these dissociated cells were incubated in a HCO_3^--free Ringer's solution (a condition that decreases basolateral membrane K^+ conductance, presumably due to cell shrinkage), the appearance of activity after excising the patch suggests that the channel was initially inhibited by some cellular factor and that this inhibition was lost after the channel was removed from communication with the cytoplasm. At present, it is not appropriate to speculate on the molecular identity of this regulatory factor. However, this large conductance channel was the only K^+ channel found (to date) in intact or excised patches of cells bathed in either HCO_3^--free Ringer's solution or one-half osmotic strength NaCl–Ringer's solution (J. W. Hanrahan and S. A. Lewis, unpublished observations).

Of the preparations studied so far, we are unable to find a common theme for the regulation of K^+ channels. Consequently, we have found reports in the literature of a K^+ channel that is directly activated by stretch; one that is Ca^{2+}-activated and in which the Ca^{2+} entry is modulated by a Ca^{2+}-permeable, stretch-activated channel; a channel that is activated by cell swelling but, upon removal of the membrane patch, quickly loses activity; and finally, a channel that is quiescent in some cell-attached patches but becomes active after excising the patch.

B. Chloride Channels

In nonepithelial cells, it has been determined that, during cell shrinkage induced by hyperosmotic solutions, there is an inhibition of both K^+ and Cl^- permeability. Concomitant with this channel inhibition, there is a stimulation of Na^+–H^+ and Cl^-–HCO_3^- exchangers. Thus, in conjunction with the Na^+,K^+-ATPase and because of reduced K^+ and Cl^- permeability, the cell gains K^+, Cl^-, and Na^+ and swells back toward normal vol-

ume. Conversely, during cell swelling induced by a hyposmotic solution, there is an increase in K^+ and Cl^- permeability and the cell loses KCl (decreasing cell osmolality) and shrinks toward control volume. Do epithelial cells also regulate their basolateral membrane Cl^- permeability in a manner similar to that described above? To date, a nonselective, Cl^--permeable channel has been reported in the basolateral membrane of the rabbit proximal tubule (Gogelein and Greger, 1986) and two different anion channels have been found in the basolateral membrane of the rabbit urinary bladder (Hanrahan *et al.*, 1985). These two channels were easily identified. One had a large conductance of 382 pS in symmetric 150 m*M* KCl, had multiple subconductance states, and was only found in excised patches after repeatedly switching the clamp potential between large positive and negative voltages. Also, this channel was only active between +20 and −20 mV; larger voltages resulted in channel inactivation. The possible physiological function of this channel is unclear. The second channel was sometimes found in intact patches or appeared after patch excision (perhaps due to the loss of a cellular regulator). Major features are summarized as follows: (1) In symmetric 150 m*M* KCl, the channel was an inward rectifier (the slope conductance decreased as the membrane potential was depolarized), with a slope conductance of 64 pS at −50 mV. (2) Ionic selectivity was $Cl^- = Br^- = I^- = SCN^- = NO_3^- > F^-$ > acetate > gluconate > $Na^+ = K^+$ (1 : 1 : 1 : 1 : 1 : 0.5 : 0.3 : 0.07 : 0.04 : 0.04) and was found to be less permeable to HCO_3^- than to Cl^-. (3) At membrane voltages more negative than −80 mV, the channel inactivated. The P_o was 0.92 from −100 to −20 mV; however, at +20 to +40 mV, the P_o decreased to 0.25. (4) Kinetic analysis yielded closed times of 0.7 and 6 msec and an open time of 25 msec. The decrease in P_o (see point 3 above) was a result of an increase in the long closed time. (5) This channel was irreversibly inhibited by 0.5 m*M* DIDS (Diisothiocyanostilbene-2, 2-disulfonic acid) (in outside-out patches) but was unaffected by Ca^{2+} over the range of 10^{-8} to 10^{-3} *M* (inside-out patches).

The only hint of regulation that we have found for this Cl^- (really an anion) channel occurs upon excising the patch: removal of the channel from contact with the cell cytoplasm caused activation of the channel (in a number of instances), suggesting loss of an inhibitory factor.

C. Sodium Ion Channels

Rather than trying to provide an in-depth summary of Na^+ channel properties, the authors recommend Chapter 3 by Palmer *et al.* in this volume. In brief, there is strong evidence that apical membrane Na^+ chan-

nels in tight epithelia are regulated by long-term modulators—such as aldosterone, which activates previously quiescent channels (Garty and Edelman, 1983), or urinary protease (kallikrein), which is released into the renal connecting duct lumen and irreversibly degrades apical Na^+ channels (Lewis and Alles, 1986)—or short-term modulators, such as stimulatory regulation by antidiuretic hormone, inhibitory regulation by serosal ouabain (Lewis *et al.*, 1976), or Cl^- replacement with an impermeant anion (Lewis *et al.*, 1985). Such short-term, reversible down-regulation of Na^+ channels was proposed to be induced by an increase in cell Ca^{2+} levels [see Taylor and Windhager (1979) for review]. Using the patch-clamp technique, Palmer and Frindt, (1987) have been unable to show a direct effect of Ca^{2+} on isolated inside-out patches from renal cortical collecting duct cells. In apparent contradiction to these results, Garty *et al.* (1987) demonstrated in toad urinary bladder vesicles that ~50% of the total Na^+ flux was dependent on an interaction between cell Ca^{2+} concentration and pH. Maximal inhibition was achieved at both high Ca^{2+} and an alkaline pH (see Chapter 3 by Palmer *et al.*, this volume), while low pH and high Ca^{2+} or low Ca^{2+} and high pH produced only minor inhibitory effects. Whether Na^+ channel regulation occurs by a direct Ca^{2+} effect, as shown in vesicle studies, or by Ca^{2+}-mediated effect (e.g., Ca^{2+}/calmodulin-activated kinase or phosphatase resulting in channel phosphorylation or dephosphorylation), as suggested by patch-clamp data, must await further studies.

D. Na^+,K^+-ATPase

From the above discussion, it is obvious that the channel activity is regulated. Since Na^+ activity is reasonably independent of net Na^+ transport, the Na^+,K^+-ATPase might be similarly regulated. Such regulation can occur by a number of mechanisms, such as: (1) Rapid recruitment by neurotransmitter of a cytoplasmic pool of Na^+,K^+-ATPase into the basolateral membrane (see Chapter 7 by Mircheff *et al.*, this volume); (2) long-term regulation by synthesis and insertion into the basolateral membrane (see Chapter 11 by O'Neil and Chapter 9 by Rossier *et al.*, this volume); (3) arginine vasopressin- and phorbol ester-induced increase in Na^+,K^+-ATPase activity in hepatocytes (Lynch *et al.*, 1986); (4) inhibition of renal bovine Na^+,K^+-ATPase by glycosylation (Garner *et al.*, 1987); (5) activation by protein kinase C agonists (phorbol esters) of Na^+,K^+-ATPase from diabetic rabbit nerve (endoneural) (Greene and Lattimer, 1986); and (6) the metabolic source of ATP (Lynch and Balaban, 1987), i.e., aerobic glycolysis or oxidative phosphorylation, which can also regulate the number of active pumps by regulation of membrane-

bound glycolytic enzymes (speculation). In this regard, phosphofructokinase kinetics can be modified by protein kinase C (Hofer *et al.*, 1985).

VII. POSSIBLE CELLULAR TRANSDUCERS

So far, we have suggested (or hypothesized) that alterations in cell volume set into action a chain of events which result in either the activation or inactivation not only of membrane channels but also of Na^+,K^+-ATPase.

Such transduction is suggestive of a so-called second messenger system. Listed below are some generally accepted second messenger systems.

1. Cyclic adenosine monophosphate (cAMP)-dependent protein kinase (i.e., kinase A) is the first we will consider. In brief, a positive stimulus to a receptor allows a G-binding protein to bind guanosine triphosphate (GTP). This activated G protein then activates adenylate cyclase, which in turn converts ATP into cAMP. cAMP binds to a holoenzyme (composed of two regulatory subunits and two catalytic subunits) and results in a release of the catalytic subunits. The catalytic subunit (active A kinase) then phosphorylates its target protein, requiring ATP as the phosphate donor. Down-regulation of this process occurs when the GTP is hydrolyzed (by the G-binding protein). This inactivates the G-binding protein and, as a consequence, the adenylate cyclase. Cellular cAMP levels can then be returned to control levels by the continued conversion of cAMP to AMP by phosphodiesterase (Reuter, 1987).

2. Phosphatidylinositol 4,5-bisphosphate (PIP_2) cascade is initiated in a manner identical to that above, i.e., stimulation of a receptor which binds to a G protein which is active upon binding GTP. This activated G protein in turn activates a phospholipase (in this instance, phospholipase C), which in turn cleaves PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Berridge and Irvine, 1984). In the presence of phosphatidylserine, DAG activates protein kinase C, which in turn phosphorylates its target protein, using ATP as the phosphate donor [see Nishizuka (1986) for review]. The IP_3 produced from PIP_2 cleavage releases Ca^{2+} from an intracellular storage site (endoplasmic reticulum). This increased cytoplasmic Ca^{2+} can then have direct effects upon membrane proteins or can activate another kinase: the Ca^{2+}/calmodulin-dependent kinase. The latter kinase can then activate cell proteins by ATP-dependent phosphorylation.

3. Three other pathways are noteworthy. One is the direct stimulation

by an external stimulus of tyrosine kinase, so called because it phosphorylates (using ATP) tyrosine residues of protein (Huganir and Greengard, 1987). Next is a guanylate cyclase, which, upon activation, produces cGMP from GTP. The cGMP activates a G kinase, which phosphorylates proteins using ATP as a phosphate source (Lincoln and Corbin, 1983). Last is the direct interaction of an activated G-protein subunit, which modulates protein function not by phosphorylation but by direct interaction between the activated G-protein subunit and the target protein (Graziano and Gilman, 1987).

What combination of the above regulatory systems and as yet undiscovered regulatory cascades will be involved in epithelial cross-talk/homocellular regulation is only now being investigated. In this regard, a recent report by Cantiello and Ausiello (1987) on LLC-PK_1 (a renal epithelial cell line of proximal tubule origin) has shown that amiloride-sensitive Na^+ uptake (across the apical membrane) is inhibited by atrial natriuretic peptide (ANP) and 8-bromo-cGMP. In a more recent report, Morhrmann *et al.*, (1987) demonstrated that stimulation of protein kinase C using OAG (1-oleoyl-2-acetylglycerol) or phorbol 12-myristate 13-acetate also inhibited amiloride-sensitive Na^+ uptake, as did nitroprusside (which activates soluble guanylate cyclase) and pertussis toxin [which ribosylates a family of G-binding regulatory proteins; see Dolphin (1987) for review]. In addition, these authors found that depleting the cells of protein kinase C by overnight incubation in phorbol ester reduced the efficacy of OAG and ANP in inhibiting amiloride-sensitive Na^+ uptake, without, however, causing a decrease in normal Na^+ conductance. The tentative conclusion drawn by these authors is that Na^+ channel activity is regulated by a G protein.

Whether a similar inhibition of K^+ conductance also occurs by a cascade of events leading to G-protein interaction with K^+ channels has not been elucidated, but an answer is being actively pursued in a number of laboratories.

VIII. REASSESSMENT OF DOUBLE-MEMBRANE MODEL

We started this chapter by presenting the classical model for transepithelial transport, the double membrane model of Koefoed-Johnsen and Ussing (1958). As we have seen, the original model is a static one, with no provisions for the alteration of transport rate. We would like to finish this chapter by presenting a modified model which incorporates many of the features discussed above. First, we will present the model and then

we will use it to explain the results of a number of recent experiments conducted on the rabbit urinary bladder.

The original model is simply modified by the incorporation of Na^+–H^+ and Cl^-–HCO_3^- exchangers in the basolateral membrane. In addition, the K^+ and Cl^- channels that contribute to the basolateral conductance are sensitive to changes in volume, such that their conductance is decreased by a decrease in cell volume. Such a model (Fig. 2) can be used to explain the results obtained from two different series of experiments, both of which result in changes in cell volume.

The first is the replacement of serosal Cl^- with an impermeant anion (in this case, gluconate). The resultant loss in K^+ and Cl^- causes the cell to shrink and V_{bl} to depolarize (Fig. 3). The depolarization is due to a reduction in both K^+ and Cl^- conductance of the basolateral membrane. The modulation of basolateral K^+ conductance has been found in other studies, but the reduction in basolateral Cl^- conductance in response to cell shrinkage is a new finding. It was determined experimentally using the following protocol: Bladders were first incubated in gluconate–Ringer's solution to shrink the cells. Then, the anion NO_3^- (which has been shown to have permeability through the anion channel equal to that of Cl^-) was used to replace gluconate in the serosal solution. Under these conditions, NO_3^- was excluded from entering the cell, and thus did not cause an increase in cell volume nor a reactivation of the basolateral membrane K^+ conductance, i.e., V_{bl} did not repolarize. When, instead of NO_3^-, Cl^- was used to replace gluconate, V_{bl} did recover, but only after a lag period of some 4–5 min (Fig. 3). It was determined that, during this lag period, cell volume was increased by the accumulation of Na^+ and Cl^- via parallel Na^+–H^+ and Cl^-–HCO_3^- exchangers. The Na^+ was then removed from the cell in exchange for K^+ by the action of Na^+,K^+-ATPase. The net result of the action of the exchangers is a gain of KCl, which leads to a volume increase and a reactivation of basolateral conductance. The inability of NO_3^- to increase cell volume can be traced to its ineffectiveness as a substitute for Cl^- on the Cl^-–HCO_3^- exchanger.

The second series of experiments involves the effects of a hyperosmotic challenge applied to the serosal solution of the bladder. The typical response to such a challenge (+66 mOsm) was an initial depolarization, followed by a recovery of V_{bl} to a new steady-state potential some 2–3 mV higher than the prechallenge value for V_{bl} (Fig. 4). The voltage recovery phase was found to be dependent on the presence of serosal Cl^- and HCO_3^- and was inhibited by amiloride and niflumic acid, but not by bumetanide.

It was concluded that the initial depolarization reflected an inactivation

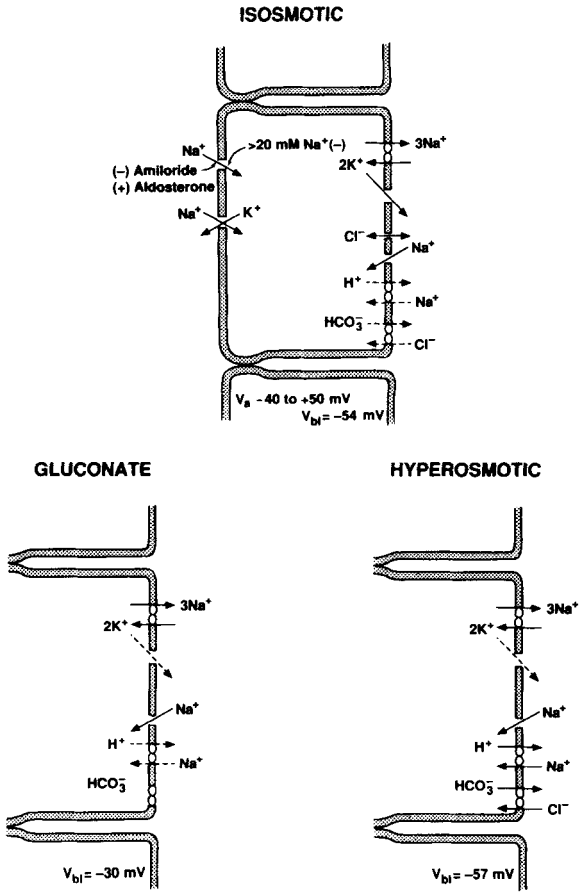

FIG. 2. Interactive models of sodium transport. (Top) Model under normal isosmotic conditions. The model in Fig. 1B has been extended by the addition of parallel $Cl^- - HCO_3^-$ and $Na^+ - H^+$ exchangers in the basolateral membrane. The exchangers may or may not be active under isosmotic conditions. (Lower left) The effect of replacement of serosal Cl^- with the impermeant anion gluconate on model parameters. Cell shrinkage causes a reduction in basolateral K^+ conductance and eliminates Cl^- conductance. Although a $Cl^- - HCO_3^-$ exchanger is activated, it is nonfunctional in the absence of Cl^-. The reduced K^+ conductance is manifested as a depolarization of V_{bl}. (Lower right) Effect of increasing the osmolarity of the serosal solution on model parameters. Cell shrinkage reduces K^+ and Cl^- conductance and activates $Cl^- - HCO_3^-$ and $Na^+ - H^+$ exchange. The increased exchanger activity leads to an accumulation of Na^+ and Cl^-. Na^+ is then exchanged for K^+ via the Na^+ pump, so the net result is an increase in K^+, Cl^-, and Na^+ and a recovery of volume. The increased pump activity produces a hyperpolarization of V_{bl}.

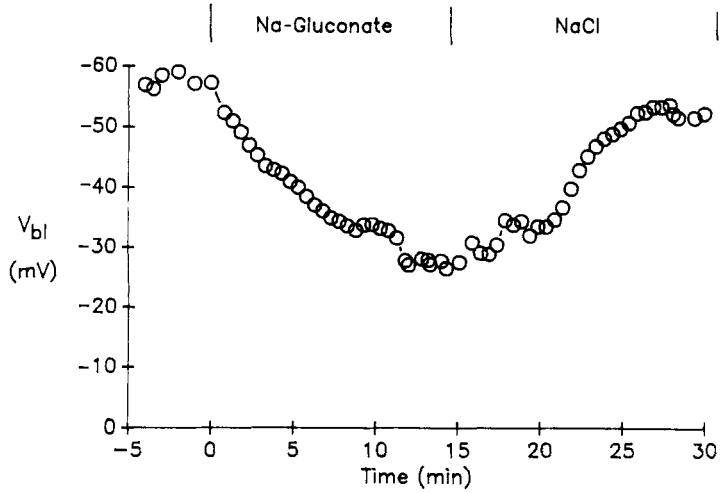

FIG. 3. The response of V_{bl} to the replacement of serosal Cl^- with gluconate in the rabbit urinary bladder. Bars indicate the period of gluconate exposure.

of K^+ and Cl^- conductance (as was the case for gluconate-induced shrinkage), while the repolarization was in fact a regulatory volume increase caused by the activation of Na^+–H^+ and Cl^-–HCO_3^- exchangers. Again, the exchangers accumulated Na^+ and Cl^-, with Na^+ being exchanged for K^+ via Na^+,K^+-ATPase.

Although serosal ouabain or amiloride does not (under control conditions) elicit a rapid basolateral membrane depolarization (Lewis *et al.*, 1978), a sizable portion of V_{bl} (~10–12 mV) in hyperosmotically challenged bladder is rapidly (1–2 min) inhibited by serosal ouabain and serosal amiloride. This suggests that, under hyperosmotic conditions, cell volume recovery is not complete and the fraction of volume recovery which occurred is maintained by the continued operation of the Na^+–H^+ and Cl^-–HCO_3^- exchangers and Na^+,K^+-ATPase.

It is interesting that apical membrane Na^+ entry is a regulating factor for basolateral membrane ionic permeability, but we must also consider the crucial role of serosal Na^+ (and Cl^-, HCO_3^-, and K^+) in channel regulation. Given these interactions, it is no longer fruitful to determine the rate-limiting step in transepithelial Na^+ transport.

IX. UNCHARTED REGIONS

Although one would like to end on a positive and infomative note, unfortunately, and as is typical of science, we end up with more questions

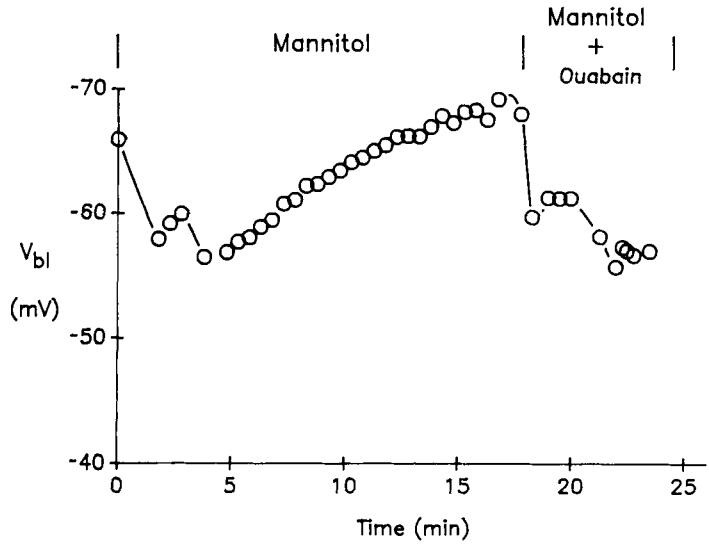

FIG. 4. The response of V_{bl} to the addition of 66 m*M* mannitol into the serosal solution of the rabbit urinary bladder. The response is characterized by an initial depolarization of V_{bl}, followed by a recovery in V_{bl} to a new steady state. Once the steady state was achieved, 10^{-4} *M* ouabain was added to the serosal solution.

than answers. We have not addressed (and at present cannot address), such pivotal questions as: (1) What is the cellular sensor that activates or inactivates channels and electroneutral transport systems? Is it volume which applies tension on the membrane, or is it cellular osmosensors? (2) Does a single regulatory cascade control all channel activities and transporters, or does the sensor regulate more than one cascade system? (3) Are individual channel or transporter populations controlled by single or multiple regulatory systems? (4) Do the cell membranes contain multiple channel populations which respond to or are regulated by a common signal? Thus, during cell shrinkage, is the K^+-channel population that is inhibited the same channel population that is activated during cell swelling? (5) How conservative among epithelia is the regulatory system? Do different epithelia contain different mechanisms for channel and transport regulation? (6) Are the regulatory themes conserved between epithelial and nonepithelial cells?

X. SUMMARY

We have attempted to summarize, in a manner that is compatible with both the cell's survival and its physiological role in plasma homeostasis,

the possible mechanisms involved in the ability of epithelial cells to regulate their individual membrane transport properties. It is obvious that, in order to elucidate these mechanisms, a clear understanding of the regulation of the individual channels and transporters is required as well as of the process by which the cell senses and communicates the need for up- or down-regulation of transport proteins.

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Chapter 6

The "Second" Sodium Pump and Cell Volume

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I. INTRODUCTION

The energy-dependent mechanisms that extrude Na^+ from the cells of higher-order animals, against large electrochemical potential differences, are responsible for counterbalancing the colloid osmotic force of intracellular macromolecular anions, to which the cell is normally impermeable (Leaf, 1956; Tosteson and Hoffman, 1960). In many cells, it has been demonstrated that the Na^+–K^+ pump is the main mechanism responsible for active Na^+ extrusion. The extruded Na^+ is exchanged with K^+ in a ratio of approximately $3Na^+$ to $2K^+$ (De Weer and Rakowski, 1984; Hoffman *et al.*, 1979; Sen and Post, 1964; Thomas, 1972). Therefore, the Na^+–K^+ pump is electrogenic and has been associated with cell volume regulation. However, the observation that the Na^+–K^+ exchange can be curtailed with cardiac glycosides without inhibition of volume regulation

in kidney cortex cells led some investigators to propose that a cardiac glycoside-insensitive sodium pump plays an important role in cell volume regulation (Daniel and Robinson, 1971; Kleinzeller and Knotková, 1964a,b; Macknight, 1968a,b; Macknight *et al.*, 1974; Maude, 1969; Whittembury and Proverbio, 1970). Since the second sodium pump hypothesis was proposed, several explanations have emerged to argue against a major role of this mechanism (if it indeed exists) in the volume regulation of kidney cortex cells (for review see Whittembury *et al.*, 1978).

In this chapter, we summarize the results of early experiments on rat kidney cortex slices which suggest the presence of a ouabain-insensitive sodium pump. We will also provide newer evidence that basolateral plasma membrane fractions from proximal tubular cells of the rat kidney have ouabain-insensitive Na^+-ATPase with different kinetic characteristics from those of Na^+,K^+-ATPase. Subsequently, we will present the results of experiments indicating two different modes of Na^+ uptake in inside-out basolateral plasma membrane vesicles associated with specific ATPase activities. Finally, we will describe the results of recent experiments dealing with the relationship between cell volume and the activity of ouabain-insensitive Na^+-ATPase.

II. SODIUM TRANSPORT IN KIDNEY SLICES

Outermost kidney cortex slices are known to be rich in proximal tubules. This preparation has an experimental advantage in that the lumens of the tubules are collapsed, so the activities of the ion-transporting mechanisms located at the basolateral plasma membrane can be evaluated easily. Employing this preparation, several lines of evidence have been obtained during the past few years that support the hypothesis of a second sodium pump. The basic experimental protocol used consists of incubating the slices for 90 min at 0°C in a medium without K^+. Under this condition, the slices gain Na^+, Cl^-, and water and lose K^+. Then, the slices are rewarmed at 25°C in the presence or absence of K^+, ouabain, or ethacrynic acid, etc. The net ion movements that occur during the rewarming period are used to evaluate the activities of the sodium pumps (for details see Whittembury and Proverbio, 1970).

A. Sodium Extrusion in Kidney Cortex Slices

Rat kidney cortex slices have been extensively used in our laboratory to study the characteristics of the second sodium pump. Table I shows the initial net fluxes of Na^+, K^+, Cl^-, and water in slices previously incu-

TABLE I
INITIAL NET FLUXES OF Na^+, K^+, Cl^-, AND WATER IN RAT KIDNEY CORTEX[a]

Rewarming medium	Initial net fluxes			
	Cell Na^+[b]	Cell K^+[b]	Cell Cl^-[b]	Cell water[c]
$5K^+$	−13.76 ± 0.39	+8.23 ± 0.31	−5.53 ± 0.18	−0.112 ± 0.003
$0K^+$	− 6.61 ± 0.32	−1.18 ± 0.13	−6.02 ± 0.13	−0.115 ± 0.002
$5K^+$ + ouabain	− 6.87 ± 0.41	−1.01 ± 0.22	−5.88 ± 0.20	−0.107 ± 0.002
$5K^+$ + ethacrynic acid	− 7.88 ± 0.26	+8.02 ± 0.18	−1.02 ± 0.25	−0.012 ± 0.003

[a]Slices were rewarmed at 25°C after preincubation for 120 min in the $0K^+$ medium. All of the incubation media (preincubation or rewarming) contained 9 m*M* sodium acetate, 15 m*M* $NaHCO_3$, 0.6 m*M* NaH_2PO_4, 2.4 m*M* Na_2HPO_4, 12 m*M* $MgSO_4$, 0.6 m*M* Na_2SO_4, 1m*M* calcium gluconate, 5 m*M* glucose. NaCl and KCl were added to obtain the following media (final concentrations): $0K^+$, 120 m*M* NaCl; $5K^+$, 115 m*M* NaCl and 5 m*M* KCl. When required, 7 m*M* ouabain or 2 m*M* ethacrynic acid was added to the $5K^+$ medium. The solutions were gassed with O_2:CO_2 (95:5, vol/vol) throughout the rewarming period. Cellular extrusion during rewarming is represented by negative values and cellular uptake is shown by positive values. The results are expressed as means ± SE of the initial slopes of six experiments. See Whittembury and Proverbio (1970) for details.

[b]Data are expressed as μmol/g solids/min.

[c]Data are expressed as g/g solids/min.

bated in a K^+-free isosmotic medium at 0°C and then rewarmed under different experimental conditions. Upon rewarming of the slices in a medium containing 5 m*M* K^+, the cells extrude Na^+, Cl^-, and water and gain K^+. Clearly, if maintenance of normal intracellular ionic contents and regulation of cell volume were due entirely to the activity of the Na^+–K^+ pump, one would expect these to be inhibited in the presence of ouabain concentrations that inhibit the Na^+–K^+ exchange. However, when the slices are rewarmed in the absence of K^+ ($0K^+$ medium) or in the presence of K^+ and ouabain ($5K^+$ medium + 7 m*M* ouabain), conditions under which the Na^+–K^+ exchange is completely blocked (there is a loss of cell K^+), a sizable amount of Na^+ is still extruded with Cl^- and water (cell volume restoration). On the other hand, rewarming with 2 m*M* ethacrynic acid in the $5K^+$ medium results in a significant inhibition of the extrusion of Na^+ accompanied with Cl^- and water, whereas the Na^+–K^+ exchange is minimally affected.

In spite of the work by Rose *et al.* (1985), who concluded that these slices are a suitable model for transport and metabolic studies, these early experiments with rat kidney cortex slices have been criticized on the grounds of uncertainty regarding the external environment of the cells and the relative insensitivity of the Na^+–K^+ pump from rat kidney tissue to ouabain (Cooke, 1978a,b, 1981; Podevin and Boumendil-Podevin,

1972). While we cannot respond fully to this criticism, it is important to note that the Na^+–K^+ pumps responsible for the the regulation of the intracellular concentration of K^+ are inhibited by ouabain, since the K^+ uptake is completely blocked (Table I). Therefore, we have to assume that the remaining Na^+–K^+ pumps, which are not inhibited by ouabain, are not associated with the regulation of the intracellular concentrations of K^+ but are responsible for cell volume regulation, regardless of whether the "ouabain-sensitive" Na^+–K^+ pumps are working. This interpretation requires the existence in the rat kidney cortex cells of atypical Na^+–K^+ pumps as well as atypical Na^+,K^+-ATPases. On the other hand, while it is true that ethacrynic acid is not an ideal inhibitor, because it penetrates the cell and progressively inhibits other cell functions (Epstein, 1972a,b; Gaudemer and Foucher, 1967; Gordon, 1968), during the first few minutes of incubation of kidney cortex or liver slices in the presence of 2 m*M* ethacrynic acid, the cellular damage induced by this agent is not significant (Russo and van Rossum, 1986; F. Proverbio and R. Marín, unpublished observations).

B. Oxygen Consumption Associated with Sodium Transport

It has long been known that there is a proportional relationship between the activity of the metabolically dependent ion-transporting mechanisms and oxygen consumption by kidney cortex slices (Allison, 1975; Kleinzeller and Knotková, 1964a; Whittam and Willis, 1963). Most of these studies were carried out at 25°C because of the difficulties of adequately oxygenating kidney cortex slices at higher temperatures (Macknight, 1968c; Mudge, 1951; Whittam and Willis, 1963; Whittembury and Proverbio, 1970). At 25°C, the slices are adequately oxygenated. Thus, we determined the oxygen consumption of rat kidney cortex slices previously loaded with Na^+, Cl^-, and water and depleted of K^+ (during the chilling period) when rewarmed at 25°C under the same conditions shown in Table I. The results are shown in Table II. It may be seen that in the presence of K^+ in the incubation medium there is a higher oxygen consumption by the slices than that in the absence of K^+. Ouabain (7 mM) in the rewarming medium produces a significant inhibition of the oxygen consumption by slices rewarmed in the presence of K^+. This effect is not seen in the absence of K^+. On the other hand, 2 m*M* ethacrynic acid affects a similar fraction of the oxygen consumption when the slices are rewarmed in the presence or absence of K^+. These results are in agreement with those shown in Table I; however, they have been criticized on the grounds of poor oxygenation of the cells in the slices (Balaban *et al.*, 1980).

TABLE II
OXYGEN CONSUMPTION BY RAT KIDNEY CORTEX[a]

Rewarming medium	Oxygen consumption (nl O_2/mg wet tissue/hr)		
	Control	+ Ouabain	+ Ethacrynic acid
$0K^+$	382 ± 10	370 ± 15	296 ± 12
$5K^+$	723 ± 20	387 ± 14	612 ± 22

[a]Slices were rewarmed at 25°C after preincubation for 120 min in the $0K^+$ medium. Experimental procedure is as described in Table I. Values are expressed as means ± SE of the initial slopes of six determinations.

Up to now, we have dealt with the results of some of the experiments that led us to propose a ouabain-insensitive Na^+ extrusion mechanism different from the classical Na^+–K^+ pump. The results of more recent studies, aimed at demonstrating the existence (and possible physiological role) of a ouabain-insensitive sodium pump at the basolateral plasma membranes of rat kidney proximal tubular cells, will be described in the following sections.

III. SODIUM-STIMULATED ATPase ACTIVITIES

Three major membrane ATPases have been described in basolateral plasma membrane fractions prepared from rat kidney cortex slices: (1) a ouabain-insensitive Ca^{2+}-ATPase, (2) a ouabain-sensitive Na^+,K^+-ATPase, and (3) a ouabain-insensitive Na^+-ATPase (del Castillo *et al.*, 1982; Kinne-Saffran and Kinne, 1974; Marín *et al.*, 1983; Parkinson and Radde, 1971; Proverbio and del Castillo, 1981; Proverbio *et al.*, 1986; van Heeswijk *et al.*, 1984). The scope of this section is circumscribed to the study of Na^+-stimulated ATPase activities.

The first demonstration of ouabain-insensitive Na^+-ATPase activity that was different from classical Na^+,K^+-ATPase activity was performed by Proverbio *et al.* (1975) in guinea pig kidney cortex microsomal fractions, aged for 10–12 days in a refrigerator. Several years later, Proverbio and del Castillo (1981) and Marín *et al.* (1983) demonstrated the same ouabain-insensitive Na^+-ATPase activity in freshly prepared basolateral plasma membranes from guinea pig and rat kidney cortex, respectively. This activity is completely inhibited by 2 m*M* ethacrynic acid or furosemide. Na^+-ATPase activity was then characterized (del Castillo *et al.*, 1982; Marín *et al.*, 1983) and demonstrated to be modulated by micromo-

TABLE III
PARTIAL CHARACTERIZATION OF Na^+-ATPase AND Na^+, K^+-ATPase ACTIVITIES

Characteristic	Na^+-ATPase	Na^+, K^+-ATPase
Mg^{2+} requirement	Yes	Yes
K_m^{MgATP} (m*M*:m*M*)	1.2:0.48	0.5:0.5
K_m^{Na} (m*M*)	8.0	16.0
K^+ requirement	No	Yes
Cation stimulation	$Na^+ > Li^+$	$K^+ > Rb^+ > NH_4^+ > Cs^+$ plus Na^+
Anion stimulation	No	No
Substrate	ATP	ATP >>>> GTP, ITP, ADP, CTP
Optimal temperature (°C)	47	52
Optimal pH	6.9	7.2
Ouabain sensitivity (7 m*M*)	0%	100%
Furosemide sensitivity (2 m*M*)	95%	5%
Ethacrynic acid sensitivity (2 m*M*)	100%	60%

ATP, Adenosine triphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate.

lar quantities of Ca^{2+} (Marín *et al.*, 1985b; Proverbio *et al.*, 1982). A summary of the main characteristics of Na^+-ATPase compared to those of Na^+,K^+-ATPase is presented in Table III. It is noticeable that many of the studied characteristics of the two ATPase activities differ.

Na^+-ATPase has also been demonstrated to be a different biochemical entity from Na^+,K^+-ATPase (Tables IV and V). It may be seen in Table IV that in purified membranes pretreated with sodium dodecyl sulfate (SDS) (1.6 μg of SDS per microgram of protein) to avoid the formation of membrane vesicles, Na^+,K^+-ATPase activity is inhibited by 7 m*M* ouabain and is insensitive to 2 m*M* furosemide. As shown in Table IV,

TABLE IV
Na^+, K^+-ATPase ACTIVITY OF PURIFIED BASOLATERAL PLASMA MEMBRANE FRACTIONS FROM RAT KIDNEY PROXIMAL TUBULAR CELLS[a]

Membrane treatment	Na^+, K^+-ATPase activity (nmol P_i/mg protein/min)		
	Control	+7 m*M* Ouabain	+2 m*M* Furosemide
SDS	1890 ± 56	18 ± 10	1840 ± 68
SDS + trypsin	8 ± 10	10 ± 10	4 ± 8
NaI + SDS	1993 ± 67	21 ± 11	1980 ± 54

[a]Membrane purification, treatments, and experimental procedure are as in Proverbio *et al.* (1986). Values are expressed as means ± SE (n = 6).

TABLE V
Na^+-ATPase Activity of Purified Basolateral Plasma Membrane Fractions from Rat Kidney Proximal Tubular Cells[a]

Membrane treatment	Na^+-ATPase activity (nmol P_i/mg protein/min)		
	Control	+7 m*M* Ouabain	+2 m*M* Furosemide
SDS	205 ± 11	198 ± 9	9 ± 3
SDS + trypsin	212 ± 10	206 ± 12	12 ± 7
NaI + SDS	6 ± 6	4 ± 6	2 ± 3

[a]Membrane purification, treatments, and experimental procedure are as in Proverbio *et al.* (1986). Values are expressed as means ± SE (n = 6).

when the membrane fractions were pretreated with SDS and then with trypsin (Forbush, 1982; Giotta, 1975), Na^+,K^+-ATPase activity was completely abolished. We can also see that pretreatment of the purified membranes with NaI (Ebel *et al.*, 1971) and then with SDS did not affect Na^+,K^+-ATPase activity or its behavior toward ouabain and furosemide. Table V presents the results of the same series of experiments shown in Table IV but carried out on Na^+-ATPase activity. It can be seen that, contrary to Na^+,K^+-ATPase activity, Na^+-ATPase activity is insensitive to ouabain (7 m*M*) and is completely inhibited by furosemide (2 m*M*). Na^+-ATPase activity is not affected by pretreatment of the membranes with trypsin and SDS, while it is abolished when the membranes are pretreated with NaI and SDS. The results presented in Tables IV and V show a different behaviors of both ATPases toward the trypsin and NaI treatments; these results suggest that Na^+-ATPase and Na^+,K^+-ATPase are two different entities.

The existence of two different Na^+-ATPase activities in basolateral plasma membranes from rat kidney proximal tubular cells is in agreement with the existence of two different sodium pumps; however, it is necessary to demonstrate that both ATPases are capable of actively transporting Na^+ across the basolateral plasma membrane.

IV. ACTIVE SODIUM UPTAKE IN BASOLATERAL PLASMA MEMBRANE VESICLES

Both Na^+-ATPases require Mg^{2+}, Na^+, and ATP at the cytoplasmic face of the basolateral plasma membrane in order to work (Marín *et al.*, 1985a). In addition, Na^+,K^+-ATPase requires K^+ at the extracellular

face of the plasma membrane. Inside-out vesicles are ideal for the investigation of active transport of Na^+ catalyzed by the Na^+-ATPases. Transport activity of Na^+-ATPase in inside-out vesicles can be evaluated by measuring Na^+ uptake (over passive values) by vesicles incubated with Mg^{2+}, Na^+, and adenosine triphosphate (ATP). On the other hand, the transport activity of Na^+,K^+-ATPase can be evaluated by incubating K^+-loaded vesicles in a medium containing Mg^{2+}, Na^+, and ATP, in the presence or absence of ouabain inside the vesicles. For the experiments described in this chapter, the inside-out vesicles were preincubated for 2 hr at 0°C in different media, in which they were later incubated, to allow the isotope (^{24}Na) to reach equilibrium. The media were complete, except for Mg^{2+}, which was added later, to start the incubations. The Na^+ uptake and ATP hydrolysis assays were carried out at 37°C according to a method already described by Marín *et al.* (1985a). Figure 1 shows the active Na^+ uptake, under steady-state conditions (upper panel), as well as the concomitant ATP hydrolysis (lower panel), by inside-out vesicles of basolateral plasma membranes, prepared from rat kidney cortex slices. The white columns represent the values obtained (active Na^+ uptake and Na^+-stimulated ATPase activity) when the vesicles were incubated in the complete medium with Na^+ but without K^+ (passive Na^+ uptake and Mg^{2+}-ATPase activity values were already subtracted). The hatched portion of the columns represents, in both cases, the stimulatory effect of adding K^+ (+ valinomycin) to the complete incubation medium with Na^+ (for details see Marín *et al.*, 1985a). In other words, the white columns represent the values related to the sodium pump and the hatched portions of the columns represent the values related to the Na^+–K^+ pump. Notice that there are two kinds of Na^+ uptake associated with two ATPase activities: (1) Na^+ uptake and ATPase activity that are K^+ independent, insensitive to 7 m*M* ouabain (inside and outside the vesicles), and totally inhibited by 2 m*M* furosemide in the incubation medium. These results represent the expression of the ouabain-insensitive sodium pump. (2) The expression of the Na^+–K^+ pump can be seen in the hatched portions of the columns. There is Na^+ uptake associated with ATPase activity that is K^+ dependent, ouabain sensitive (ouabain inside the vesicles), and furosemide insensitive.

The results presented in Fig. 1 constitute a clear demonstration that, besides the Na^+–K^+ pump, there is a K^+-independent sodium pump in the basolateral plasma membranes of rat kidney proximal tubular cells.

V. CELLULAR VOLUME AND ATPase ACTIVITY

Eukaryotic cells can regulate their volume by way of ion-transport systems located in the plasma membranes (Cala, 1983; Grinstein *et al.*, 1984;

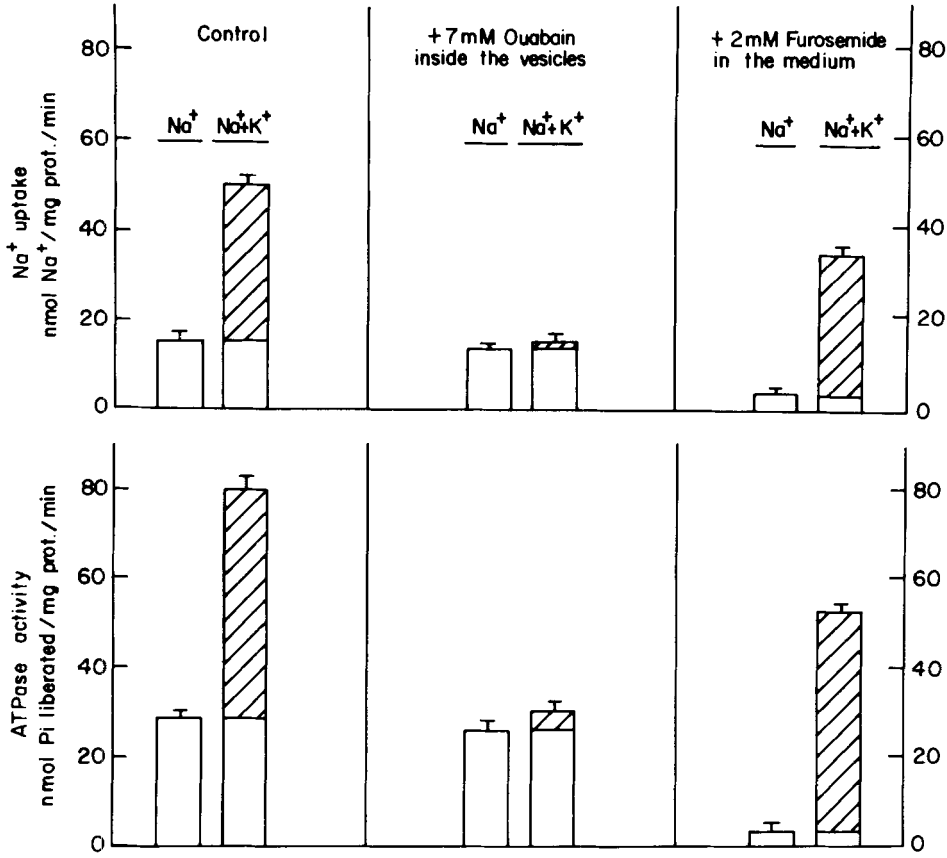

FIG. 1. Active Na^+ uptake and ATPase activity by inside-out basolateral plasma membrane vesicles. (Upper panel) Na^+ indicates the difference in Na^+ uptake by vesicles incubated with $Na^+ + ATP + Mg^{2+}$ and $Na^+ + ATP$. $Na^+ + K^+$ (hatched portion of the columns) indicates the difference in Na^+ uptake by vesicles incubated with $Na^+ + ATP + Mg^{2+} + K^+$ + valinomycin and $Na^+ + ATP + Mg^{2+}$ + valinomycin. The concentrations of the ligands were 10 m*M* Tris–HCl (pH 7.2); 50 m*M* NaCl, with 50 μCi/ml ^{24}Na; 5 m*M* $MgCl_2$; 20 m*M* KCl; 5 m*M* ATP; 6.25 μg of valinomycin per milligram of protein; and, when required, 7 m*M* ouabain and 2 m*M* furosemide. Osmolarity inside the vesicles was 300 mOsm. Osmolarity of the incubation media was adjusted to 300 mOsm with sucrose. Values are expressed as means ± SE (n = 8). (Lower panel) Na^+-stimulated ATPase activity (over Mg^{2+}-ATPase activity) of the vesicles, incubated as indicated above. Values are expressed as means ± SE (n = 8).

Hoffmann, 1982; Kregenow, 1981; Siebens, 1985). If the Na^+–K^+ or sodium pumps are associated, in some way, with the regulation of cell volume, their activity might be modulated by cell swelling or shrinkage.

Rat kidney cortex slices were prepared as mentioned above. The slices were used for determinations of cell water content or ATPase activities of their homogenates (Pérez-González *et al.*, 1980; Whittembury and Proverbio, 1970). The cell water determinations were carried out with either freshly prepared slices or with slices preincubated under specific conditions to vary their cell water content. The cell water contents of the slices were determined by measuring their tissue water contents and their inulin spaces, as previously described (Whittembury and Proverbio, 1970). For ATPase assays, the slices (freshly prepared or preincubated) were washed once in a medium containing 250 m*M* sucrose, 0.5 m*M* dithiothreitol, 0.2 m*M* phenylmethylsulfonyl fluoride, and 20 m*M* Tris–HCl (pH 7.2). The slices were then homogenized in 3 vol of the same medium per gram of tissue. The ATPase assays were carried out as described elsewhere (Marín *et al.*, 1985a, 1986).

A. Cell Water Content and Sodium-Stimulated ATPase Activities

The cell water contents of slices incubated under different conditions are shown in Fig. 2. It can be seen that cell water content, and hence cell

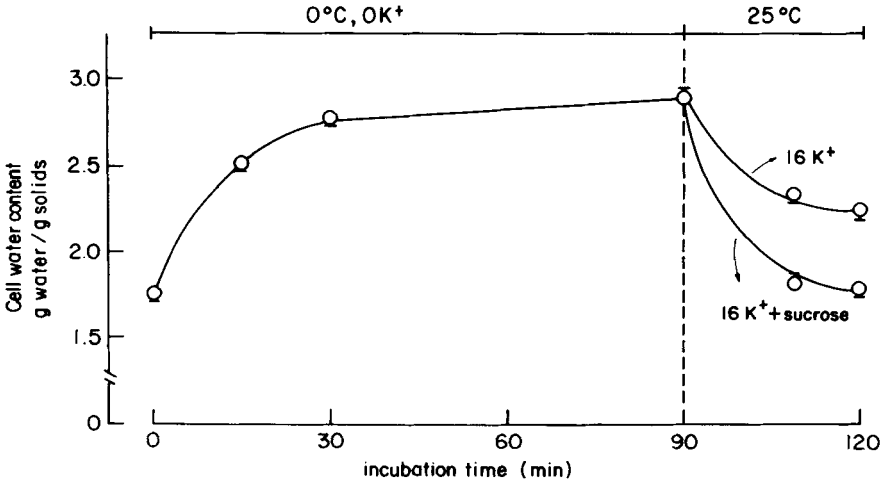

FIG. 2. Time course of the cell water content of rat kidney cortex slices incubated for 90 min at 0°C in the $0K^+$ medium. After the chilling period, the slices were rewarmed at 25°C in a medium containing 16 m*M* K^+ or 16 m*M* K^+ + 100 m*M* sucrose. For details of the experimental technique, see Whittembury and Proverbio (1970). Values are expressed as means ± SE (n = 6).

volume, increases upon incubation of the slices at 0°C in a medium without K^+. When the swollen slices are then rewarmed at 25°C in a medium containing 16 m*M* K^+, the cell water content reaches values intermediate between those of freshly prepared slices (0-min preincubation time) and those of swollen slices (90-min preincubation time). Addition of 100 m*M* sucrose to the rewarming medium (to increase the osmolarity) produces a complete reversal of the cell water content of the swollen slices, which reached values similar to those of the freshly prepared slices.

Figure 3 shows the Na^+ATPase and Na^+,K^+-ATPase activities of homogenates of slices preincubated under the same conditions shown in Fig. 2. Notice that the Na^+,K^+-ATPase activity is not affected by changes in the cell water content of the slices (cf. Fig. 2). This is not the case for the Na^+-ATPase, which is activated by increasing the cell water content of the slices. The Na^+-ATPase activity of homogenates of freshly prepared slices is low (8 ± 2 nmol P_i/mg of protein/min), and represents ~10% of the Na^+,K^+-ATPase activity. Homogenates of swollen slices show Na^+-ATPase activity similar to that of the Na^+,K^+-ATPase (80 ± 3 nmol P_i/mg of protein/min). Partial reversal of the cell volume (by rewarming the swollen slices in 16 m*M* K^+ medium) produces an intermediate decrease in the level of Na^+-ATPase activity. Complete reversal of

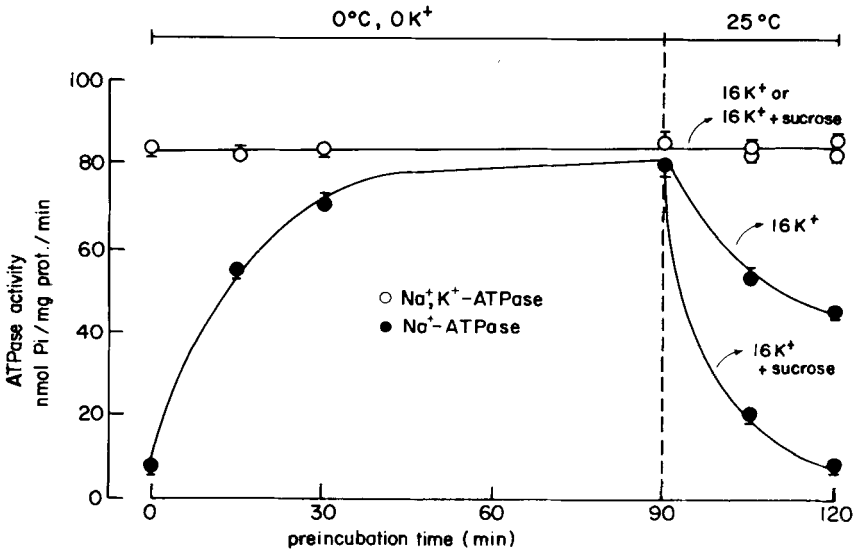

FIG. 3. Na^+, K^+-ATPase and Na^+-ATPase activities of homogenates of rat kidney cortex slices incubated as indicated in Fig. 2. For details of the experimental technique see Whittembury and Proverbio (1970) and Proverbio *et al.* (1986). Values are expressed as means ± SE (n = 6).

the cell volume (by rewarming the swollen slices in 16 m*M* K^+ + 100 m*M* sucrose medium) returns Na^+-ATPase activity to control values (i.e., those of freshly prepared slices).

B. Characterization of "Activated" Na-ATPase

Homogenates from swollen slices ("activated" Na^+-ATPase) were utilized to study the characteristics of Na^+-ATPase activity. All of the tested characteristics were found to be strictly similar to those shown in Table III for the "not activated" Na^+-ATPase. These results constitute a clear indication of the presence of one enzymatic system, working under different "states of modulation." This state of modulation is determined by the relative volume of the cells when the tissue is homogenized.

VI. CONCLUSIONS

The results presented in this chapter involving studies of Na^+ transport (slices, inside-out vesicles), oxygen consumption (slices), and ATPase activities (slice homogenates, purified basolateral plasma membranes, inside-out vesicles) clearly indicate that in addition to the Na^+–K^+ pump, the basolateral plasma membranes of rat kidney proximal tubular cells possess a second sodium pump with different characteristics. This sodium pump extrudes Na^+ actively from the cells along with Cl^- and water. It does not require K^+, is insensitive to even 10 m*M* ouabain, and is inhibited by 2 m*M* ethacrynic acid or furosemide.

The results presented in this chapter, indicating a clear modulation of the Na^+-ATPase activity by cell volume, strongly suggest that this system plays an important role in cell volume regulation. However, it is not easy to explain how changes in cell volume can modulate this activity. A modulator attached to the basolateral plasma membrane, able to detect changes in cell volume (perhaps through changes in plasma membrane conformation or architecture), could be responsible for this effect. Experiments designed to clarify this point are in progress. While the physiological importance of this ouabain-insensitive Na^+ transport system has yet to be established, this mechanism might function to readjust cell volume to normal when perturbed by transport stress.

Finally, it is important to mention that ouabain-insensitive Na^+-ATPase activity has been shown to be present in several other tissues—for example, gills of squid (Proverbio *et al.*, 1988), rainbow trout (Pfeiler, 1978; Pfeiler and Kirschner, 1972), and sea bass (Borgatti *et al.*, 1985); rat and guinea pig small intestine (Luppa and Müller, 1982; del Castillo

and Robinson, 1985a,b; del Castillo and Whittembury, 1987; Tosco *et al.*, 1988); and rat myocardial sarcolemma (Scelza *et al.*, 1987).

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Chapter 7

Rapid Stimulation-Associated Changes in Exocrine Na^+,K^+-ATPase Subcellular Distribution

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I. INTRODUCTION

Many of the fluid-transporting epithelia sustain abrupt changes in Na^+ entry rates during their normal daily patterns of activity. In the small intestine (Hudson and Schultz, 1984) and in the gallbladder (Moran *et al.*, 1986), the rate of pumping by the basolateral membrane localized Na^+,K^+-ATPase increases to match increases in the rate of Na^+ entry. Since steady-

state cytosolic Na^+ activities remain essentially unchanged, the cell must in some way be capable of sensing changes in Na^+ influx rate and adjusting theNa^+,K^+-ATPase pump parameters appropriately (see Chapter 2 by Schultz, this volume). The mechanisms underlying this phenomenon of homocellular regulation remain largely matters of conjecture.

Current concepts about the mechanism of NaCl secretion in exocrine gland acini suggest key roles for coupled Na^+–Cl^- entry and for Na^+,K^+-ATPase-driven Na^+ extrusion (Silva *et al.*, 1977). Since transepithelial flux rates and Na^+ influx rates can change rapidly in response to increased levels of hormonal or neural stimulation, it seems likely that exocrine acinar cells would also possess efficient homocellular mechanisms for regulating their cytosolic Na^+ activities. The thesis of this chapter is that the recruitment of Na^+, K^+-ATPase pump units mobilized from preformed cytoplasmic pools is an important aspect of this process. We will begin by reviewing the roles of coupled Na^+–Cl^- influx mechanisms and of Na^+,K^+-ATPase in exocrine secretion and by summarizing published evidence that a variety of cell types contain substantial cytoplasmic pools of Na^+,K^+-ATPase. We will then summarize a subcellular fractionation analysis of the rat exorbital lacrimal gland which indicates that the cytoplasmic pools of Na^+,K^+-ATPase are concentrated in the Golgi complex. Finally, we will review the use of analytical cell fractionation methods in a direct demonstration of stimulation-associated redistribution of Na^+,K^+-ATPase between cytoplasmic structures and the basolateral plasma membranes of lacrimal gland acinar cells.

II. ROLE OF Na^+,K^+-ATPase IN SODIUM CHLORIDE SECRETION

Na^+,K^+-ATPase functions in concert with several other transporters to produce the NaCl-rich primary secretions that are characteristic of the acini of many of the mammalian exocrine glands. Na^+,K^+-ATPase is expressed in the acinar cell basolateral plasma membranes in parallel with coupled Na^+–Cl^- entry mechanisms (Silva *et al.*, 1977) and K^+ channels. Na^+,K^+-ATPase maintains high cytoplasmic K^+ activities and low Na^+ activities by exchanging intracellular Na^+ for extracellular K^+. The coupled Na^+–Cl^- entry mechanisms use the energy of the inwardly directed Na^+ electrochemical potential gradient to accumulate Cl^- at activities well above those predicted for electrochemical equilibrium. The K^+ channels provide an exit pathway for K^+ taken up by Na^+,K^+-ATPase. The acinar cell apical membranes are believed to contain Cl^- conductance pathways which, when activated, would allow Cl^- to exit the cell and generate a lumen-negative transepithelial potential. Finally, the para-

cellular pathway is presumed to have sufficient cation permeability to sustain a secretory flux of Na^+ equivalent to the net transepithelial Cl^- flux (Silva *et al.*, 1977).

One can easily imagine a mechanism in which the transepithelial electrolyte flux rate is regulated at the level of the apical Cl^- conductance pathway. In this simple scheme, any secretagogue-dependent increase in the apical Cl^- conductance would be accompanied by a decrease in cytosolic Cl^- activity. Decreased cytosolic Cl^- activity would, in turn, increase the driving force for electroneutral Na^+–Cl^- entry. Any increase in cytosolic Na^+ activity would increase the Na^+,K^+-ATPase pump rate, and the consequent increase in cytosolic K^+ activity would accelerate K^+ efflux via the basolateral K^+ channels. There is now good evidence that secretagogues accelerate the efflux of Cl^- from exocrine acini (Martinez and Cassity, 1985; Saito *et al.*, 1985; Melvin *et al.*, 1987). Marty *et al.* (1984) have found that lacrimal acinar cells contain Ca^{2+}-dependent Cl^- channels, and Greger *et al.* (1985) have shown that, in the rectal gland, cyclic adenosine monophosphate (cAMP)-activated Cl^- channels are present in the apical plasma membrane. However, there are also a number of indications that the activities of the other transport pathways in the secretory mechanism are regulated in some way.

The best studied of the regulatory phenomena is activation of the basolateral K^+ channels. The ability of secretagogues to cause K^+ efflux was recognized long before the role of K^+ in the overall secretory mechanism was understood (Burgen, 1956). Recent applications of patch-clamping techniques, reviewed by Petersen (1986), indicate that increases of cytosolic Ca^{2+} activate K^+ channels.

The results of a series of studies with isolated acini, dispersed acinar cells, and intact lacrimal glands indicate that rates of coupled Na^+–Cl^- influx and of Na^+,K^+-ATPase-driven Na^+efflux through the acinar cell basolateral membrane are also regulated. Fluid production by the rat lacrimal gland increases from an unstimulated rate of 0.1 μl per minute to a maximal rate of 1.2 μl per minute upon administration of 10 μ*M* acetylcholine (Alexander *et al.*, 1972). This implies that stimulation should be accompanied by a large increase in the rate of Na^+-coupled Cl^- influx. Precise measurements of Na^+ and Cl^- unidirectional flux rates have not been published, but time courses of $^{22}Na^+$uptake suggest that the Na^+ influx rate increases 10- to 12-fold following stimulation (Parod *et al.*, 1980). Measurements with ion-selective microelectrodes confirm that, as would be predicted, the stimulation of secretion leads to a decrease in cytoplasmic Cl^- activity, i.e., from 31 m*M* to 20 m*M* (Saito *et al.*, 1985). Na^+-coupled Cl^- influx appears to be mediated largely by a parallel array of Na^+/H^+ antiporters (Parod *et al.*, 1980; Saito *et al.*, 1987; Mircheff *et*

al., 1987) and Cl^-/HCO_3^- antiporters (Lambert *et al.*, 1988), so the driving force for entry should be independent of any change in membrane potential difference or cytosolic K^+ activity. The stimulation-associated decrease in cytosolic Cl^- activity would appear to be too small to account for the greater than tenfold acceleration of Na^+ influx. In fact, because the cytoplasmic Na^+ activity increases from ~6 m*M* to 14 m*M* upon stimulation (Saito *et al.*, 1987), the driving force for electroneutral Na^+–Cl^- influx actually decreases rather than increases. The regulatory mechanisms underlying the acceleration of coupled Na^+–Cl^- influx are not known, but examples of the regulation of Na^+/H^+ antiport in several other cell types (Grinstein and Rothstein, 1986) suggest the possibility that the lacrimal Na^+/H^+ antiporters might be activated by messengers of the phosphatidylinositol cascade.

The sustained secretagogue-dependent elevation of cytosolic Na^+ activity contrasts with the transient nature of the Na^+ activity increases that have been observed when transepithelial Na^+ fluxes were stimulated in the *Necturus* small intestine (Hudson and Schultz, 1984) and the rabbit gallbladder (Moran *et al.*, 1986). However, as in the absorptive epithelia, it appears that increased saturation of the Na^+,K^+-ATPase in place in the membrane might not be sufficient to account for the necessary acceleration of the Na^+,K^+-ATPase pump rate. According to the empirical relationship reported by Lewis and Wills (1983), the observed change in lacrimal acinar cytosolicNa^+ activity should increase the pump rate from 0.07 J_{max} to 0.50 J_{max}. This sevenfold acceleration would compensate for 60–70% of the secretagogue-induced increase in the Na^+ influx rate. It seems possible, then, that some additional mechanism operates to further accelerate the Na^+,K^+-ATPase pump rate.

In principle, two different kinds of mechanisms have been found to be involved in regulating transport through cell membranes: activation of transporters already in place in the plasma membrane and insertion of additional transporters which have been mobilized from cytoplasmic pools. The next sections will review several lines of evidence which have pointed to the conclusion that various cell types contain substantial cytoplasmic pools of Na^+,K^+-ATPase, such as would be required for a recruitment mechanism.

III. FUNCTIONAL AND IMMUNOCYTOCHEMICAL EVIDENCE FOR CYTOPLASMIC Na^+,K^+-ATPase POOLS

A. Indirect Suggestions

A number of observations in the literature could be explained satisfactorily in terms of the existence of cytoplasmic pools of Na^+,K^+-ATPase.

For example, prolonged stimulation frequently leads to the formation of watery vacuoles in the apical cytoplasm of secretory epithelial cells (Mills and Quinton, 1981; Leslie and Putney, 1983), and Quinton (1981) has already suggested that this phenomenon might reflect the response of a vacuolar membrane-associated Na^+,K^+-ATPase to secretagogue-induced acceleration of Na^+ influx. More recently, Paccolat *et al.* (1987) noted a disparity between aldosterone-induced changes in, on one hand, Na^+,K^+-ATPase subunit biosynthetic rates and transcellular Na^+ fluxes and, on the other hand, total immunochemical pools of Na^+,K^+-ATPase activity. Within 15–24 hr after 100 n*M* aldosterone is added to A6 cells, short-circuit currents increase up to tenfold, and rates of $Na+,K^+$-ATPase α- and β-subunit synthesis increase up to fourfold. In contrast, the total immunochemical pools of the Na^+,K^+-ATPase subunits do not change significantly during the response to aldosterone. This result could be explained if the basolateral membrane-expressed pool represents a small fraction of the total Na^+,K^+-ATPase of the cell and if, in addition to increasing the Na^+,K^+-ATPase biosynthetic rate, aldosterone increases the fraction of the total which is expressed in the basolateral membranes.

B. Pools Involved in Na^+,K^+-ATPase Turnover

The first clear-cut indication that cells must possess cytoplasmic Na^+,K^+-ATPase pools was published a number of years ago, when Vaughan and Cook (1972) reported that HeLa cells are capable of replacing Na^+,K^+-ATPase pump units that have been inactivated by ouabain. When HeLa cells were incubated with 1 μ*M* [^{3}H]ouabain long enough to allow binding to 60% of the available sites, incubated in ouabain-free medium for 5 hr, then reexposed to [^{3}H]ouabain, they bound an additional amount equal to 100% of the total initial number of [^{3}H]ouabain binding sites. Thus, it appeared that the cells had inserted a new set of ouabain binding sites into their surface membranes. Subsequent studies indicated that surface-bound [^{3}H]ouabain was internalized and accumulated in lysosomes, from which it was slowly released (Cook *et al.*, 1982).

The simplest interpretation of the [^{3}H]ouabain binding data is that the Na^+,K^+-ATPase subunits turn over in a cycle of internalization, degradation, and replacement by newly assembled peptides (Cook *et al.*, 1982). The alternative interpretation is that the Na^+,K^+-ATPase units recycle, releasing [^{3}H]ouabain into the lysosomes before returning to the plasma membranes. In the first case, the cells should contain newly synthesized peptides in transit to the plasma membranes, as well as recently internal-

ized peptides in transit to the lysosomes. In the second case, several different cytoplasmic compartments should contain the recycling Na^+,K^+-ATPase peptides. Turnover of the Na^+,K^+-ATPase subunits is by now, of course, well-documented (e.g., Paccolat *et al.*, 1987; Bowen and McDonough, 1987); the possibility that the subunits also recycle has not yet been evaluated.

While studies of the turnover of [^{3}H]ouabain binding sites clearly pointed to the existence of cytoplasmic Na^+,K^+-ATPase pools, they could not provide direct information either about the magnitude of these pools or about their subcellular localization. One approach to evaluating the relative magnitudes of the surface-expressed and cytoplasmic pools has been based on the use of antibodies to Na^+,K^+-ATPase.

C. Surface and Cytoplasmic Pools of Na^+,K^+-ATPase Immunoreactivity

Fambrough and co-workers have employed two different immunochemical methods for measuring surface-expressed and cytoplasmic Na^+,K^+-ATPase in cultured cells. The first involves binding of labeled antibodies to fixed cells and fixed cells which have been permeabilized by treatment with saponin. The saponin-dependent increment in antibody binding represents the cytoplasmic pool of Na^+,K^+-ATPase. In cultured chick myotubes, the cytoplasmic pool represented ~60% of the total Na^+,K^+-ATPase (Wolitzky and Fambrough, 1986). Similar measurements indicated that the cytoplasmic pool accounted for ~31% of the Na^+,K^+-ATPase of cultured chick sensory neurons (Tamkun and Fambrough, 1986). The second method involves two sequential steps of immunoprecipitation of pulse-labeled Na^+,K^+-ATPase peptides. In the first step, antibodies are bound to surface-exposed Na^+,K^+-ATPase in intact cells, then, after solubilization, collected on a protein A–Sepharose column. In the second step, Na^+,K^+-ATPase peptides that were not retained by the column, i.e., the peptides that were not expressed in the surface membrane, are immunoprecipitated. Analysis of chick sensory neuron preparations by this method indicated that the cytoplasmic pool accounted for 30–55% of the total Na^+,K^+-ATPase (Tamkun and Fambrough, 1986).

The availability of antibodies to the Na^+,K^+-ATPase peptides is making it possible to study the subcellular distribution of Na^+,K^+-ATPase immunoreactivity; preliminary results with lacrimal acinar cells indicate that the Golgi complex is a major locus (Wood and Mircheff, 1986). Approaches based on analytical subcellular fractionation methods have already provided evidence that this is the case.

IV. DELINEATION OF CYTOPLASMIC NA^+,K^+-ATPase POOLS

A. Subcellular Approach

1. Basic Premises

Subcellular fractionation methods are widely employed for preparative purposes, but the use of rigorous analytical procedures to generate comprehensive pictures of subcellular organization is much less common. Therefore, it is appropriate to briefly review some of the basic premises of analytical subcellular fractionation.

When cells are disrupted, some organelles, such as the mitochondria and the nuclei, remain intact, while others are broken into small fragments, usually in the form of vesicles. The endoplasmic reticulum almost invariably vesiculates, and the plasma membranes and the Golgi complex usually vesiculate to various extents. In principle, it should be possible to resolve any mixture of subcellular particle populations by employing a suitable set of physical separation procedures. The number of different particle populations that will have to be resolved will depend, in part, on the uniformity with which structures such as the plasma membrane and the Golgi saccules are disrupted. It seemed that the analytical problem would be simpler if these organelles were entirely represented either by populations of sheets or by populations of vesicles than if they were represented by mixtures of both sheets and vesicles. Since one of the goals of subcellular fractionation studies was to obtain samples of plasma membrane vesicles to be used for the characterizing transport mechanisms, it seemed appropriate to select cell disruption conditions that would favor complete vesiculation of the plasma membranes. One drawback of this strategy has been that the conditions selected for plasma membrane vesiculation also cause Golgi saccules, lysosomes, and secretory vesicles to fragment and form smaller particles, so that their distinctive morphologies are lost, and their content proteins are released to the soluble phase.

As outlined below, separation procedures are available which are capable of resolving a large number of membrane vesicle populations. These vesicle populations are usually visualized through the activities of various biochemical markers that are associated with them (DeDuve, 1964). Inferences about the subcellular origins of membrane populations of interest can often be drawn on the basis of the spectra of biochemical markers that they contain. However, because of the extensive traffic of material among the endoplasmic reticulum, the Golgi complex, plasma membranes, and lysosomes, very few membrane constituents are strictly localized to one organelle or another, and membrane identifications based

on markers should usually be regarded as working hypothesis, ultimately to be tested against independent criteria.

2. Methods

Many of the membrane vesicle populations are quite heterodisperse with respect to size and, consequently, sedimentation coefficient, so differential sedimentation is used primarily to generate three basic fractions, which represent adjacent windows on a continuum related to membrane sedimentation coefficient. Particles that sediment at 1000 *g* for 10 min include nuclei, intact secretory granules, and connective tissue fragments. Particles that sediment between 1000 *g* for 10 min and 250,000 *g* for 75 min include the plasma membrane vesicles, mitochondria, vesicle populations derived from the Golgi complex, the endoplasmic reticulum, disrupted secretory granules and lysosomes, and various other populations of microsomal vesicles.

Equilibrium density gradient centrifugation is used to focus the mitochondria and microsomal vesicle populations of the high-speed pellet fraction into a few well-defined windows. In practice, it is most convenient to analyze the entire low-speed supernatant fraction on a density gradient in a high-capacity zonal rotor, then to use high-speed centrifugation to harvest the membranes from each density gradient fraction. With the exception of the endoplasmic reticulum vesicles, most of the individual populations of microsomal membranes that have been delineated have reasonably discrete density distributions. On the other hand, a given region of the density gradient might well contain samples of as many as five different membrane populations.

Several less common separation procedures have been used to resolve microsomal membrane populations which equilibrate in the same density window. These include free-flow electrophoresis, density perturbation with digitonin, and phase partitioning (Mircheff, 1983). Affinity-based separations also have been suggested, but for the purposes of comprehensive analytical subcellular fractionation, it seemed more appropriate to concentrate on developing a few continuous separations rather than an extensive series of essentially discontinuous separations. Partitioning in aqueous polymer two-phase systems (e.g., Albertsson *et al.*, 1982) was selected as the most generally useful procedure, since one can often manipulate membrane-partitioning behavior by modifying the composition of the two-phase system.

B. Analytical Fractionation of Rat Exorbital Lacrimal Gland

The strategies outlined above were originally developed in the course of studies with freshly obtained tissue from the rat exorbital lacrimal

gland (Mircheff and Lu, 1984). It has more recently become of interest to begin fractionation analyses with lacrimal tissue samples that are in controlled states of secretory activity. This was provided by the lacrimal gland fragment preparation described by Putney *et al.*, (1978). In the experiments described below, lacrimal gland fragments were incubated for 85 min in a modified Krebs–Ringer bicarbonate (KRB) buffer. Alternatively, they were allowed to equilibrate in KRB buffer for 55 min, then stimulated during an additional 30-min incubation in KRB buffer containing 10 μM carbachol. At then end of the *in vitro* incubation, the fragments were rapidly transferred to ice-cold isolation buffer, homogenized, and subjected to the membrane isolation procedures (Yiu *et al.*, 1988).

1. Interpreting Marker Density Distributions

Figure 1 presents the distributions of several standard biochemical markers among the high-speed pellets obtained by high-speed centrifugation of sorbitol density gradient fractions (Yiu *et al.*, 1988). Most of the succinate dehydrogenase, a marker for the mitochondrial inner membranes, is focused in a well-defined peak centered about fraction 17. The other markers have multimodal distribution patterns. The complexity of those distributions indicates that each of the markers is associated with several membrane populations, which can be distinguished on the basis of differences in their modal densities.

The interpretation of the marker distribution patterns in Fig. 1 must take into account the likelihood that each density window might contain samples of several different membrane populations. The implications of this concept are illustrated by the relationship between galactosyltransferase and Na^+,K^+-ATPase. As a first approximation, the Na^+,K^+-ATPase density distribution might be interpreted in terms of the superposition of the distributions of major Na^+,K^+-ATPase-containing membrane populations centered in windows I, II, and IV, and a minor Na^+,K^+-ATPase-containing population in window VI. Similarly, the galactosyltransferase distribution might be interpreted in terms of the superposition of at least four different galactosyltransferase-containing membrane populations. Two populations, which together account for most of the galactosyltransferase activity, are centered in density windows III and IV; a third population, which also accounts for an appreciable fraction of the galactosyltransferase activity, is centered in density window II; and a fourth population, which represents only a minor fraction of the total activity, appears to be centered in window VI. The same question arises for each window of the density gradient: Does one membrane population contain both markers, or are the markers associated with two different populations that happen to have similar density distributions? Our answers de-

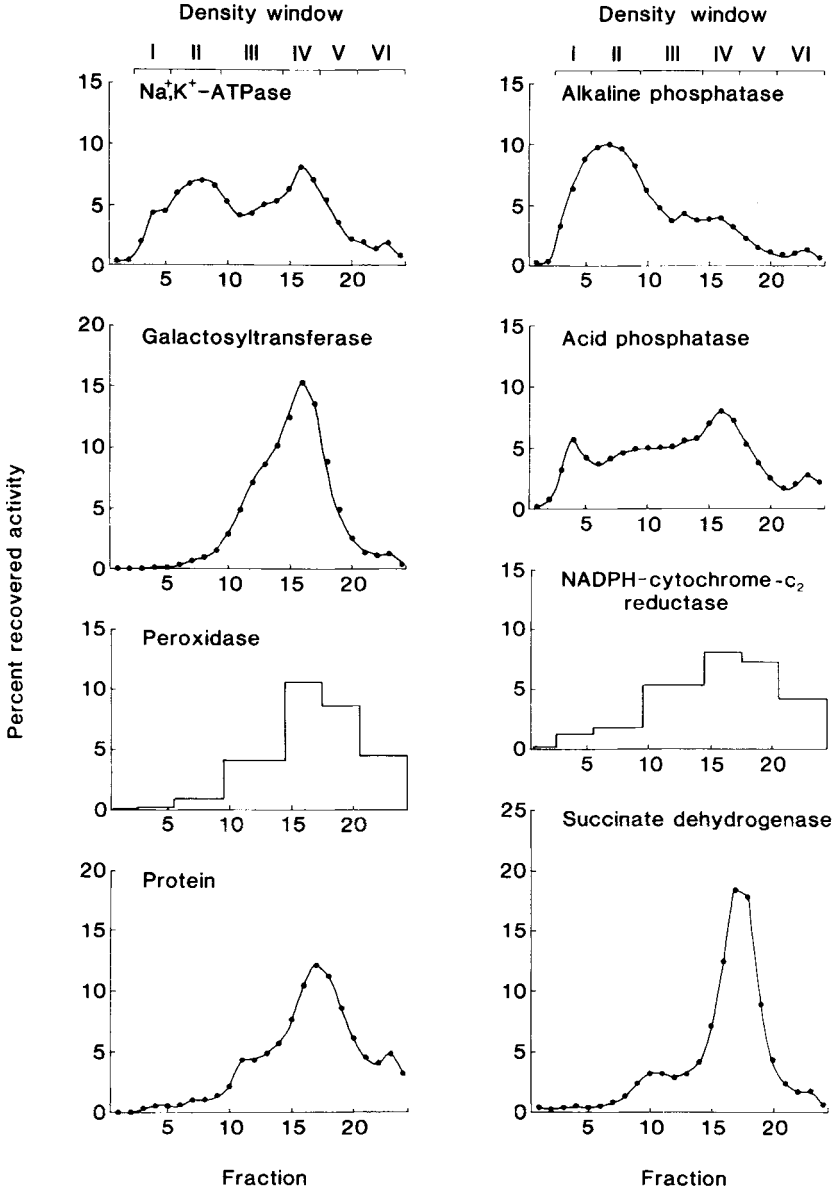

FIG. 1. Density distributions of biochemical markers from lacrimal gland fragments. Values given are percentages of the total activities recovered in the pellets obtained by high-speed centrifugation of the density gradient fractions. A low-speed supernatant fraction was analyzed by density gradient centrifugation in a Beckman Z-60 zonal rotor essentially as described by Yiu *et al.* (1988) and by Mircheff and Lu (1984).

pend on our ability to use phase partitioning to analyze the contents of each density window.

2. Phase-Partitioning Analyses of Density Windows I and II

The marker distribution patterns generated by phase-partitioning analyses (not shown) indicate that density window I is dominated by a single membrane population, and that the biochemical properties measured in density window I are approximately those of its major population. This population, provisionally designated population *a*, has several characteristics which assure us that it has been derived from the acinar cell basolateral membrane. Its cumulative enrichment factor for Na^+,K^+-ATPase is 22, a value that compares well with the enrichment factors of most epithelial basolateral membrane preparations. Its cumulative enrichment factor for alkaline phosphatase, another enzyme frequently found in plasma membranes, is 27. As indicated in Fig. 1, window I is largely free of the mitochondrial and endoplasmic reticulum markers, succinate dehydrogenase and NADPH–cytochrome-c_2 reductase, and the cumulative enrichment factor for the Golgi *trans* saccule marker, galactosyltransferase, is only 1.0.

Like density window I, denisty window II is dominated by a single major membrane population, which we shall provisionally designate population *d*. As in earlier studies (Mircheff *et al.*, 1983; Mircheff and Lu, 1984), phase-partitioning analyses confirm that population *d* accounts for most of the Na^+,K^+-ATPase and protein in window II and that it is also the major locus of the galactosyltransferase activity equilibrating in this density window.[1] Population *d* has a Na^+,K^+-ATPase cumulative enrichment factor of 21. This value is similar to the corresponding value for population *a*, and in earlier studies with fresh lacrimal gland tissue (Mircheff *et al.*, 1983), the Na^+,K^+-ATPase-rich membranes equilibrating in window II were identified as a major basolateral membrane-derived population. However, it has become clear that population *d* differs from population *a* in several important respects. Its cumulative enrichment factors for alkaline phosphatase and galactosyltransferase are 17 and 4, respectively, so that, in comparison with population *a*, it is characterized by a fourfold lower ratio of Na^+,K^+-ATPase to galactosyltransferase and by a sixfold lower ratio of alkaline phosphatase to galactosyltransferase.

We are considering two alternative working hypotheses to account for

[1]Density window II is also the locus of an Na^+,K^+-ATPase- and alkaline phosphatase-containing vesicle population believed to represent the acinar cell apical plasma membrane; this population accounts for extremely small fractions of the marker activities and protein (Mircheff *et al.*, 1983).

the quantitative differences between populations *a* and *d*. The first is that these populations have been derived from distinct microdomains of the acinar cell basolateral membranes. The second is that population *d* has been derived, not from the basolateral membranes, but from a cytoplasmic structure, perhaps a domain of the Golgi complex, which is involved in the assembly, internalization, or recycling of basolateral membrane constituents. The precedent for the second hypothesis is the demonstration by Fishman and Cook (1982, 1986) that each of 7 major plasma membrane-expressed sialoglycoconjugates of HeLa cells has a cytoplasmic pool which is roughly twice as large as the pool exposed at the cell surface. Subcellular fractionation analyses suggest that the cytoplasmic pool is related to the Golgi complex (Fishman and Cook, 1986), and ultrastructural studies of membrane recycling pathways in various cell types support this suggestion (e.g., Farquhar and Palade, 1981, Pastan and Willingham, 1985).

3. Phase-Partitioning Analyses of the Window IV Microsomal Fraction

While windows I and II are each dominated by a single major membrane population, the marker density distribution patterns in Fig. 1 would suggest that density window IV must contain elements of at least three different populations, i.e., mitochondria (marked by succinate dehydrogenase), endoplasmic reticulum membranes (marked by NADPH–cytochrome-c_2 reductase), and Golgi membranes (marked by galactosyltransferase). The mitochondria can be separated from the other membrane populations, which have microsomal sedimentation properties, by differential rate centrifugation through a column of 17.5% sorbitol (Mircheff and Lu, 1984). Initial phase-partitioning analyses of the microsomal fraction from density window IV confirmed that it did, in fact, contain separate endoplasmic reticulum-derived and Golgi complex-derived populations (Mircheff and Lu, 1984). These analyses also revealed the presence of a third population, marked by relatively high contents of acid phosphatase and alkaline phosphatase, whose subcellular origins are unknown. More recent phase-partitioning analyses, performed under a variety of conditions, indicate that the microsomal fraction from density window IV is even more heterogeneous than had been anticipated in the initial studies.

Figure 2 depicts the marker distribution patterns that are generated when the microsomal fraction, at a starting protein load of 0.4 mg/ml in the upper phase, is analyzed in a two-phase system containing 5% dextran T500 and 3.5% polyethylene glycol 8000, pH 8.0. Since the galactosyltransferase dis-

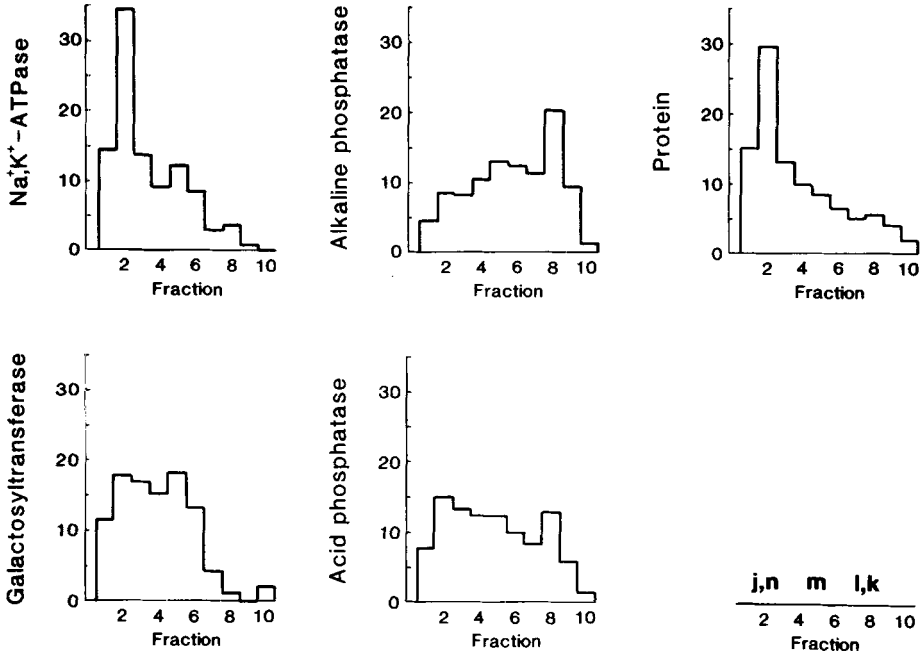

FIG. 2. Phase-partitioning analysis of the microsomal fraction from density window IV. The sample protein load was 0.4 mg/ml in the upper phase of a dextran–polyethylene glycol two-phase system with pH 8.0. A 40-step phase-partitioning analysis was performed essentially as described by Mircheff and Lu (1984). The partitioning fractions were pooled into groups of four and concentrated by high-speed centrifugation prior to marker determinations. The lower right-hand panel gives the approximate positions of the membrane populations that have been delineated. Populations *l* and *k*, which overlap between fractions 7 and 9, can be resolved by phase-partitioning analysis in a phase system with pH 7.3; population *l* contains discernible, but minor, Na^+,K^+-ATPase and galactosyltransferase activities. Population *m* is fairly well resolved from the other populations, while populations *j* and *n* overlap between fractions 1 and 3.

tribution is clearly bimodal, the window IV microsomal fraction must contain at least two different galactosyltransferase-rich membrane populations. One of these, population *m*, which partitions between fractions 3 and 6, is fairly well resolved from the other populations; it is characterized by enrichment factors for galactosyltransferase, Na^+,K^+-ATPase, and alkaline phosphatase of 10.7, 1.6, and 0.9, respectively; it accounts for roughly one half of the galactosyltransferase activity and ~25% of the Na^+,K^+-ATPase activity in the microsomal fraction from density window IV. Of all of the membrane populations that have been resolved from the lacrimal

fragment preparation, population *m* has the largest cumulative enrichment factor for galactosyltransferase; this suggests that population *m* represents *trans* saccules of the Golgi complex.

We can discern the characteristics of the second major galactosyltransferase-rich population by comparing the marker distributions in Fig. 2 with the distributions that are generated when the starting sample protein load is increased to 1.2 mg/ml in the upper phase of the pH 8.0 phase system. As depicted in Fig. 3, increasing the sample protein load causes population *m* to shift farther to the right, so that it is now superimposed on populations *k* and *l*. The other galactosyltransferase-rich population, designated population *n*, has also been shifted toward the right, so that it now overlaps populations *k*, *l*, and *m*. Most of the Na^+,K^+-ATPase is also shifted rightward; thus, ~75% of the Na^+,K^+-ATPase activity of the window IV microsomal fraction is associated with population *n*. The best estimates of the biochemical properties of population *n* are probably ob-

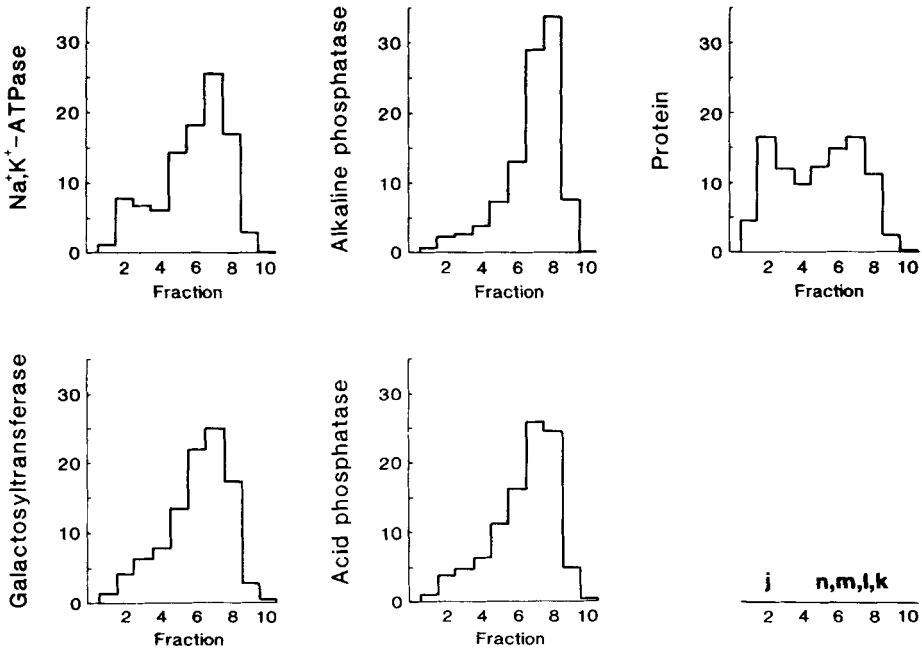

FIG. 3. Phase-partitioning analysis of the microsomal fraction from density window IV. Phase partitioning was performed as in the analysis described in Fig. 2, but the sample protein load was increased to 1.2 mg/ml in the upper phase. Populations *n*, *m*, *l*, and *k* all overlap, but population *j* is now well resolved.

tained by sampling partitioning fraction 5; at this point, the galactosyltransferase, Na^+,K^+-ATPase, and alkaline phosphatase cumulative enrichment factors are 3.0, 1.1, and 0.4, respectively. The excess of galactosyltransferase enrichment over the Na^+,K^+-ATPase and alkaline phosphatase enrichments suggests that population *n* has been derived from the Golgi complex. The relative disparity between the enrichment factors for galactosyltransferase in populations *m* and *n* suggests that population *n* represents some domain other than the *trans* saccules.

The rightward shift of population *n* leaves population *j*, which accounts for ~40% of the protein in the microsomal fraction, relatively well resolved from the other membrane populations. In population *j*, the cumulative enrichment factors for galactosyltransferase, Na^+,K^+-ATPase, and alkaline phosphatase, are 1.4, 0.5, and 0.1, respectively. Furthermore, we have confirmed that population *j* accounts for most of the NADPH–cytochrome-c_2 reductase activity in the microsomal fraction from density window IV (M. E. Bradley, unpublished observations; Mircheff and Lu, 1984). These characteristics confirm that population *j* has been derived from the endoplasmic reticulum.

4. Phase-Partitioning Analyses of the Other Density Windows

Multiple partitioning analyses of density windows III and V under the same set of conditions described above resolve several additional populations which, like populations *m* and *n*, are particularly notable as coinciding peaks in the paritioning distributions of galactosyltransferase and Na^+,K^+-ATPase. Window III contains three different membrane populations that are rich in both galactosyltransferase and Na^+,K^+-ATPase. They differ from each other, and from populations *m* and *n*, with respect to their partitioning behavior in one phase system or another and with respect to their marker cumulative enrichment factors. Window V, which contains elements of population *n* spilling over from window IV, is the locus of two additional Na^+,K^+-ATPase- and galactosyltransferase-containing populations that are not represented in density window IV. Windows III and V also contain elements of the endoplasmic reticulum population, population *j*.

As a group, the Na^+,K^+-ATPase- and galactosyltransferase-containing membrane populations from windows III–V are characterized by relatively high cumulative enrichment factors for galactosyltransferase (ranging from 3.0 to 10.7, with a mean of 5.7), low cumulative enrichment factors for Na^+,K^+-ATPase (ranging from 0.7 to 3.9, with a mean of 1.9), and lower cumulative enrichment factors for alkaline phosphatase (ranging from 0.4

to 2.7, with a mean of 1.2). The most likely explanation for the diversity of the physical and biochemical characteristics of these populations is that they represent different domains of the Golgi complex, or vesicles which mediate traffic between the Golgi complex and other organelles.

5. Divergence between Na^+,K^+-ATPase and Other Basolateral Membrane Constituents

There is a striking divergence between the subcellular distributions of Na^+,K^+-ATPase and other basolateral membrane constituents. As is apparent from the marker density distribution patterns depicted in Fig. 1, the membrane populations which equilibrate in density windows I and II are characterized by a relative excess of alkaline phosphatase over Na^+,K^+-ATPase, while the populations which equilibrate in density windows IV and V are characterized by a marked relative excess of Na^+,K^+-ATPase over alkaline phosphatase. Analyses of the density distributions of a number of other basolateral membrane constituents, including Na^+-amino acid transporters (Mircheff *et al.*, 1983), Na^+/H^+-antiporters (Mircheff *et al.*, 1987), Cl^-/HCO_3^- antiporters (Lambert *et al.*, 1988), and muscarinic acetylcholine receptors (Peters *et al.*, 1986) indicate that the alkaline phosphatase density distribution is the more typical. In other words, Na^+,K^+-ATPase is unique among the basolateral membrane expressed activities surveyed to data in having a large pool associated with membranes related to the Golgi complex.

While the work of Fishman and Cook (1982, 1986) suggests that many plasma membrane constituents may exist in large cytoplasmic pools, there are relatively few examples of Golgi-associated pools of specific plasma membrane constituents. Perhaps the best known of these is the insulin-dependent glucose transporter of adipocytes (Kono *et al.*, 1982). The analogy between the Na^+,K^+-ATPase and glucose transporter subcellular distributions raised in our minds the possibility that the Golgi-associated Na^+,K^+-ATPase pool might represent a reserve that could be mobilized to the plasma membranes to compensate for the acceleration of Na^+ influx that occurs at the onset of stimulated fluid secretion.

V. Stimulation-Associated Recruitment of Na^+,K^+-ATPase to Lacrimal Acinar Cell Basolateral Plasma Membranes

A. Stimulation-Associated Marker Redistributions

Once the Na^+,K^+-ATPase subcellular distribution has been surveyed, the analytical fractionation approach was used to determine whether

Na^+,K^+-ATPase is mobilized from one or another of the cytoplasmic pools and inserted into the basolateral plasma membranes during the response of the lacrimal acinar cell to carbachol stimulation (Yiu *et al.*, 1988). This was done by comparing the Na^+,K^+-ATPase density distributions from resting lacrimal fragments and from fragments that had been stimulated for 30 min with 10 μM carbachol. Release of peroxidase, a macromolecular secretory product of the acinar cell, was monitored as a measure of secretory activity. Addition of 10 μM carbachol accelerated peroxidase release by factors of 10–14, confirming that the stimulus–secretion coupling mechanisms of the cell had remained intact during preparation and *in vitro* equilibration of the lacrimal fragments (Yiu *et al.*, 1988).

1. Na^+,K^+-ATPase

The Na^+,K^+-ATPase density distributions from four resting and four stimulated lacrimal fragment preparations are summarized in Fig. 4. Stimulation for 30 min led to a 41% increase in the Na^+,K^+-ATPase activity of density window I; this difference was significant at the $p < 0.05$ level. Stimulation also led to a highly significant 32% decrease in the Na^+,K^+-ATPase activity of density window V. In a separate series of experiments, 5-min

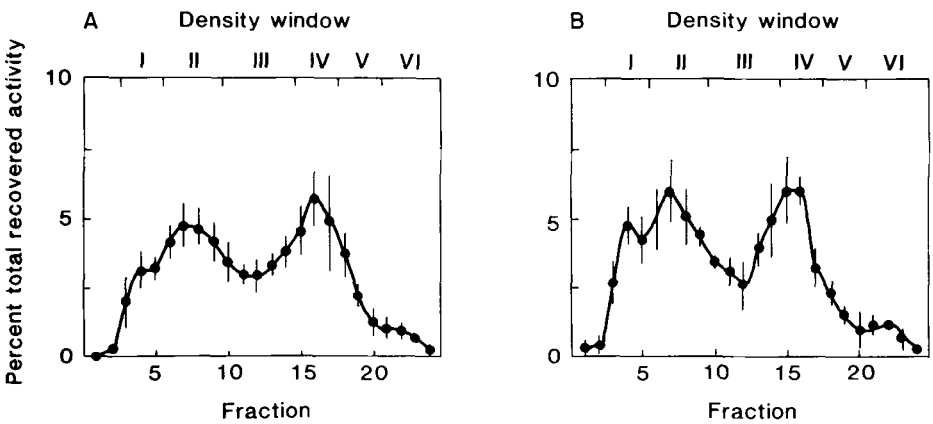

FIG. 4. Density distributions of Na^+,K^+-ATPase from resting (A) and stimulated (B) lacrimal gland fragment preparations. Values given are percentages of the total activities recovered in all fractions generated by the fractionation analysis; bars indicate standard deviations from four replicate experiments. The 30-min stimulation with 10μM carbachol increased the Na^+,K^+-ATPase content of density window I by 41% and decreased the Na^+,K^+-ATPase content of window V by 32%. (From Yiu *et al.*, 1988. Reprinted by permission of Springer-Verlag.)

stimulation after a 55-min equilibration period increased the Na^+,K^+-ATPase activity in density window I 1.3-fold (Lambert *et al.*, 1988).

The stimulation-associated increase in the Na^+,K^+-ATPase activity of window I reflects a redistribution of activity within the density gradient rather than an activation of latent pump units, since the total Na^+,K^+-ATPase activity recovered in all fractions from the stimulated preparations is identical to the total recovered from the resting preparations. The net increase in window I represents 3.5% of the total Na^+,K^+-ATPase activity, while the net decrease in window V represents 2.3% of the total activity. Within the variability of the measurements, these quantities are equivalent, although it remains possible that relatively small changes in the Na^+,K^+-ATPase activity of other fractions might also contribute to the increased activity in window I.

Preliminary phase-partitioning analyses indicate that stimulation causes no change in the number of membrane populations that equilibrate in density window I. Thus, the net increase in the Na^+,K^+-ATPase activity of window I results from the insertion of additional pump units into population *a* rather than the appearance of a new Na^+,K^+-ATPase-containing population. As noted above (Section IV,B,4), the microsomal fraction from density window V contains elements of three different Na^+,K^+-ATPase- and galactosyltransferase-containing membrane populations, all of which appear to have been derived from the Golgi complex. Density window V microsomal fractions from stimulated preparations have not yet been analyzed by phase partitioning, so it is not clear which of its Golgi complex-derived populations represents the source of the Na^+,K^+-ATPase that is recruited to population *a*.

The 40% increase in Na^+,K^+-ATPase activity is accompanied by no change in the alkaline phosphatase activity and a statistically insignificant increase in the protein content of density window I. These results indicate that the redistribution process is selective, i.e., that Na^+,K^+-ATPase is inserted into population *a* in excess of other membrane proteins. Furthermore, the Na^+,K^+-ATPase cumulative enrichment factors in the three Na^+,K^+-ATPase- and galactosyltransferase-rich populations that are represented in density window V, i.e., the group of populations from which Na^+,K^+-ATPase is recruited, are 10- to 30-fold lower than in population *a*. Therefore, the Na^+,K^+-ATPase pump units which are mobilized must be segregated from other constituents of the donor structure before they are inserted into the basolateral plasma membrane.

2. Other Markers

The stimulation-associated recruitment of Na^+,K^+-ATPase to population *a* is selective, but it is not absolutely specific. Stimulation with carba-

chol for 30 min leads to a statistically significant 20% increase in the muscarinic acetylcholine receptor (MAChR) activity of window I evaluated by [^{3}H]quinuclidinylbenzilate binding (Bradley *et al.*, 1987). This redistribution appears to be accompanied by decreases in the MAChR contents of windows II–V which are, individually, too small to be statistically significant.

Translocation of Na^+,K^+-ATPase to population *a* is also, as illustrated in Fig. 5, accompanied by increases in the acid phosphatase contents of density windows I–III. As is the case for Na^+,K^+-ATPase, the change in the acid phosphatase density distribution pattern is the result of movement of activity between fractions rather than of activation of latent enzymes. The source of the acid phosphatase activity which is added to windows I–III appears to be a population of microsomal membranes that remains in the supernatant fraction during high-speed centrifugation of the density gradient fractions (Yiu *et al.*, 1988). Preliminary phase-partitioning analyses of density window I samples indicate that acid phosphatase is inserted into population *a*. We still have to learn whether the acid phosphatase that is added to windows II and III is associated with new membrane populations or inserted into one of the Na^+,K^+-ATPase- and galactosyltransferase-rich or acid phosphatase- and alkaline phosphatase-rich membrane populations that have already been delineated.

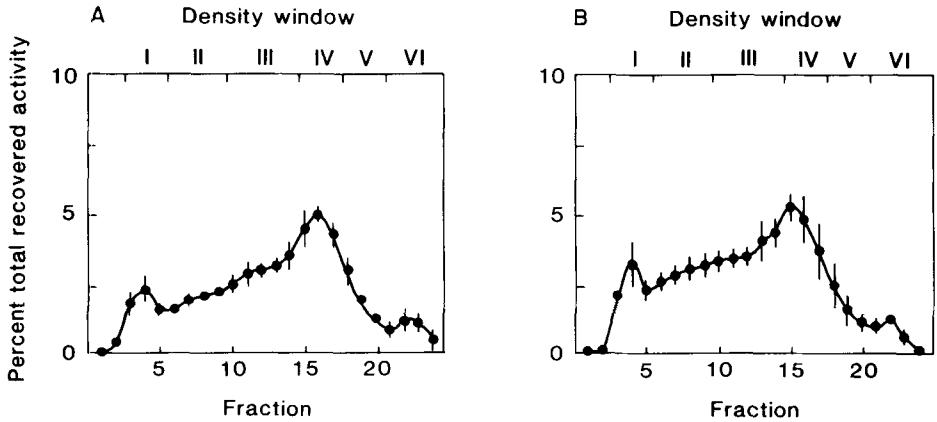

FIG. 5. Density distributions of acid phosphatase from resting (A) and stimulated (B) lacrimal gland fragment preparations. As in Fig. 4, values given are percentages of the total activities recovered in all fractions generated in four replicate fractionation analyses; error bars not shown are smaller than symbols. The 30-min stimulation with 10 μM carbachol increased the acid phosphatase contents of density windows I, II, and III by 33, 50, and 25%, respectively; these increases can be accounted for by decreases in the acid phosphatase contents of the high-speed supernatant fractions of windows IV and V. (From Yiu *et al.*, 1988. Reprinted by permission of Springer-Verlag.)

B. Na^+,K^+-ATPase Recruitment and Membrane Dynamics

Recruitment of Na^+,K^+-ATPase results in a remodeling of basolateral membrane composition that is congruent with the stimulation-induced change in lacrimal acinar cell electrolyte transport function. Depending on whether population *d* has been derived from a cytoplasmic membrane compartment or from a basolateral membrane microdomain which is relatively unaffected by the remodeling phenomenon, the stimulation-associated redistribution of Na^+,K^+-ATPase yields either a 1.4-fold or a 1.2-fold increase in the basolateral membrane Na^+,K^+-ATPase J_{max}. Thus, it would seem that increases in the degree of saturation of the pumps play the major role and that recruitment of additional pumps is a secondary factor in the homocellular regulation of acinar cytoplasmic Na^+ activity.

It is possible that basolateral membrane remodeling occurs in the context of an overall acceleration of basolateral membrane recycling. The basis of this suggestion is the demonstration that stimulation leads to a marked increase in fluid phase endocytosis at the lacrimal acinar cell basal-lateral plasma membrane (Oliver and Hand, 1981; Oliver, 1982). The redistributions of MAChR and acid phosphatase which occur at the same time as, but not in spatial parallel with, the recruitment of Na^+,K^+-ATPase may be hints of the complexity of the traffic between the Golgi complex, other cytoplasmic domains, and the basolateral membranes which is triggered by receptor activation.

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Chapter 8

Differential Subunit and Isoform Expression Involved in Regulation of Sodium Pump in Skeletal Muscle

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I. INTRODUCTION

In a variety of cellular systems, there is evidence that a rise in intracellular sodium ion concentration leads to up-regulation of the sodium pump.

Because of their many experimental advantages, tissue culture systems have played a major role in the development and testing of this concept. Lamb and McCall (1972) and Vaughan and Cook (1972) demonstrated that up-regulation of the sodium pump in HeLa cells could be triggered by partially inhibiting the sodium pump. The rationale was that, as a consequence of sodium pump inhibition, intracellular sodium would rise, triggering up-regulation. Recent work by Pressley and colleagues (1986), using ARL 15 cells, has provided perhaps the most rigorous documentation of the ion changes and time course of up-regulation. Other conditions that should lead to a rise in intracellular sodium—for example, reduction in extracellular potassium ion concentration (Boardman *et al.*, 1974; Pollack *et al.*, 1981; Kim *et al.*, 1984)—likewise result in up-regulation of the sodium pump. For excitable cells, the influx of sodium ions can be augmented by opening the voltage-sensitive sodium channels in their plasma membranes with pharmacological agents. Kim *et al.* (1984) used this method to elicit up-regulation of the sodium pump in cardiac myocytes in tissue culture; Wolitzky and Fambrough (1986) used the same approach to trigger up-regulation of sodium pumps in tissue-cultured skeletal muscle. The molecular basis for sodium pump regulation in skeletal muscle continues to be a major focus of our research.

Recently, it has become clear that the sodium pump is actually encoded in sets of genes, different ones of which are expressed in different tissues. This has greatly complicated analysis of sodium pump regulation, for now one must deal with the problem of multiple isoforms as an aspect of regulation. To this end, our laboratory put aside the analysis of sodium pump regulation in skeletal muscle and turned to an analysis of the genetic basis of the sodium pump and to production of a set of monoclonal antibodies useful for discriminating among molecular forms of each subunit. As an offshoot of this work, we turned to expression systems in which selected sodium pump genes can be expressed and the behavior of the resultant subunits studied. With the information, tools, and new ideas from these studies, we have returned now to analysis of sodium pump regulation in skeletal muscle. This chapter is a progress report.

II. ISOFORMS OF AVIAN SODIUM PUMP α AND β SUBUNITS

In 1979, Sweadner published convincing evidence for multiple molecular forms of the sodium pump α subunit, based on slight differences in electrophoretic mobility and differences in affinity for strophanthidin, and she reported selective expression of the "α^+" form, especially in rat brain (Sweadner, 1979). Subsequent research by Specht and Sweadner

(1984) and by Sweadner and Gilkeson (1985) has further refined these observations. In producing monoclonal antibodies to α and β subunits of the sodium pump, we have found that different monoclonal antibodies recognize different tissue distributions of sodium pumps (Fambrough and Bayne, 1983; Fambrough *et al.*, 1987). Molecular biological studies, especially in rats (Shull *et al.*, 1986a; Hara *et al.*, 1987; Herrera *et al.*, 1987), mice (Kent *et al.*, 1987), humans (Shull and Lingrel, 1987), and chickens (Fambrough *et al.*, 1987) have confirmed and extended the concept of molecular forms (isoforms).

A. α-Subunit Isoforms

1. Structures Deduced from cDNAs

cDNAs encoding the α subunit of the sodium pump have now been cloned and sequenced from humans (Kawakami *et al.*, 1986b), pigs (Ovchinnikov *et al.*, 1986), rats (Shull *et al.*, 1985), fish (Kawakami *et al.*, 1985), and birds (Takeyasu *et al.*, 1988). Only for rats have the nucleotide sequences of cDNAs encoding multiple isoforms been published [αI, αII, and αIII (Shull *et al.*, 1986a); αI and αIII (Hara *et al.*, 1987); αI and most of αIII (Herrera *et al.*, 1987)]. However, there is less direct evidence for a similar set of isoforms in mice (Kent *et al.*, 1987) and in humans (Shull and Lingrel, 1987), and in these cases, it is clear that each α-subunit isoform is encoded by a separate gene. There is still less than unanimity about nomenclature, but it appears that most research groups will adopt a nomenclature in which αI signifies the predominant α-subunit isoform in mammalian kidney and heart, αII signifies the isoform found in abundance in brain and skeletal muscle, and αIII signifies an isoform found principally in brain. In this nomenclature, the published α-isoform cDNA sequences for pigs (Ovchinnikov *et al.*, 1986), humans (Kawakami *et al.*, 1986b), and chickens (Takeyasu *et al.*, 1988) are most similar in sequence to the rat αI isoform.

One of the most important results coming from our molecular biological work on α subunits in the chicken is the identification of a set of α-subunit isoforms that correspond one-for-one with the αI, αII, and αIII isoforms of the rat (Fig. 1). The partial deduced amino acid sequences for α-subunit isoforms (Fig. 1) show some infrequent regions of strong sequence dissimilarity, whereas the overall sequence similarity is exceedingly high. One of these regions of dissimilarity is just N-terminal of the fluorescein isothiocyanate (FITC)-binding site (Lys-507 in rat αI). In this region, each of the rat α-subunit isoforms has quite a different sequence than the others. However, there is high sequence similarity, even in this region, between pairs of chicken and rat α subunits. The same pairwise covariance

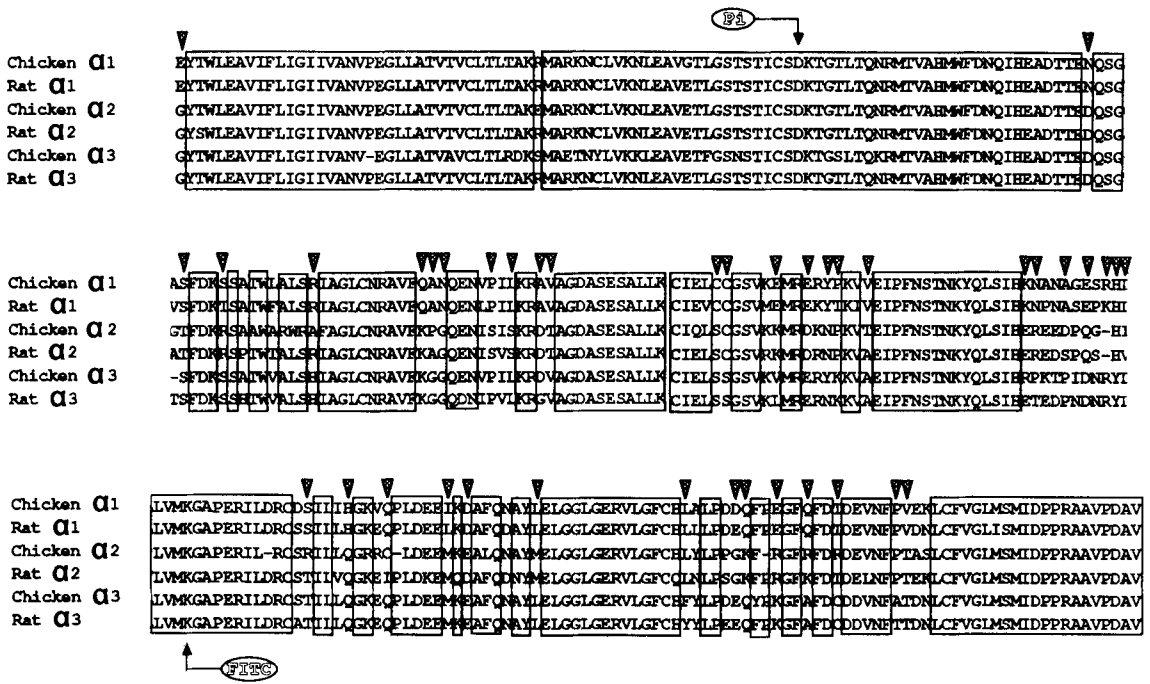


FIG. 1. Amino acid sequence similarities suggest a 1 : 1 correspondence between rat and chicken α-subunit isoforms. Partial amino acid sequences, deduced from nucleotide sequences of encoding cDNAs are presented, including the phosphorylation site (Pi) and the FITC binding site. Regions of sequence identity for all forms are boxed. Positions at which pairs of rat and chicken sequences show selective sequence identity are indicated by crosshatched triangles. In the regions of low sequence similarity, such as the region next to the FITC binding site, there is high sequence similarity between single rat and chicken α-subunit isoforms, consistent with the hypothesis that these similarities indicate cross-species isoform homologies.

from other α sequences occurs at many other positions, indicating strong pairwise similarity that allows one to define αI, αII, and αIII isoforms in the chicken that correspond to the rat α-subunit isoforms.

The chicken α-subunit isoforms are also encoded in separate genes. We have cloned the entire αI gene and a large portion of the αII and αIII genes from chicken genomic libraries. It remains likely that additional α-subunit genes occur in both the chicken and the rat.

There is other evidence for a correspondence between pairs of chicken and rat α-subunit isoforms. Probably most important, the corresponding cDNAs encoding each "pair" of rat and chicken α-subunit isoforms have strong sequence similarities in their 3′ and 5′ untranslated regions. These similarities are unlikely to have arisen from convergent evolution, since these regions should not be under the same evolutionary selection pressure as the coding sequences. Rather, the sequence similarities in coding and noncoding regions of corresponding cDNAs suggest that the set of genes encoding the α-subunit isoforms has existed since before the divergence of mammals and birds. The maintenance of such a gene set through evolution should, then, signify some true selective advantage of this particular gene set for vertebrates. Additional supporting evidence for a vertebrate-wide set of α-subunit isoforms comes from studies on the tissue-specific expression of α-subunit isoforms (see next section).

2. Differential Expression of α-Subunit Isoforms

a. Evidence from mRNA Studies. When isoform-specific cDNA probes are used to detect mRNAs on RNA blots from various tissues, a semiquantitative picture of distribution of expression of α-subunit isoforms is obtained. Table I shows tabulated data from such analyses for the rat (data of Young and Lingrel, 1987) and the chicken. There is a high level of congruence between data for the rat and the chicken, suggesting some biological significance for the tissue-specific isoforms that was important enough to be maintained through at least 300 million years of evolution (the estimated age of the last common ancestor of birds and mammals).

b. Evidence from Immunological Studies. For high-resolution analysis of the distribution of α-subunit isoforms in tissues and even within single cells, antibody rather than cDNA probes are of most utility. Having a set of monoclonal antibodies (mAb) to the α subunit, we employed a bacterial expression system for mapping epitopes to specific α-subunit isoforms. Fragments of each cDNA encoding an α-subunit isoform were cloned in frame into the expression vector pEMBL (Dente *et al.*, 1983) and the plasmids were grown in *Escherichia coli* to yield expression of β-galacto-

TABLE I
COMPARISON OF DISTRIBUTION AND SIZE OF mRNAs ENCODING THE Na^+, K^+-ATPase[a]

Tissue	α-Subunits mRNA						β-Subunit mRNA	
	Rat αI	Chicken αI	Rat αII	Chicken αII	Rat αIII	Chicken αIII	Rat	Chicken
Brain	+ +	+	+ + +	+ + + +	+ + + +	+ + + +	+ + +	+ + +
Skeletal muscle	+	+[b]	+ + + +	+ + +[b]	–	+[b]	+	+
Heart	+ +	+ + +	+	–	–	–	+	+
Kidney	+ + + +	+ + + +	±	–	–	–	+ + + +	+ + + +
Stomach/ gizzard	+	+ +	±	–	±	–	+ +	±
Liver	±	+	–	–	–	–	±	–
Size (kb)	3.7	5.5, 4.0	5.3, 3.4	4.0, 3.8	3.7	4.0, 3.8	2.7, 2.35, 1.8, 1.4	2.5

[a]Relative abundance: + + + +, + + +, + +, +, ±, –. Data concerning rats are from Young and Lingrel (1987).
[b]Only one size was detected.

sidase fusion proteins with each α subunit segment. Colony lysates were generated on nitrocellulose and probed with monoclonal antibodies that were known to react with α subunit on protein blots (Western blots). Figure 2 illustrates some of the results, identifying mAb 7C as αI specific, mAb 5 as reacting with αI and αIII isoforms, and mAb 6B as reacting with all three isoforms (αI, αII, and αIII).

In Fig. 3, cryosections of chicken skeletal muscle tissue have been labeled with fluorescent antibodies, demonstrating the distribution of binding sites for mAbs 7C and 5. At this resolution, it is already clear that an epitope found only on isoform αI is localized at high concentration on peripheral nerve axon and occurs in low abundance in a subset of muscle fibers, whereas an epitope recognized by mAb 5 occurs on α subunits in peripheral nerve axons and all skeletal muscle fibers. These data complement the mRNA blot data and give further information as to cell-type-specific expression of isoforms.

B. β-Subunit Isoforms

Only a single isoform of the β subunit has so far been identified from cDNA cloning. This same cDNA type, defined by sequence similarities in noncoding as well as coding sequences, has been reported for fish (Noguchi *et al.*, 1986), chickens (Takeyasu *et al.*, 1987), pigs (Ovchinnikov *et al.*, 1986), rats (Schull *et al.*, 1986b; Mercer *et al.*, 1986), dogs (Brown *et al.*, 1987), and humans (Kawakami *et al.*, 1986a).

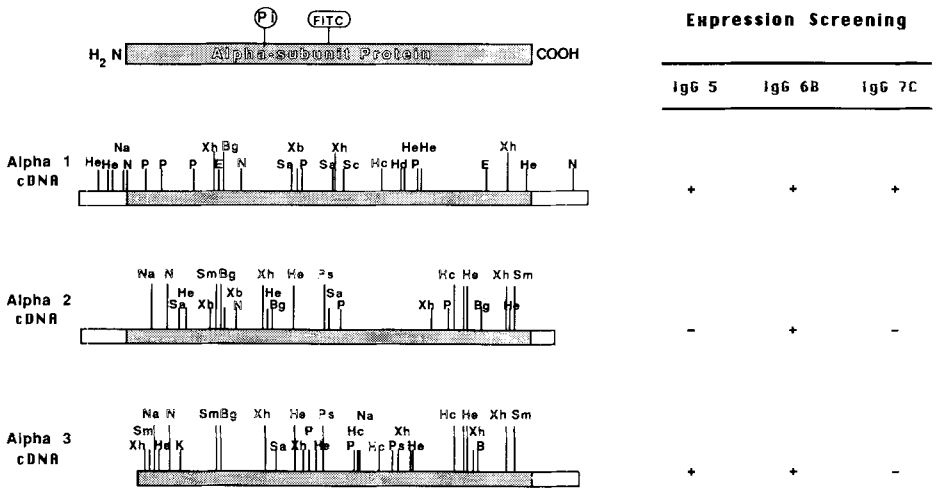


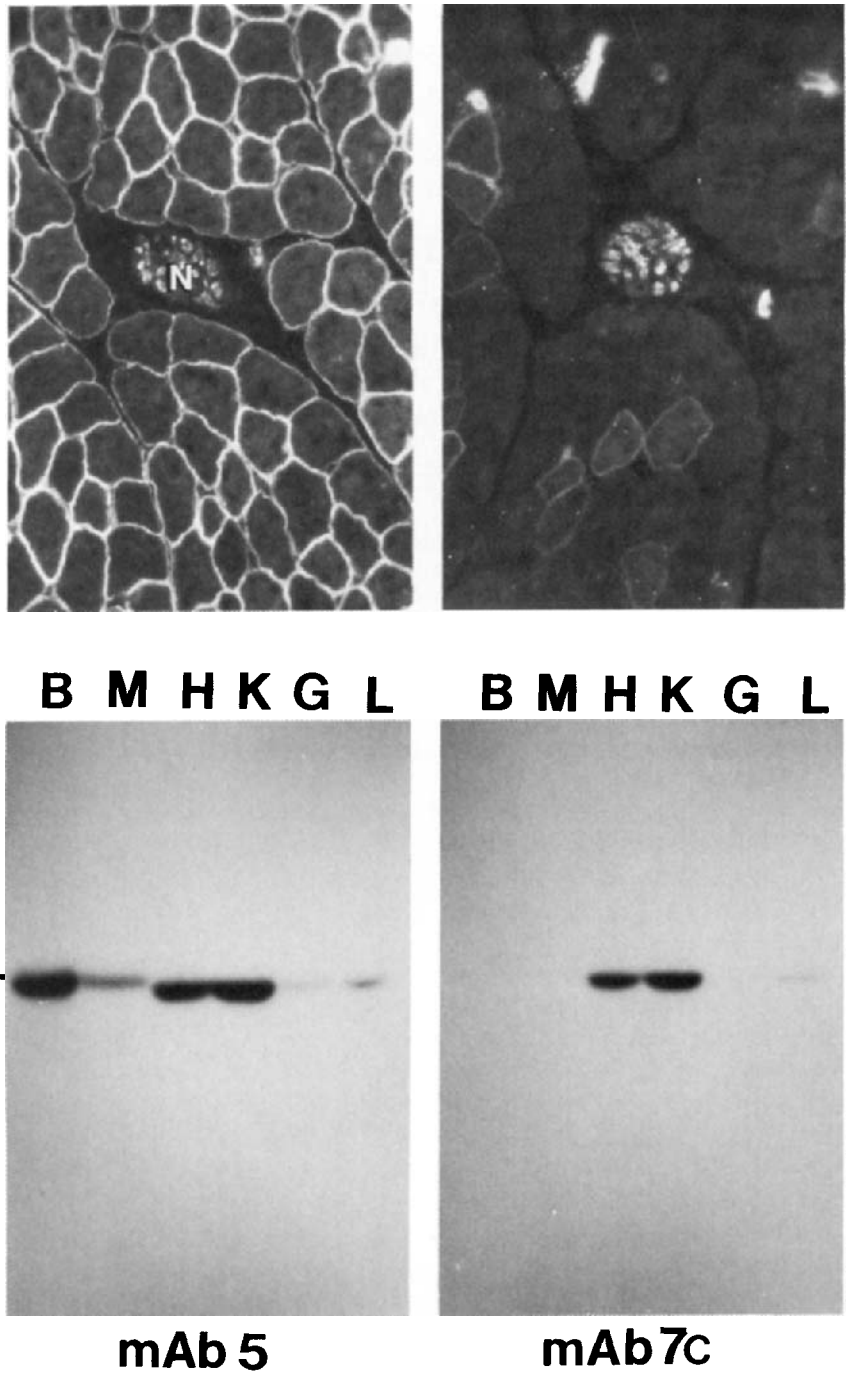
FIG. 2. Mapping restriction sites and encoded epitopes for chicken α-subunit cDNAs. At left are symbolized the three characterized chicken α-subunit cDNAs. The regions encoding α subunits are shaded, and restriction endonuclease sites are indicated (He, *Hae*II; Na, *Nae*I; N, *Nco*I; P, *Pvu*II; Xh, *Xho*I; E, *Eco*RI; Bg, *Bgl*I; Sa, *Sac*I; Xb, *Xba*I; Sc, *Sca*I; Hc, *Hin*cII; Hd, *Hin*dIII; Sm, *Sma*I; Ps, *Pst*I; K, *Kpn*I; B, *Bam*HI).Note the large number of shared restriction sites between cDNAs encoding chicken αII and αIII. At right is a table indicating which of three monoclonal antibodies bind to each of the α-subunits encoded by the cDNA. To obtain these data, each cDNA was expressed either as a full-length cDNA with SV-40 early promoter in mouse cells or as partial-length cDNA fused to the bacterial β-galactosidase gene so that a fusion protein was expressed in *Escherichia coli*.

1. STRUCTURAL STUDIES

Figure 4 illustrates the sequence similarities across several classes of vertebrates, documenting occurrence of stretches of very high sequence similarity, especially in the cytoplasmic and membrane-spanning domains of the β subunit. The only structural evidence for other β-subunit isoforms so far is the mention (without data) of a second N-terminal amino acid sequence for β subunit from the chicken (Takeyasu *et al.*, 1987).

2. DIFFERENTIAL EXPRESSION OF β-SUBUNIT ISOFORMS

Table I gives the relative amount of β-subunit mRNA expressed in different chicken and rat tissue. In each species, the β-subunit mRNA was detected by Northern blot analysis, with cloned cDNA as probe. As we discuss below, this cDNA may detect only one member of a multigene family of β-subunit encoding genes, so the low values may reflect expres-


N
B M H K G L
B M H K G L
α-
mAb 5
mAb 7c

sion in those instances of some other isoform of β-subunit mRNA. One point that is clear from the data in Table I is that there is not a 1 : 1 correspondence in levels of expression of α-subunit isoforms and particular β-subunit isoforms. Rather, the tissue distribution of expression of the cloned β-subunit type is a different distribution than that of any particular α-subunit mRNA. This suggests that some of the molecular heterogeneity in the sodium pump arises from assembly of various combinations of α- and β-subunit isoforms.

Several other lines of evidence suggest that there should be at least three β-subunit isoforms. First, Fambrough and Bayne (1983) demonstrated that chick fibroblasts display $\sim 5 \times 10^5$ ouabain binding sites per cell yet have only about one twenty-fifth as many β-subunit molecules per cell that react with mAb 24 [a monoclonal antibody specific for the β subunit (Tamkun and Fambrough, 1986)]. This suggests the occurrence of a second β-subunit isoform in chick fibroblasts. Second, RNA blot analysis in the rat and in the chick (Table I) shows very low levels of expression of β-subunit mRNA in liver, whereas there is a high level of basolateral expression of β-subunit protein on chicken hepatocytes (R. Baron and D. Fambrough, unpublished observations). Protein blots of membrane proteins fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), probed with different monoclonal antibodies to chicken β subunit, also show a high level of expression of β subunit in liver, but the same antibodies fail to identify a β subunit in chick fibroblasts. Cryosections of skeletal muscle tissue labeled with different anti-β-subunit monoclonal antibodies and examined by immunofluorescence microscopy also display two labeling patterns (Fig. 5).

These observations together suggest the occurrence of at least three isoforms of the β subunit: one well characterized, a second occurring in liver and having some epitopes in common with the first, and a third in fibroblasts that has not been identified with either antibodies or cDNA probes. It remains possible that the β subunit is encoded by a single gene and that the apparent molecular heterogeneity has other explanations. However, it seems much more likely that there is a small gene family encoding the β subunit, just as there is for the α subunit.

FIG. 3. Antigenic heterogeneity of the sodium pump α subunit. (Top) Immunofluorescence labeling of chicken muscle cryosections with monoclonal antibodies. Cross sections of chicken sartorius muscle labeled with monoclonal antibody (as indicated) and fluorescent second antibody. N, Peripheral nerve bundle. (Bottom) Autoradiographs showing binding of monoclonal antibodies 5 and 7C to α subunit on protein blots of membrane proteins from different tissues (brain, muscle, heart, kidney, gizzard, and liver, respectively). Monoclonal antibodies followed by ^{125}I-labeled second antibody were used to identify the α subunit of the sodium pump on protein blots after SDS-PAGE.

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                1                                                  50
Torpedo         MAREKSTDDGGGWKKFLWDSEKKQVLGRTGTSWFKIFVFYLIFYGCLAGIFIGTIQVMLLTISDFEPKYQDRVAPP
Chicken         MARGKAKDGDGNWKKFIWNSEKKELLGRTGGSWFKILLFYVIFYGCLAGIFIGTIQVMLLTVSEFEPKYQDRVAPP
Sheep           MARGKAKEE-GSWKKFIWNSEKKEFLGRTGGSWFKILLFYVIFTGCLAGIFIGTIQVMLLTISEFKPTYQDRVAPP
Human           MARGKAKEE-GSWKKFIWNSEKKEFLGRTGGSWFKILLFYVIFYGCLAGIFIGTIQVMLLTISEFKPTYQDRVAPP
                                                  ———————— M1 ————————

                          100                                                        150
Torpedo         GLSHSPYAVKTEISFSVSNPNSYFNHVNGLKELLKNYNESKQDGNTPFEDCGVIPADYITRGPIEESQGQKRVCRF
Chicken         GLTQVPQVQKTEISFTVNDPKSYDPYVKNLEGFLNKYSAGEQTDNIVFQDCGDIPTDYKERGPYNDAQGQKKVCKF
Sheep           GLTQIPQIQKTEIAFRPNDPKSYMTYVDNIDNPLKKYRDSAQKDDMIFEDCGNVPSELKDRGEFNNEQGERKVCRF
Human           GLTQIPQIQKTEISFRPNDPKSYEAYVLNIVRFLEKYKDSAQRDDMIFEDCGDVPSEPKERGDFNHERGERKVCRF

                                                  200
Torpedo         LLQWLKNCSGIDDPSYGYSEGKPCIIAKLNRILGFYPKPPKNGTDLPEALQANYNQYVLPIHCQAKKEEDKVRIGT
Chicken         KREWLENCSGLQDNTFGYKDGKPCILVKLNRIIGFKPKAPENES-LPSDLAGKYNPYLIPVHCVAKRDEDADKIGM
Sheep           KLEWLGNCSGINDETYGYKEGKPCVIIKLNRVLGFKPKPPKNES-LETYPVMKYNPYVLPVQCTGKRDEDKEKVGS
Human           KLEWLGNCSGLNDETYGYKEGKPCIIIKLNRVLGFKPKPPKNES-LETYPVNKYNPNVLPVQCTGKRDEDKDKVGN

                          250                                                        300
Torpedo         IEYFGMGGVGGFPLQYYPYYGKRLQKNYLQPLVGIQFTNLTHNVELRVECKVFGDNIAYSEKDRSLGRFEVKIEVKS
Chicken         VEYYGMGGYPGFALQYYPYYGRLLQPQYLQPLVAVQFTNLTYDVEVRVECKAYGQNIQYSDKDRFQGRFDIKFDIKSS
Sheep           IEYFGLGGYPGFPLQYYPYYGKLLQPKYLQPLLAVQFTNLTMDTEIRIECKAYGENIGYSEKDRFQGRFRVKIEVKS
Human           VEYFGLGNSPGFPLQYYPYYGKLLQPKYLQPLLAVQFTNLTMDTEIRIECKAYGENIGYSEKDRFQGRFDVKIEVKS
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FIG. 4. Deduced amino acid sequences of β subunits from fish, bird, and mammal samples. The β subunit is presumed to be oriented in the plasma membrane with N-terminal residues 2–35 in the cytoplasm, residues 36–62 spanning the lipid bilayer (————M1————), and the rest of the polypeptide chain on the exterior of the cell. *Torpedo* electric tissue β-subunit sequence is from Noguchi *et al.* (1986), chicken brain sequence is from Takeyasu *et al.* (1987), sheep kidney sequence is from Shull *et al.* (1986b), and human HeLa cell sequence is from Kawakami *et al.* (1986a).

III. ANALYSIS OF SUBUNIT FUNCTION AND REGULATION THROUGH EXPRESSION OF AVIAN SODIUM PUMP cDNAs IN TISSUE-CULTURED MOUSE CELLS

The sodium pump of mouse L cells is relatively resistant to inhibition by cardiac glycosides such as ouabain: In buffered saline with 2 m*M* potassium, $^{86}Rb^{+}$ uptake by the mouse L cell sodium pump is 50% inhibited by $\sim 2 \times 10^{-4}$ *M* ouabain, whereas in avian fibroblasts and myotubes under these conditions, the sodium pump is 50% inhibited at 2×10^{-6} *M* ouabain (Takeyasu *et al.*, 1988). Thus, if functional avian sodium pumps could be expressed in mouse L cells, the avian pump could be identified by its ouabain sensitivity. Expression of avian sodium pump subunits in mouse cells could also be studied with the avian-specific monoclonal antibodies to each subunit.

A. α-Subunit Expression

A cDNA encoding the avian αI subunit isoform was ligated into an expression vector so that expression was under control of the SV-40 early

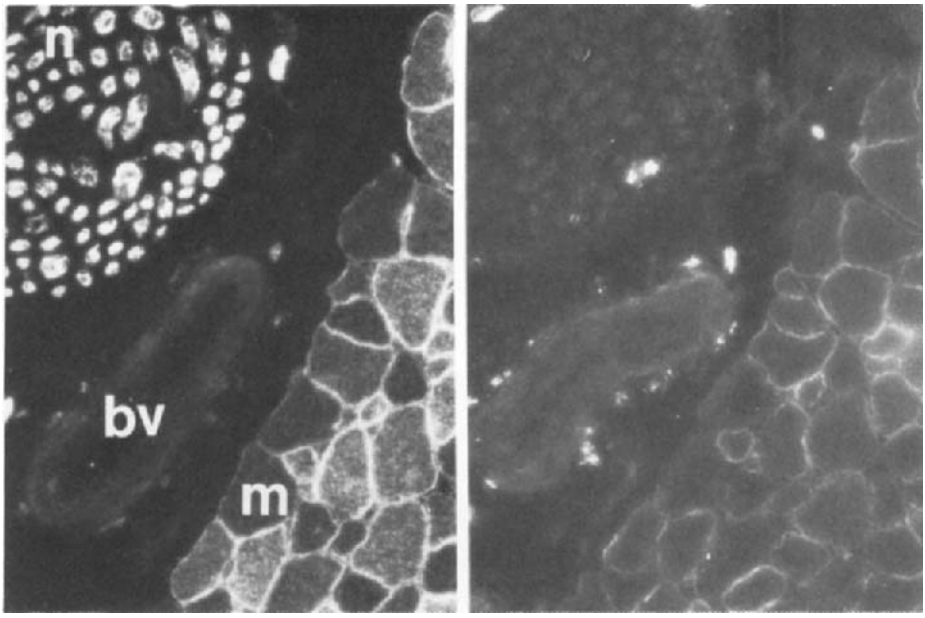

FIG. 5. Antigenic heterogeneity of the sodium pump β subunit. Immunofluorescent labeling of chicken muscle cryosections with mAb 24 (left) and mAb 9 (right). n, Peripheral nerve bundle; bv, blood vessel; m, skeletal muscle fiber.

promoter. This construct was introduced into mouse Ltk^- cells together with the herpes virus thymidine kinase gene by cotransfection by the calcium phosphate precipitation method, and tk^+ colonies were selected in hypoxathine–aminopterin–thymidine (HAT) medium. Clones of mouse L cells expressing the avian sodium pump αI subunit were identified by the following criteria: (1) expression of high-affinity ouabain-binding sites ($K_d = 2.5 \times 10^{-7} M$), (2) expression of $^{86}Rb^+$ uptake sensitive to ouabain ($IC_{50} = 2 \times 10^{-6} M$), (3) expression of a ~100,000 molecular weight polypeptide immunoprecipitable with avian-specific, anti-α-subunit mAb 5, and (4) immunofluorescence labeling with mAb 7C, specific for the αI isoform of avian sodium pump α subunit. These results showed that ouabain sensitivity could be conferred on ouabain-resistant cells simply by expression of an α subunit of a ouabain-sensitive species, and thus implied that the β subunit is not directly of importance in ouabain sensitivity (Takeyasu *et al.*, 1988).

B. β-Subunit Expression

In earlier experiments (Takeyasu *et al.*, 1987), the avian β-subunit cDNA was ligated into the same expression vector and expressed in

mouse L cells in the same manner. In the case of the β subunit, however, the results were somewhat different. Expression was confirmed by demonstration that the selected cells expressed high levels of β subunit immunoprecipitable by avian-specific mAb 24 and by immunofluorescent labeling of the cells with fluorescent monoclonal antibody. Mouse cells expressing high levels of chicken β subunit showed no high-affinity ouabain binding or ouabain-sensitive $^{86}Rb^+$ uptake.

C. Coexpression of α and β Subunits

1. Subunit Assembly

Before discussing subunit assembly in the mouse L cell expression system, a quick review of our earlier work on subunit assembly in chicken myotubes and sensory neurons is in order (Fambrough, 1983; Wolitzky and Fambrough, 1986; Tamkun and Fambrough, 1986). Using monoclonal antibodies specific for the avian β subunit, newly synthesized β subunit was isolated from cells labeled with [^{35}S]methionine in tissue culture and characterized by SDS–PAGE and fluorography. Subunit assembly was defined by the coprecipitation of β and α subunits from detergent solution with monoclonal antibody to the β subunit, since the α subunit should not precipitate unless it was already associated with the β subunit. Rapid pulse and pulse-chase experiments (Fig. 6) demonstrated that subunit assembly is very fast (probably occurring in the endoplasmic reticulum) and precedes the conversion of N-linked oligosaccharides on the β subunit from high-mannose to complex type. In fact, experiments in which tunicamycin was used to prevent N-glycosylation of the β subunit showed that no aspect of glycosylation was necessary for subunit assembly or for transport of the assembled α–β complexes to the plasma membrane (Tamkun and Fambrough, 1986).

When avian α or β subunit is expressed singly in mouse cells, it is likely that interspecies hybrid α–β complexes are formed. This was directly shown for β-subunit expression by demonstrating coprecipitation of mouse α subunit with avian β subunit with anti-β subunit mAb 24 (Takeyasu *et al.*, 1987). Interspecies hybrid complex formation is expected, given the high degree of sequence similarity between β subunits of various vertebrates, particularly in the cytoplasmic and transmembrane domains (Fig. 4).

2. Role of the β Subunit in Sodium Pump Transport to the Plasma Membrane

One striking difference between the α- and β-subunit-expressing mouse cells is the cellular distribution of the avian sodium pump subunit. In the

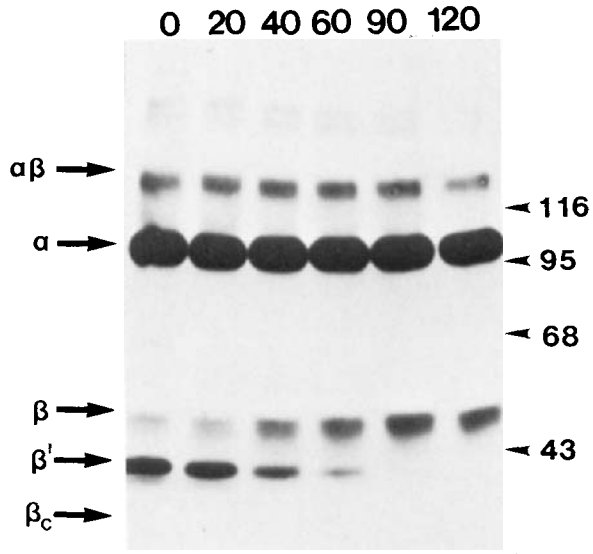

FIG. 6. Biosynthesis and assembly of the sodium pump in chicken skeletal muscle in tissue culture. Cultured myotubes were labeled for 30 min with [^{35}S]methionine and then chased for the indicated times (minutes) in medium with nonradioactive methionine. The sodium pump was purified by immune precipitation with a monoclonal antibody to the β subunit and analyzed by SDC–PAGE and fluorography. The positions of the α subunit, mature β subunit, and high-mannose intermediate form of the β subunit (β′) are indicated at left. Positions of four molecular-weight markers (in kilodaltons) are on the right. The conversion of β′ to the mature β subunit form involves modification of the N-linked oligosaccharide chains, a set of events that occurs in the Golgi apparatus. Coprecipitation of α subunit with β subunit at all time points is evidence for assembly of the subunits early in the biosynthesis/intracellular transport process. β_c, Unglycosylated β subunit. (Data are from Wolitzky and Fambrough, 1986.)

case of α, a large fraction of the molecules remains intracellular, apparently in the endoplasmic reticulum (Fig. 7A), whereas the avian β subunit is localized principally on the plasma membrane (Fig. 7B). This difference could be explained by hypothesizing that transport of the α subunit to the plasma membrane occurred only after formation of α–β complexes. When avian α subunit is expressed in the mouse cells, this α subunit competes with the mouse α subunit for a limited quantity of mouse β subunit. Unassembled avian and mouse α subunit remain in the endoplasmic reticulum. On the other hand, transport of β subunit to the plasma membrane may not require assembly; alternatively, unassembled β subunit may be degraded very rapidly by the cells so that it does not accumulate to any great extent. The important aspect of this hypothesis is that it assigns to the β subunit a role in intracellular transport of the α subunit rather than any direct role in ion transport.

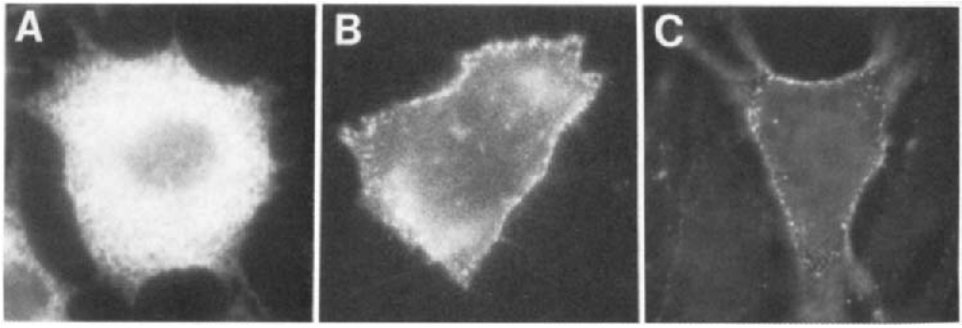

FIG. 7. Expression of avian α subunit (A), β subunit (B), and both α and β subunits (C) in mouse L cell lines following transfection with avian cDNAs. Cell lines selected for expression of the avian subunit were grown on coverslips. The cells were labeled by single or double fluorescent antibody techniques. A single cell is shown in each panel. (A) the α subunit is localized largely in the endoplasmic reticulum, although functional avian sodium pump could be assayed on the surface of such cells (Takeyasu *et al.*, 1988). (B) The labeling is virtually all on the plasma membrane. Cells of this line were shown to assemble avian β subunits with mouse α subunits (Takeyasu *et al.*, 1987). (C) Cells expressing both avian subunits (labeling with anti-α-subunit mAb 7C shown here) have virtually all α–β complexes in the plasma membrane. These data are consistent with a role of the β subunit in intracellular transport of newly synthesized α subunit to the plasma membrane.

To test the possible function of the β subunit in α-subunit transport to the plasma membrane, we have been constructing mouse cell lines expressing different levels of chicken α and β subunits. One of these, constructed by transfecting an α-subunit expression plasmid into a mouse cell line expressing avian β subunit [line Ltk-β3 (Takeyasu *et al.*, 1987)], efficiently transfers virtually all of the α–β complexes to the plasma membrane (Fig. 7C). Other cell lines with various levels of α- and β-subunit expression contain different intracellular pools of avian α and/or β subunits. These preliminary results suggest that not only is the β subunit needed for transport of α subunit to the plasma membrane, but the ratio of α and β and perhaps also the total level of expression affect the efficiency of this transport. Suggestive evidence for a role of β subunit in α-subunit transport to the plasma membrane has also come from studies by Noguchi *et al.* (1988) on expression of *Torpedo* sodium pump subunit mRNAs in *Xenopus* oocytes.

IV. VERATRIDINE-INDUCED UP-REGULATION OF SODIUM PUMP IN PRIMARY MUSCLE CULTURES

Chick embryo myoblasts plated into collagen-coated Petri dishes will undergo one or two rounds of cell devision and then fuse to form multinu-

cleate myotubes. The expression of fusion competence occurs approximately synchronously with the differentiation of myoblasts to express muscle contractile protein and muscle plasma membrane properties. At this time, there is a marked rise in the expression of the sodium pump, measured as binding sites for mAb 24. After a few days, the level of sodium pump expression plateaus at ~2–4 × 10^5 ouabain binding sites on the plasma membrane for each nucleus in the multinucleate myotubes; the number of mAb 24 sites is comparable to the number of ouabain binding sites, suggesting that the β subunit recognized by mAb 24 is the major β subunit in these cells (Fambrough and Bayne, 1983). Recent mRNA characterization in our lab supports the immunological data in indicating that the major β subunit expressed in primary chick muscle cultures is that encoded by the cloned cDNA (Takeyasu *et al.*, 1987) and the major α subunit is the αI type.

A. Kinetics of Up-Regulation and Down-Regulation

In differentiated myotubes, the level of plasma membrane sodium pump can be increased in a dose-dependent manner by maintenance of the myotubes in medium containing veratridine (Wolitzky and Fambrough, 1986). The effect is specific for the differentiated myotubes, because they alone display voltage-sensitive sodium channels. As expected, the veratridine effect, which should be mediated by a stimulation of sodium influx through veratridine-poisoned sodium channels, is completely blocked by blockade of the sodium channels with tetrodotoxin (TTX). The calcium ionophore A23187 did not mimic the action of veratridine, suggesting that sodium rather than calcium ions are important in the stimulus for up-regulation (Wolitzky and Fambrough, 1986). What further steps there are in the signal transduction mechanism remains a central question.

The time course of up-regulation induced by veratridine is illustrated in Fig. 8. There is an initial lag, followed by a period of relatively rapid up-regulation to a new plateau level that is reached after ~24–36 hr. The new level is 60–100% above the basal level. At any time during up-regulation, blockade of sodium influx with TTX causes a down-regulation back to the basal level (Fig. 8). Down-regulation begins quickly and occurs with a half-time of about 3 hr.

B. Mechanisms of Up-Regulation and Down-Regulation

These regulatory responses appear to be effected by rather complex mechanisms. We had supposed that up-regulation would involve elevated biosynthetic rate, reduced degradation rate, or a redistribution of sodium pumps from intracellular storage to surface expression, while down-regu-

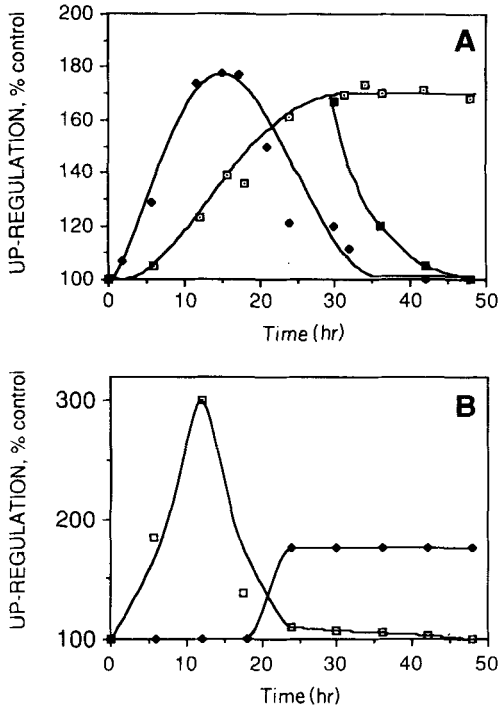

FIG. 8. Kinetics of up- and down-regulation of the sodium pump in tissue-cultured chicken skeletal muscle. (A) Up-regulation was induced by adding veratridine to the medium, and the time course (⊡) was measured as the increase in cell surface binding sites for ^{125}I-labeled monoclonal antibody to the β subunit. Down-regulation (■) was initiated by removing veratridine and blocking the sodium channels with TTX. Relative rates of biosynthesis of sodium pump α–β complexes were assayed by pulse-labeling experiments (◆). (Data are adapted from Wolitzky and Fambrough, 1986.) (B) Levels of α-subunit mRNA (◆) and β-subunit mRNA (□) during up-regulation of the sodium pump in chicken myotubes. Experiments were comparable in design to those illustrated in (A). RNA was isolated at each time point and Northern blots were probed with ^{32}P-labeled cDNAs encoding α and β subunits. Of the α-subunit probes, only those encoding the αI isoform showed significant binding. During the early phase, there is a rise in β-subunit mRNA without any change in α-subunit mRNA.

lation might be mediated by reversal of one of these mechanisms. In fact, the situation is far more complicated. The simplest aspect of this regulation seems to be down-regulation from the up-regulated state.

1. Down-Regulation by Selective Endocytosis

Down-regulation induced in up-regulated myotubes by blockade of sodium ion influx through the voltage-sensitive sodium channels is a rela-

tively rapid phenomenon that occurs without appreciable lag phase. Changes in biosynthetic rate cannot begin to account for it, and only an extremely rapid degradation of a portion of sodium pump molecules could accomplish down-regulation through a degradative mechanism. In fact, quantification of total sodium pump molecules in the myotubes before and after down-regulation reveals no change. The disappearance of cell surface sodium pumps is mirrored by a corresponding increase in intracellular sites (available for mAb binding only after the myotubes are made permeable with saponin). Thus, down-regulation is quantitatively accounted for by a rapid interiorization of a subset of the plasma membrane sodium pump molecules.

The fact that down-regulation brings the system back to the basal level of sodium pump expression in the plasma membrane shows that the myotubes have an uncanny ability to judge the appropriate level of sodium pump expression (even though this level may represent some excess capacity for ion transport). We do not understand this precision, but we can imagine a simple mechanism the cells could possibly use to ensure correct expression level. That is, the cells might, during up-regulation, insert into the plasma membrane a set of sodium pump molecules marked in some chemical way as distinct from the basal population. Then, during down-regulation, these marked molecules could be recognized and selectively removed. While this mechanism is easy to imagine, there is no evidence either for or against this idea.

If down-regulation involves selective interiorization of sodium pumps, can this process be reversed to yield rapid re-up-regulation? The answer appears to be affirmative. Although the experiments are difficult to perform, our preliminary evidence suggests that, after up-regulation and then down-regulation, myotubes stimulated a second time with veratridine re-up-regulate without any lag period and with a rapid time course, approximately mirroring the kinetics of down-regulation. Such behavior would be consistent with retrieval sodium pumps from an intracellular membrane pool.

2. Mechanisms of Up-Regulation

a. Intracellular versus Surface Expression. During up-regulation, there is a large increase in sodium pump molecules in the plasma membrane (Fig. 8). Myotubes also contain a large number of sodium pumps in some intracellular membrane system(s), accounting for slightly more than 50% of total sodium pump molecules (Wolitzky and Fambrough, 1986). During up-regulation, the size of the intracellular pool of sodium pump molecules increases very slightly. It is clear that up-regulation in-

volves somewhat selective augmentation in the population of sodium pumps in the plasma membrane.

b. Accelerated Biosynthesis. During up-regulation of the sodium pump in veratridine-stimulated cells, the rate of sodium pump biosynthesis increases to about double the control rate (Figs. 8 and 9), and this

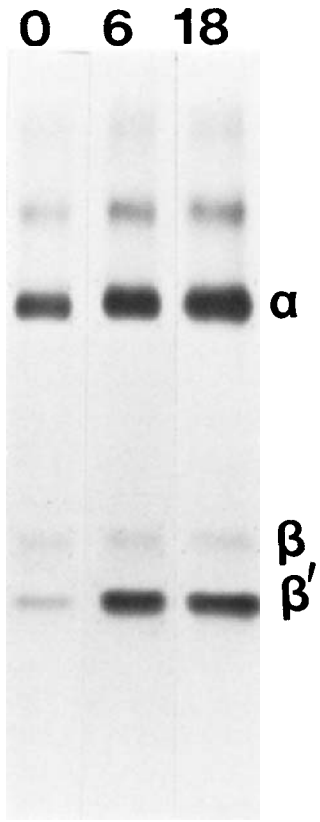

FIG. 9. Evidence for overexpression of β subunit during veratridine induction in chicken skeletal muscle. Myotubes were stimulated with veratridine for 0, 6, or 18 hours. During the last hour of stimulation, the cells were labeled with [^{35}S]methionine. After labeling, cells were rinsed and extracted with detergent solution, and the sodium pump was isolated by immune precipitation with a monoclonal antibody to the β subunit and analyzed by SDS–PAGE and fluorography. During up-regulation, there is an increase in the rate of incorporation of [^{35}S]methionine into both α and β subunits, but there is a disproportionate labeling of the high-mannose intermediate form of the β subunit (β′; see Fig. 6), suggesting overexpression of this subunit, its N-glycosylation in the endoplasmic reticulum, and its accumulation there.

can quantitatively account for the time course and magnitude of the up-regulation response.

These measurements of biosynthetic rate were made by pulse labeling of myotubes with [^{35}S]methionine for 1 hr followed by isolation of immune-precipitable sodium pump (anti-β subunit mAb 24) and quantification of incorporated radioactivity. Analysis of incorporation of radioactivity into α subunits after SDS–PAGE of purified sodium pumps yielded the same results. However, SDS–PAGE and fluorography revealed a second aspect of biosynthesis. During up-regulation, there was a dramatic overproduction of β subunits (Fig. 9) that appeared in the immune precipitates as a high-mannose intermediate form of β (that is, the form found in the endoplasmic reticulum). We interpret this finding as evidence that the myotubes, during up-regulation, overproduce β subunits that do not assemble with α subunits. This finding also suggests that subunit assembly may be an important point of sodium pump regulation in myogenic cells (see Section IV,B,2,e, below).

c. Slowed Degradation Rate. Figure 8 also illustrates the decline in biosynthetic rate that occurs late in the up-regulation response. Myotubes with up-regulated sodium pumps return to approximately basal levels of sodium pump biosynthesis, but now this basal level supports the expression of nearly double the basal number of molecules. This can only be accomplished by compensatory change in sodium pump degradation rate, and this slowing of the degradation rate has been measured quantitatively in pulse-chase labeling experiments (Wolitzky and Fambrough, 1986). The mechanisms that regulate degradation rates of membrane proteins remain virtually unexplored. Consequently, this aspect of sodium pump regulation remains mysterious.

d. Transcriptional Control. Recently, we have been measuring levels of α- and β-subunit mRNAs during up-regulation (J. Taormino, unpublished observations). As expected from the subunit biosynthesis results discussed above, the level of expression of β-subunit mRNA increases severalfold in the early phase of up-regulation, while α-subunit mRNA does not change significantly until much later (Fig. 8B). Transcription rate studies by nuclear run-on suggest that the increased levels of β-subunit mRNA reflect activation of the β-subunit gene.

e. Control of Subunit Assembly. Both the subunit biosynthesis studies (section IV,B,2,b) and the subunit mRNA studies (Section IV,B,2,d) support the hypotheses that sodium pump expression in cultured myotubes is limited in the basal condition by the supply of β subunits and that increased β-subunit production, supported by increased β-gene transcription during up-regulation, permits the assembly of more α–β complexes

and consequently leads to increased numbers of sodium pumps in the plasma membrane.

This hypothesis is consonant with our studies on the expression of avian sodium pump subunits in mouse cells (Section III). If the β subunit is indeed required for transport of α subunits from the endoplasmic reticulum to the plasma membrane, then regulation of β-subunit production should be an effective mechanism for cellular regulation of the sodium pump. This mechanisms is attractive in that it does not require parallel, coordinate regulation of unlinked (Shull and Lingrel, 1987; Kent *et al.*, 1987) α- and β-subunit genes.

V. REGULATION OF SODIUM PUMP IN OTHER SYSTEMS

The sodium pump is a central player in much of cell physiology. Besides its direct roles in setting sodium and potassium ion levels and its electrogenic contribution to the transmembrane potential, the sodium pump is indirectly involved in the host of transport activities that depend on sodium and/or potassium ion gradients and on transmembrane potential. These processes, in turn, have profound effects on cell function, both through their regulation of second messengers, such as calcium ions and protons, and through their roles in intermediary metabolism. In each of the 150 or so types of cells in the vertebrate body, regulation of the sodium pump should be of major consequence. Since each cell has its unique physiology and unique structure, it is actually to be expected that regulatory controls on the sodium pump might be as numerous and nearly as varied as the number of cell types. The structural differences among the different isoforms of each subunit may be crucial to correct regulation. Some of these differences may be related to specific interactions between sodium pump isoform and other cellular components involved in spatial distribution of sodium pumps (for example, basolateral versus apical in polarized epithelia) or involved in modulation of transport rate. In this vein, the work of Lytton and colleagues (Lytton, 1985; Lytton *et al.*, 1985) may be seminal in showing selective regulation of a particular isoform of the sodium pump in the response of adipocytes to insulin.

In summary, the new data on sodium pump diversity, coming from immunological and molecular biological studies, show us important levels of complexity that must be addressed in determining the mechanisms of sodium pump regulation. Not only do these new technologies reveal heretofore unappreciated levels of complexity, but also, fortunately, they make possible new research strategies by which to approach the problems. While advances in cell and molecular biology facilitate research on

sodium pump regulation, it is our hope that discoveries related to regulation of the sodium pump will reciprocally contribute to advancing the fields of cell and molecular biology. Particularly promising possibilities for such a happening are in the areas of membrane protein subunit assembly and membrane protein targeting.

ACKNOWLEDGMENT

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Chapter 9

Transepithelial Sodium Transport and Its Control by Aldosterone: A Molecular Approach

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I. INTRODUCTION

Mineralocorticoid hormones increase sodium reabsorption and promote potassium and hydrogen secretion in a variety of high-resistance (tight) epithelia, such as the distal colon (Bridges *et al.*, 1987) or the distal nephron (Marver and Kokko, 1983). The kidney plays a major role in

extracellular sodium homeostasis. The cortical collecting tubule (CCT) of the mammalian nephron, as well as its counterpart in amphibians, the urinary bladder, are the sites where final adjustment of the ionic composition of the urine takes place. Therefore, one can expect the hormones that regulate hydromineral balance to act at these sites.

Aldosterone, the major steroid hormone modulating Na^+ reabsorption, indirectly regulates the blood pressure. Indeed, primary hyperaldosteronism is frequently associated with hypertension, and hypotension is observed in adrenal insufficiency. The study of the molecular mechanisms involved in Na^+ reabsorption and its modulation by aldosterone is expected to shed some light on the pathogenesis of certain forms of hypertension.

Epithelial cell lines derived from the amphibian kidney (A6 cells) or the urinary bladder (TBM cells), which express a high degree of differentiation in culture and maintain aldosterone responsiveness, represent ideal systems to analyze the mode of action of aldosterone on Na^+ transport. In this review, we shall restrict ourselves to such *in vitro* systems, since more general aspects of the mode of action of aldosterone on Na^+ transport have been reported elsewhere (Garty, 1986; Marver and Kokko, 1983; Morris and Brem, 1987; Rossier *et al.*, 1985; Sariban-Sohraby and Benos, 1986).

II. MECHANISM OF ACTION OF ALDOSTERONE: A MODEL

Twenty years ago, Edelmann and colleagues (Garty, 1986; Marver and Kokko, 1983) proposed a model for the mechanism of action of aldosterone on Na^+ transport. They postulated that aldosterone allosterically activates a cytoplasmic receptor which, in turn, modulates the expression of specific genes. Based on more recent experimental evidence, the model has been refined, and a revised version of the classical model of Edelmann is depicted in Fig. 1 (Truscello *et al.*, 1986). Aldosterone crosses the plasma membrane of epithelial cells sealed by tight junctions. The hormone binds to a soluble receptor located in the cytoplasm. Binding induces a conformational change that allows the hormone–receptor complex to move into the nucleus, to bind to chromatin acceptor sites, and to regulate the expression of specific genes. Analysis by two-dimensional gel electrophoresis of biosynthetically labeled proteins from aldosterone-sensitive tissues has shown that many proteins are induced or repressed by the steroid hormone (Truscello *et al.*, 1986), but almost none have been identified and characterized so far.

In principle, aldosterone could regulate Na^+ transport at three different levels: (1) on the genes encoding the sodium transporters, i.e., the amilo-

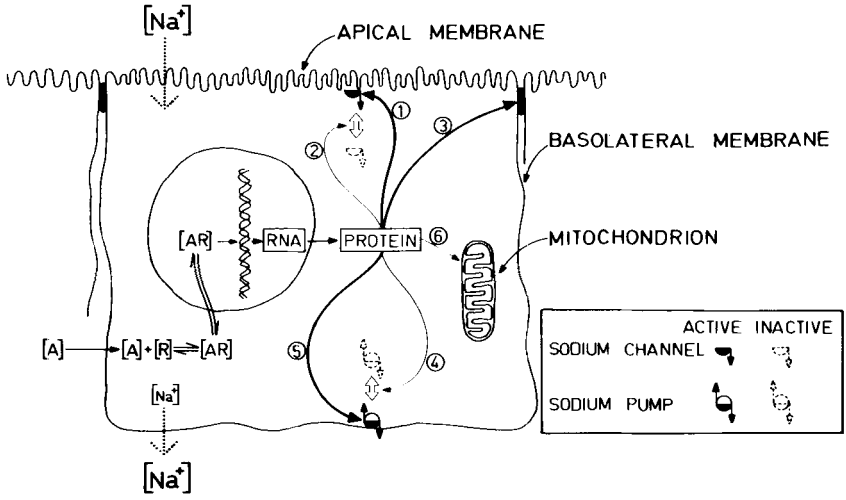


FIG. 1. Model of the mechanism of action of aldosterone. Aldosterone (A) crosses the basolateral plasma membrane of an epithelial cell and interacts with its cytosolic (soluble) receptor (R). Upon binding, the complex (AR) is activated, moves into the nucleus, binds to chromatin acceptor sites, and induces or represses transcription of specific genes. Induced or repressed proteins are, in principle, able to modulate proteins at different sites (sites 1–6). For an explanation, see text.

ride-sensitive Na^+ channel restricted to the apical plasma membrane (site 1) or the ouabain-sensitive sodium pump associated with the basolateral cell surface (site 5); (2) on genes encoding regulatory proteins able to modify the activity of preexisting sodium pumps or channels (sites 2, 4, and 6); (3) aldosterone could modulate the tightness of the epithelium by acting directly or indirectly on the proteins present in the junctional complex and thus influencing the paracellular pathway (site 3). So far, the only aldosterone-induced proteins that have been characterized are the two subunits of Na^+,K^+-ATPase (Geering *et al.*, 1985; Rossier, 1984; Verrey *et al.*, 1987, 1988).

III. CONTROL OF SODIUM TRANSPORT BY ALDOSTERONE *IN VITRO*

A. Toad Urinary Bladder System

In the urinary bladder of the toad *(Bufo marinus)*, aldosterone (0.8–800 n*M*) stimulates Na^+ transport with half-maximal stimulation at ~6.5 n*M*

At low hormone concentrations (0.8–8 n*M*), the increase in Na^+ transport occurs after a latent period of ~45 min, paralleled by a fall in transepithelial resistance. This constitutes the early response (see Fig. 2). At higher hormone concentrations (30–800 n*M*), an additional resistance-independent increase in sodium transport is observed within 2.5–8 hr. This constitutes the late response (Geering *et al.*, 1985). The action of mineralocorticoid hormones is believed, as shown in Fig. 1, to be initiated by stereospecific binding to a soluble receptor. Specific binding sites have been characterized in a number of target cells including those of the toad urinary bladder.

In almost all target cells, two types of receptors have been identified, a high-affinity (0.5–4 n*M*), low-capacity (4–30 fmol mg^{-1} protein) type I receptor and a low-affinity (25–60 n*M*), high-capacity (160–250 fmol mg^{-1} protein) type II receptor (Corvol *et al.*, 1981; Funder, 1985). Occupancy of type I receptor by aldosterone is believed to mediate the Na^+ transport response, and therefore this receptor is also termed the mineralocorticoid receptor. Type II receptor, which exhibits properties of a glucocorticoid receptor, was not thought to be involved in the Na^+ transport response. Recently, however, we have demonstrated *in vitro*, using the toad urinary bladder, that ~50% of the overall sodium transport response was caused by occupancy of type II receptors (Geering *et al.*, 1985). The early response, however, with its typical drop in electrical resistance, requires occupancy of type I receptors. From our data, we conclude that both receptors have to be occupied by aldosterone for a complete Na^+ transport response. For methodological reasons, these observations have not yet been extended to the mammalian CCT.

B. Culture of Sodium-Transporting Epithelial Cell Lines

Cell heterogeneity in the urinary bladder may explain the complexity of the physiological response and account for the presence of distinct aldosterone receptors. The epithelial layer of the *Bufo marinus* bladder consists of five cell types. The granular, mitochondrial-rich, and goblet cells, sealed at their apex by tight junctions, form a continuous layer facing the urinary space (Fig. 3A). The basal and microfilament-rich cells are situated between the upper cell layer and the basal lamina (Kraehenbuhl *et al.*, 1979). Whether cooperation between distinct cell types is required for Na^+ transport and its hormonal regulation has not been established.

The development of aldosterone-responsive amphibian cell lines has allowed examination of the role of different cell types in the regulation of Na^+ transport. The TBM cell line, established by Handler (1983), retains *in vitro*, when cultivated on porous supports, a Na^+ transport response

identical to that observed in the intact bladder. At confluency, the cells form a bilayer. The upper cells with typical tight junctions contain granules similar to those present in the granular cells of the bladder (Fig. 3B). The cells situated between the supports and the superficial cell layer do not form tight junctions, but appear to be chemically coupled to the upper cells. The TBM cells have recently been cloned by limiting dilution. The cells at confluency form a bilayer and maintain their physiological proper-

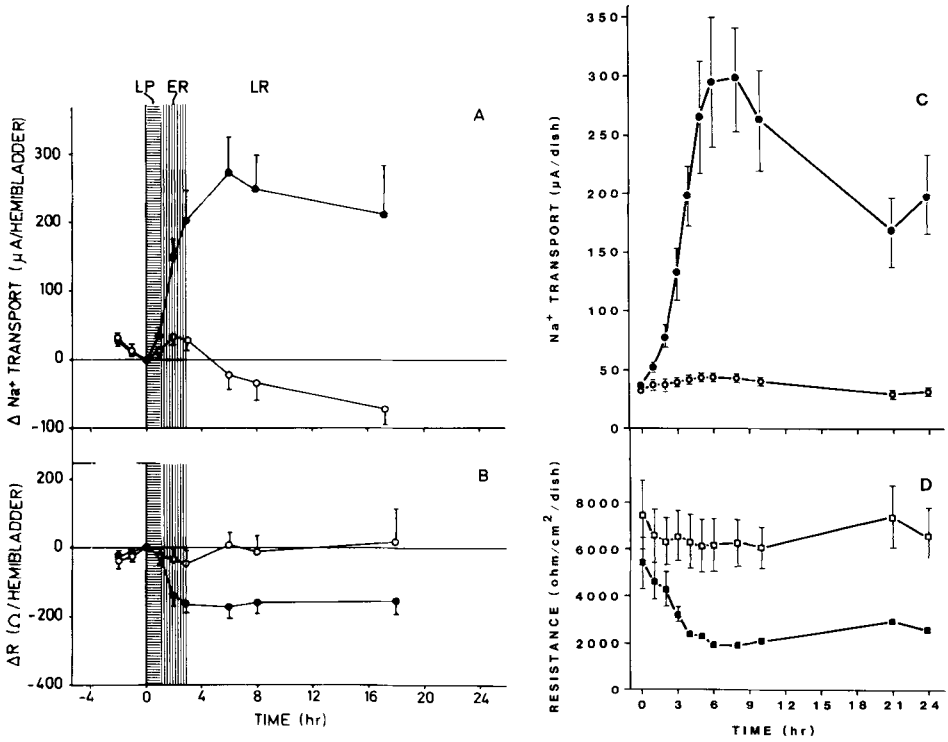
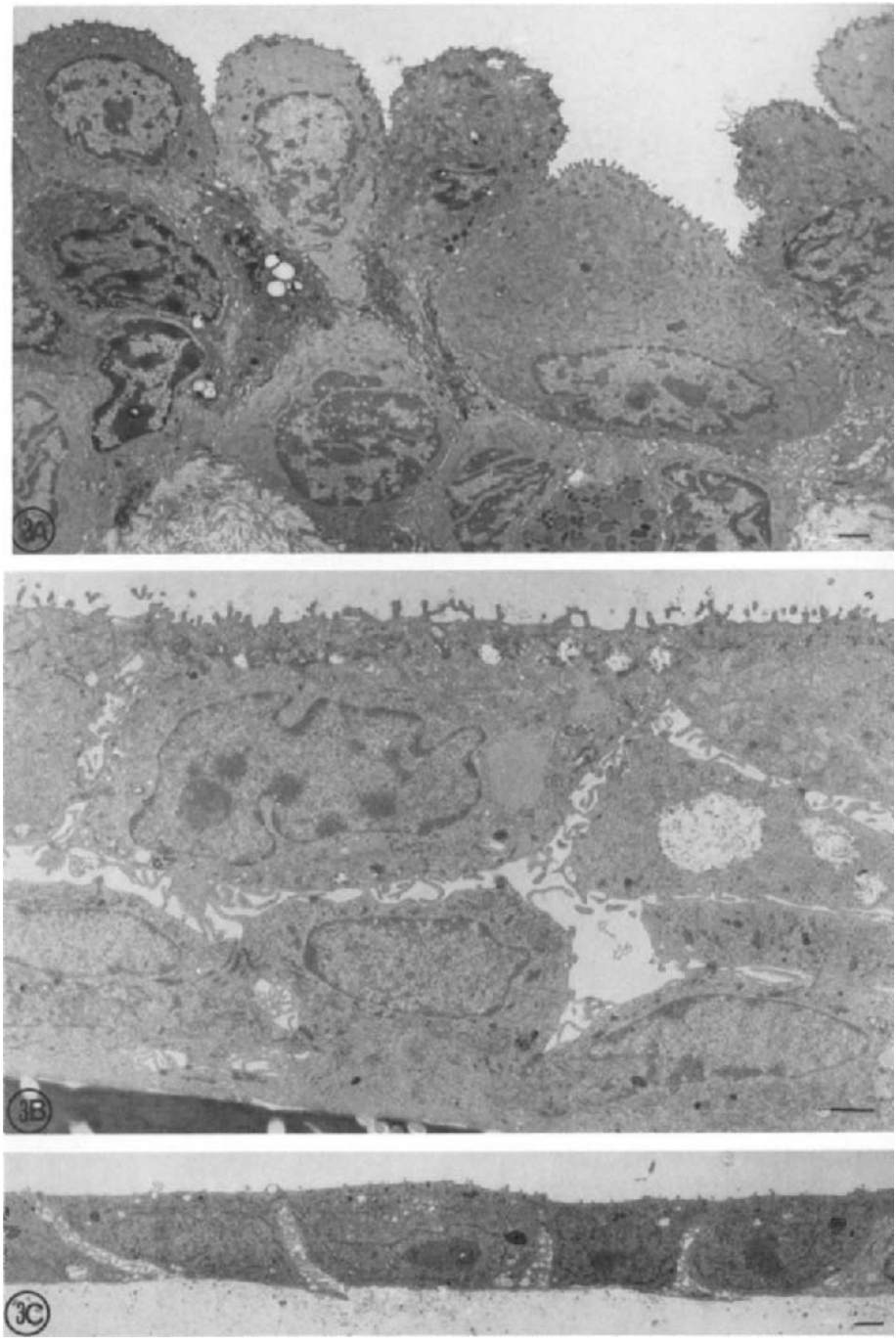


FIG. 2. Effect of aldosterone on transepithelial Na^+ transport. Time course of the effect of aldosterone on transepithelial Na^+ transport (top panels) and electrical resistance (bottom panels) in toad urinary bladders [(A) and (B)] or in TBM cells [(C) and (D)] incubated *in vitro* at 25°C. At time zero, aldosterone (80 n*M*) or the diluent was added (mucosal and serosal sides). (A) and (C): Data are expressed as the mean $\Delta SCC \pm SEM = (SCC_t - SCC_o)$ (expressed in microamperes). Aldosterone (●); control (○). (B) and (D): Data are expressed as the mean $\Delta R \pm SEM = (R_t - R_o)$ (expressed in ohms). (B) Aldosterone (●); control (○). (D) control (□); aldosterone (■). LP, Latent period; ER, early response; LR, late response; SEM, standard error of the mean; SCC, short circuit current; R, resistance; 0, value at time 0; *t*, value at time *t*. For an explanation, see the text.


3A
3B
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ties with characteristic early and late responses to aldosterone, as well as heterogeneity in aldosterone binding sites.

A similar physiological response to aldosterone has been observed in A6 cells, a cell line derived from the kidney of *Xenopus laevis*. In contrast to TBM cells, A6 cells form monolayers at confluency (Fig. 3C). Thus, the complexity of the physiological response and the presence of distinct aldosterone binding sites in A6 cells do not seem to require cooperation between distinct cell types. In TBM cells, cytodifferentiation of clonal cells into two distinct cell types seems to preceed expression of the differentiated phenotype. Whether cooperation between the two cell types is required for function remains to be established.

IV. STRATEGY FOR CLONING SODIUM PUMP AND SODIUM CHANNEL USING ANTIBODY RECOGNIZING A SHARED EPITOPE

A. Purification of Sodium Pump and Preparation of Polyclonal Antibodies

Identification, characterization, and isolation of the genes and the gene products regulated by aldosterone is a prerequisite for understanding in molecular terms how the hormone influences Na^+ transport. In principle, an increase in Na^+ transport induced by aldosterone could result from an increase in the number of channels and/or pumps mediating ion translocation, from their activation, or from both. As a first step to elucidate the mechanism of action of aldosterone, we decided to purify the sodium pump and the Na^+ channel and to isolate their corresponding genes.

FIG. 3. Morphology of Na^+-transporting tissues or cell lines. (A) Electron micrograph of the epithelial layer of the urinary bladder of *Bufo marinus*. The upper layer facing the urinary space consists of granular cells, mitochondria-rich cells, and goblet cells (not shown), which form a continuous sheet with typical tight junctions sealing the apex of each cell. The lower layer consists of microfilament-rich cells and basal cells, which can be distinguished by a distinct microfilament content, as revealed by immunocytochemistry. Bar, 1 μm. (B) Electron micrograph of clonal TBM cells. The cells were plated at low density and grown for 12 days on collagen type I-coated polycarbonate filters. At day 12, the cells transport Na^+ and respond to aldosterone, forming bilayers. The upper layer consists of granular cells, with granules present in the apical subluminal cytoplasm. The cells are sealed by tight junctions, forming a continuous sheet. The lower cells have no granules and no tight junctions. They are reminiscent of the microfilament-rich cells of the bladder. Bar, 1 μm. (C) Electron micrograph of cloned A6 cells. The cells were plated at low density and grown for 12 days on collagen type I-coated polycarbonate filters, at which time they become aldosterone responsive. They form a monolayer with typical tight junctions. The lateral plasma membrane presents numerous interdigitations. Bar, 1 μm.

Since protocols for the purification of the sodium pump were available (Rossier, 1984), we first isolated the amphibian sodium pump and purified its two subunits.

As a source for the enzyme, we choose the kidney, which contains high amounts of this plasma membrane protein (10^6–10^7 molecules per cell, which corresponds to 0.5% of the protein content of the plasma membrane). The catalytic α subunit (M_r 98K) and the glycoprotein β subunit (M_r 40K–60K) from the kidney of *Bufo marinus* were purified to homogeneity. Polyclonal antibodies raised in rabbits against each subunit served to analyze the biosynthesis of Na^+,K^+-ATPase and its regulation by aldosterone. We found that the sodium pump is a late aldosterone-induced protein and that its rate of synthesis is stimulated two- to fourfold by the hormone. The response is mineralocorticoid specific, since the competitive inhibitor spironolactone completely abolishes the response. The effect is mediated by type I receptor and the induction is sensitive to transcription inhibitors, such as actinomycin D and sodium butyrate. Thus, it is likely that aldosterone acts at the transcriptional level. There is no requirement for an increase in intracellular Na^+ consecutive to the opening of the Na^+ channel, because amiloride and its analogs do not suppress the aldosterone-induced increase in synthesis of Na^+,K^+-ATPase (Rossier, 1984).

A similar experiment was performed in the TBM and A6 cell lines (Paccolat *et al.*, 1987; Verrey *et al.*, 1987 and unpublished observations). Since the two cell surface domains, the apical and basolateral plasma membranes, are freely accessible when A6 or TBM cells are cultivated on collagen-coated polycarbonate filters, we examined whether the aldosterone-induced increase in the rate of Na^+,K^+-ATPase synthesis found in the two cell lines was reflected by a parallel increase in sodium pump density at the cell surface. Following selective cell surface radioiodination and immunoprecipitation, we showed that the density of both subunits increased two- to fourfold in the basolateral membrane after a 12- to 24-hr aldosterone stimulation (Ernst *et al.*, 1986). To our surprise, however, the anti-α-subunit antibody precipitated proteins from the apical plasma membrane that were clearly distinct from the Na^+,K^+-ATPase α subunit.

Whereas the pattern of cell surface expression of the two Na^+,K^+-ATPase subunits following aldosterone stimulation was the same in A6 and TBM cells, the apical pattern was different in the two cell lines. In TBM cells, there was a significant increase in the amount of protein recovered after stimulation, whereas, in A6 cells, aldosterone did not alter the pattern. These biochemical data were confirmed immunocytochemically as shown in Fig. 4. The anti-α-subunit antibody weakly labeled the apical cell surface of TBM cells under resting conditions, while the baso-

lateral cell surface remained unstained. Six hours after aldosterone stimulation, there was a marked increase in the labeling density of the apical membrane in some cells. Six hours later, the staining of the basolateral cell surface increased. The apical labeling pattern was not observed in TBM cells only, but it was found in tissues from *Bufo marinus* able to respond to aldosterone. These include the absorptive cells of the distal colon, the granular cells of the urinary bladder (Fig. 5), and the tubular cells of the distal nephron.

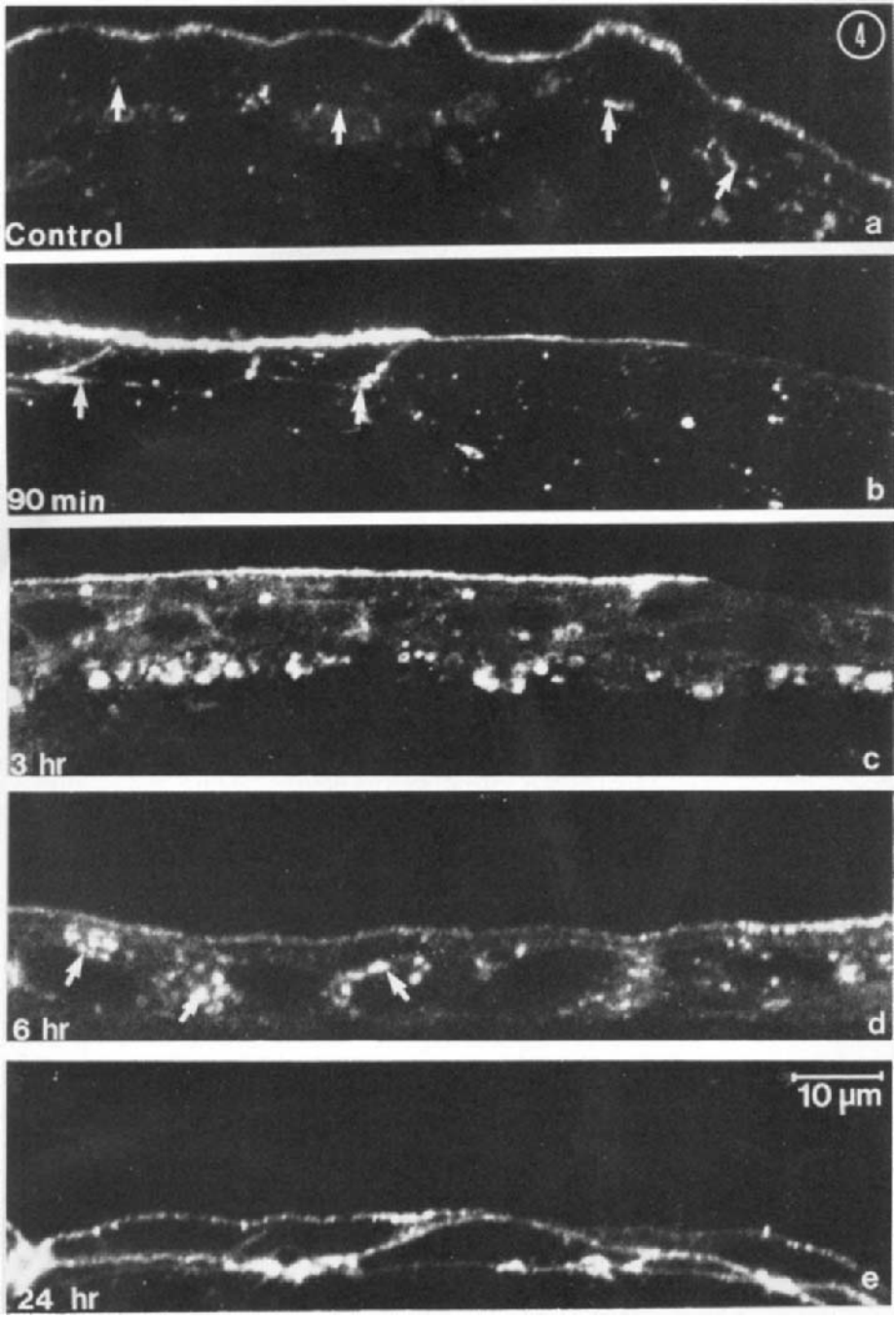
Three possibilities can be envisaged to explain the apical labeling. First, the distribution of the α subunit is uniform over the cell surface, whereas the functional pump consisting of α,β-heterooligomers is restricted to the basolateral membrane. This is unlikely, because the 98 kDa α-subunit polypeptide is recovered from the radioiodinated basolateral membrane and not from the apical membrane (Verrey *et al.*, 1989). Second, the anti-α-subunit serum contains a subpopulation of antibodies directed against a contaminating protein expressed only on the apical membrane of tight epithelia. One has to postulate that the α subunit and the apical protein share common physiochemical properties in order to explain their copurification. Third, the antibody recognizes a common epitope shared by the apical protein and the α subunit of the sodium pump.

Because the distribution of the apical labeling is restricted to those epithelia that express an amiloride-sensitive Na^+ channel, we proposed that the antibody recognizes a structure common to one of the components of the Na^+ channel and to the α subunit of the sodium pump.

B. Identification of Three Distinct cDNA Clones Using Antipump Antibodies and Expression cDNA Libraries

To identify and characterize the antigen associated with the apical plasma membrane and recognized by the polyclonal anti-α-subunit antibody, we screened expression cDNA libraries, constructed from *Xenopus laevis* A6 cell poly(A)$^+$ RNA, with the anti-α- and anti-β-subunit antibodies (Verrey *et al.*, 1989). cDNAs for each subunit were identified and the complete nucleotide sequence and the deduced amino acid sequence were determined.Both subunits show extensive similarity with the corresponding mammalian, avian, and fish sequences. A third cDNA, termed 3a, was identified which, on Northern blot analysis, hybridized to a 5.7-kb mRNA distinct from the 3.9-kb α-subunit mRNA. The partial 0.6-kb cDNA was sequenced and its primary structure was compared to that of the α subunit. Surprisingly, no similarity was detected. This rules out the presence of a common contiguous epitope shared by the two gene products.

Immunological cross-reactivity was also observed with antibodies


Control
a
90 min
b
3 hr
c
6 hr
d
10 µm
24 hr
e

raised against the fusion proteins encoded by the two distinct clones. The antibody directed against the α-subunit fusion protein reacted with the 3a fusion protein and, conversely, the anti-3a fusion protein antibody recognized the α-subunit fusion protein. These data suggest that the 3a and α-subunit proteins share a common discontiguous epitope, the nature of which will become clear once the tridimensional structure of the two proteins is established.

With this new generation of antibodies, we analyzed the biosynthesis and the cell surface distribution of the three gene products (Verrey *et al.*, 1989). We demonstrated that the α and β subunits of Na^+,K^+-ATPase were restricted to the basolateral cell surface, while the third protein (3a) was associated with the apical membrane. In biosynthetic labeling experiments, we found that the 3a gene product was synthesized as a 185-kDa protein which, during maturation along the secretory pathway got associated with several polypeptides (M_r 150K, 130K, 80K, and 55K) (unpublished observation).

A similar complex pattern was observed when apically radiolabeled proteins were immunoprecipitated with the anti-3a fusion protein antibody. The pattern was similar to that reported by Benos for the amiloride-sensitive Na^+ channel purified from A6 cells (Sariban-Sohraby and Benos, 1986). In collaboration with Benos, we demonstrated that the anti-3a antibody recognized the purified Na^+ channel both in an enzyme-linked immunosorbent assay (ELISA) and by immunoprecipitation. Kleyman has developed a procedure for the identification and the purification of the amiloride binding component of the Na^+ channel (Kleyman *et al.*, 1989; Kleyman and Cragoe, 1989). The channel was first labeled with a photoactivatable analog of amiloride. Following detergent solubilization, the amiloride binding component of the channel was recovered using an

FIG. 4. Immunofluorescence micrographs of TBM cells using a polyclonal anti-α-subunit antibody. TBM cells were plated at high density and grown for 5 days in serum-containing medium. Aldosterone (300 n*M*) was added to the culture medium and at different times the cells were fixed. Frozen sections (0.5 μm) were prepared and incubated with biotinylated, affinity-purified, anti-α-subunit antibodies and fluoresceinated streptavidin. (a) Resting conditions in the absence of aldosterone: The apical plasma membrane of the cells in the upper layer is stained (arrows). There is no visible labeling of the basolateral plasma membrane of the upper cells or the membrane of the lower cells. (b) After 90 min of aldosterone treatment, the apical labeling over some upper cells increases dramatically (arrows). (c) After 3 hr, the apical labeling extends to more upper cells. Some basolateral labeling can be seen, as well as staining of the membrane of the lower cells. (d) Six hours after the addition of aldosterone, there is an increase in labeling of intracellular structures in both the upper and lower cells (arrows). (e) After 24 hr, the basolateral membrane of the upper cells and the plasma membrane of the lower cells is clearly labeled.

anti-amiloride antibody. Under reducing conditions, a unique polypeptide of 130 kDa was identified (Kleyman *et al.*, 1989). In contrast, under non-reducing conditions, the immunoprecipitation pattern was similar to that obtained with the anti-3a fusion protein antibody.

These data suggest that the 3a gene product is one of the components of the Na^+ channel, which consists of several distinct polypeptides (Sariban-Sohraby and Benos, 1986). Whether the 3a gene product is the amiloride-binding protein (Barbry *et al.*, 1987) and by itself can mediate Na^+ translocation remains to be established. Currently, we are screening for

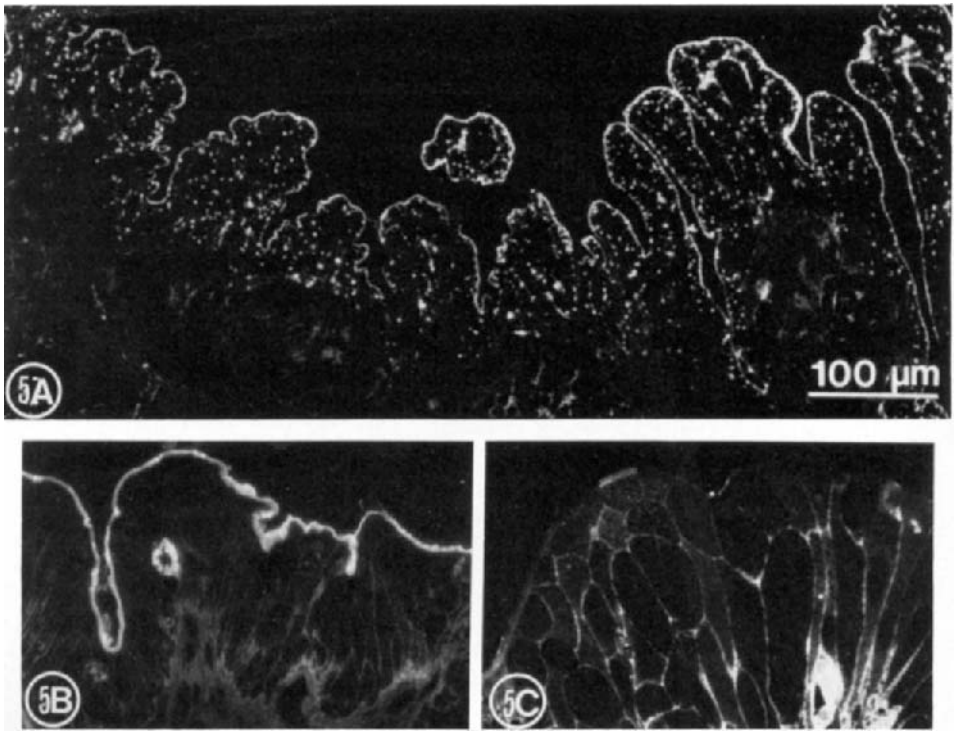


FIG. 5. Immunofluorescence micrographs of *Bufo marinus* bladder and intestinal cells. Urinary bladder (A), distal colon (B), and jejunum (C) were fixed in 0.5% glutaraldehyde–3% paraformaldehyde. Frozen sections (0.5 µm) were prepared and processed as described in Fig. 4. (A) The anti-α-subunit antibody labels the apical membrane intensively and, to a lesser extent, the basolateral plasma membrane of the granular cells. (B) The brush border of the absorptive cells in the distal colon is intensively labeled. The staining of the basolateral membrane is less intensive. (C) In the absorptive cells of the jejunum, there is no labeling associated with the brush border, while the labeling density of the basolateral membrane is similar to that seen in the distal colonic cells.

full-length cDNAs coding for the 3a gene product. We intend to synthesize the corresponding mRNA and express it in *Xenopus laevis* oocytes to test whether amiloride-sensitive Na^+ fluxes can be established.

V. ROLE OF SODIUM CHANNEL IN EARLY MINERALOCORTICOID RESPONSE

The density of the conductive Na^+ channel in the apical membrane has been shown to be very low (400 sites per cell). The ion selectivity is extremely high with a Na^+–K^+ permeability ratio as high as 1000 : 1 in toad bladder cells, in contrast to A6 cells (Palmer, 1987). The Na^+ channel, which controls entry of Na^+ into the cell at the apical cell surface, represents the rate-limiting step in transepithelial Na^+ transport. Therefore, the channel is an ideal target for hormonal regulation. It has been shown that the early mineralocorticoid response corresponds to an increase in apical Na^+ permeability (Garty, 1986). Whether this increase is due to membrane insertion of channels from a preexisting cytoplasmic pool, or to chemical modifications of cell surface-expressed channels is not known.

Methylation (Sariban-Sohraby and Benos, 1986), as well as phosphorylation/dephosphorylation by specific protein kinases/phosphatases, has been proposed as a means to regulate the activity of preexisting channels. Changes in cytosolic calcium concentration are known to modulate channel activity (Garty, 1986). It has not been shown so far whether aldosterone directly affects gene expression of the Na^+ channel. Transcriptional stimulation during the early response is unlikely, but during the late response, a transcriptional effect may participate in the adaptation of the tissue to a new steady state of Na^+ transport. Such a dual role of aldosterone on apical Na^+ channel activity has recently been proposed by Asher and Garty (1989). Once the identity of the 3a gene product as a component of the Na^+ channel is established, it will be possible to examine directly whether aldosterone modulates transcription of the channel gene during the late response and whether posttranslational modification of the channel is responsible for changes in its activity.

VI. ROLE OF Na^+,K^+-ATPase IN LATE MINERALOCORTICOID RESPONSE

Na^+,K^+-ATPase is a plasma membrane enzyme that couples the free energy contined within adenosine triphosphate (ATP) molecules to the

translocation of Na^+ and K^+ across the plasma membrane (Rossier *et al.*, 1987). The enzyme consists of an α,β-heterodimer which constitutes the minimal functional unit. In contrast to the channel, the cell surface density of the pump is high (10^6–10^7 pumps per cell). The primary structure of the α subunit (116 kDa), deduced from cDNA sequencing, is highly conserved (95% homology at the amino acid level) between all vertebrates. In contrast, the β subunit (40–60 kDa), a glycoprotein, is less conserved (60%) throughout evolution. Five genes coding for the catalytic subunit and two genes encoding for the β subunit have been described so far. Three distinct isoforms have been identified for the α subunit (αI, αII, αIII). αI is the housekeeping gene found in most cells, including A6 cells (Verrey *et al.*, 1989) and it is particularly abundant in the kidney. αII is a brain isoform and its affinity for ouabain is higher than that of the αI isoform. αIII is a major brain and muscle species, which has not been functionally characterized (Rossier *et al.*, 1987).

The existence of isoforms, and their tissue-specific expression, raise the interesting possibility of tissue-specific regulation of the pumps by various factors (for instance, ouabain or endogenous ouabain) or hormones (Rossier *et al.*, 1987). In mineralocorticoid-responsive cells (kidney, bladder, CCT, colon) aldosterone and related corticoids can modulate Na^+,K^+-ATPase activity and, in parallel, the number of ouabain-sensitive sodium pumps. In CTT, a specific mineralocorticoid effect occurs within hours. This response is independent of Na^+ transport, since the induction is maintained in the presence of amiloride (Barlet-Bas *et al.*, 1988).

Expression of the Na^+,K^+-ATPase gene and its regulation by aldosterone was examined in both A6 and TBM cells. Aldosterone acts at the transcriptional level by increasing, within 45 min, three- to fourfold the relative number of RNA polymerase II molecules transcribing the α- and β-subunit genes (Verrey *et al.*, 1988). The increase in the transcription rate can account for the two- to fourfold accumulation of both α- and β-subunit mRNAs during a 6-hr aldosterone treatment (Verrey *et al.*, 1987). The accumulation of α- and β-subunit mRNAs induced by aldosterone is paralleled by a 1.7- to 2.8-fold increase in relative rates of α- and β-subunit synthesis (Paccolat *et al.*, 1987; Verrey *et al.*, 1987). The expression of the Na^+,K^+-ATPase subunit at the cell surface also increased three- to fivefold 12–24 hr after the addition of aldosterone (Ernst *et al.*, 1986). The expression of ouabain-sensitive sodium pumps determined by an equilibrium binding assay using tritiated ouabain was not markedly altered by aldosterone (Pellauda *et al.*, unpublished observation). In collaboration with Caplan, we labeled the pump at the surface with photoactivatable ouabain, followed by immunoprecipitation with anti-ouabain

antibodies, and we showed an increase in the number of active pumps after aldosterone stimulation. This suggests that a significant portion of the pool of Na^+,K^+-ATPase induced by aldosterone is not present at the cell surface as an active enzyme. It is possible that ouabain itself changes the Na^+,K^+-ATPase pool expressed at the cell surface.

VII. CONCLUSIONS

Aldosterone regulates Na^+ transepithelial transport in tight epithelia. Like other steroid hormones, aldosterone via a specific cytoplasmic receptor regulates the transcription of specific genes and thus induces or represses a large number of proteins, the aldosterone-induced (AIP) or aldosterone-repressed (ARP) proteins. The Na^+ response induced by aldosterone consists of an early phase, with an increase in Na^+ transport concomitant to a fall in transepithelial resistance, and a late phase, with a further resistance-independent increase in Na^+ transport. Aldosterone regulates the expression of genes involved in the early and late responses. No early AIP or ARP has been isolated, but it is likely that they correspond to regulatory proteins that modulate the activity of the amiloride-sensitive Na^+ channel. The gene expression of the Na^+ channel does not seem to be regulated by aldosterone during the early response. Whether the Na^+ channel is a late AIP involved in adaptation has not been established. The two Na^+,K^+-ATPase subunits are late AIPs, which are regulated by aldosterone mainly at the transcriptional level.

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Chapter 10

Involvement of Na^+,K^+-ATPase in Antinatriuretic Action of Mineralocorticoids in Mammalian Kidney

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I. INTRODUCTION

Although mineralocorticoids have long been known to enhance the reabsorption of sodium and the secretion of potassium and protons in the kidney, the exact sites and mechanisms of these actions in the nephron are not clearly established as yet. Indeed, most of our knowledge on the mechanism of control of sodium transport by mineralocorticoids derives from studies on frog skin or toad bladder. These studies have been very fruitful, especially since these epithelia can be mounted in Ussing chambers, allowing electrophysiological and flux measurements. However, the mechanism of action of aldosterone might not be similar in these epithelia and in the kidney, in particular since the latter has a much higher capacity

of sodium transport. This problem was circumvented in part by the development of established cell lines (A6 cells) derived from the kidney of *Xenopus laevis* (Rafferty, 1969). When grown on collagen-coated filters mounted on plastic rings, these cells develop as a polarized monolayer which can be studied like a natural planar epithelium. On this model, Rossier *et al.* very elegantly analyzed the actions of aldosterone on both sodium transport and genomic expression of the sodium pump (see Chapter 9 by Rossier *et al.*, this volume), but here again there might be marked differences between amphibian and mammalian cells.

Therefore, we have decided to evaluate the action of aldosterone on the mammalian kidney. The main problem raised by such a study is the heterogeneity of the kidney, in particular, as most kidney cells are unresponsive to aldosterone (Marver and Kokko, 1983). Thus, our first attempt has been to determine the kidney cells responsible for aldosterone actions by localizing aldosterone receptors in the nephron. A second problem is linked to possible cross-reactivity of aldosterone with other corticosteroid receptors, mainly with glucocorticoid receptors (Fanestil and Park, 1981; Marver, 1984), present in large amounts in the kidney. Consequently, much attention had to be given to the mineralocorticoid specifity of the observed effects of aldosterone. To this aim, the segregation of mineralocorticoid and glucocorticoid responses in separate nephron segments has been very useful.

Two-dimensional gel electrophoresis revealed that the genomic expression of a large number of proteins is altered by aldosterone (Truscello *et al.*, 1986). However, only a few of these proteins are functionally characterized. In our attempt to study the biochemical events underlying the antinatriuretic action of aldosterone, we focused our attention on Na^+,K^+-ATPase. This protein, which is embedded in the basolateral membrane of epithelial cells, catalyzes the active extrusion of sodium ions from the intracellular pool to the serosal medium against potassium ions. Thereby, it plays a key role in sodium reabsorption across the whole epithelial wall.

There are several approaches to overcoming kidney heterogeneity, which include autoradiography or immunocytochemistry on kidney slices, and kidney tissue fractionation, such as by density gradient centrifugation or sequential sieving of kidney cell suspensions. We used kidney tissue microdissection and developed specific microanalytical methods suitable for studying single nephron segments. The present review mainly focuses on: (1) the advantages and limitations of this methodological approach to studying aldosterone action on target cells of the mammalian kidney and (2) the similarities and differences of aldosterone action between mammalians and amphibians.

II. LOCALIZATION OF ALDOSTERONE RECEPTORS ALONG THE NEPHRON

The first step in studying aldosterone action in target cells exclusively has been to define the kidney tubular cells responsive to aldosterone. Therefore, mineralocorticoid receptors were localized along the nephron by measuring specific [^{3}H]aldosterone binding in microdissected segments of nephron (Doucet and Katz, 1981b).

To obtain the large number of samples necessary for such studies requires that interstitial kidney tissue be partially hydrolyzed with collagenase prior to microdissection. This treatment may alter the biochemical properties of tubules, especially due to the large variations in collagenase and proteolytic contaminant activities among different batches of enzyme. This pitfall is circumvented in part by protecting cells against proteolysis with large amounts of serum albumin during collagenase hydrolysis. Nevertheless, each new batch of collagenase used for microdissection must be tested to determine that it has no effect on biochemical properties. Under these conditions, large numbers of well-defined pieces of tubule can be microdissected (0.2–2.0 mm of length) from nearly all nephron segments. The different nephron segments schematized on Fig. 1A are characterized according to topographical and morphological criteria (Morel *et al.*, 1978).

The main restriction to the microdissection approach lies in the minute amount of protein (between 20 and 250 ng), contained per millimeter of tubular length, depending on the nephron segments and animal species. This required the development of highly sensitive biochemical assays, such as radiochemical techniques. Furthermore, protein content cannot be conveniently determined on each of the samples to serve as a reference for biochemical activities. Consequently, tubular length, which can be automatically determined by computerized processing of the microscopic image of each sample (Khadouri *et al.*, 1987), was chosen as a reference. When necessary, data could be calculated per milligram of protein from the known relationships between total protein content and tubular length for the different nephron segments (Vandewalle *et al.*, 1981b; El Mernissi *et al.*, 1983).

Due to the paucity of hormone receptors (5–25 $\times$ 10^{-18} mol/mm) and the low specific activity of available [^{3}H]aldosterone (80 Ci/mmol), detection of aldosterone receptors by direct measurement of [^{3}H]aldosterone binding to isolated nephron segments required that several tubule segments be pooled (~5–20 mm in total length) in each sample in order to obtain significant counts per minute. This precluded the detection of [^{3}H]aldosterone binding in tubular segments which have a low protein

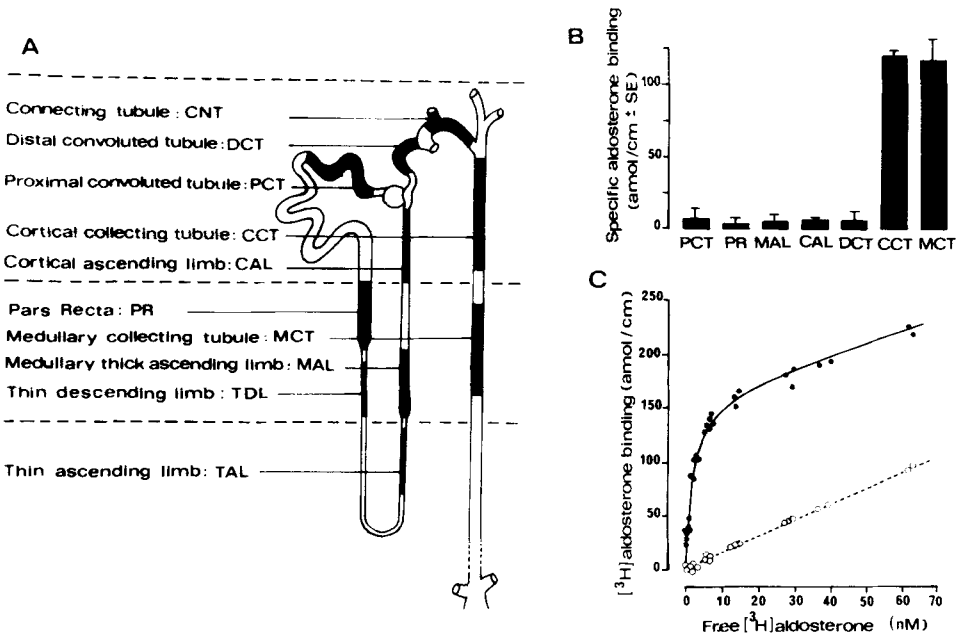

FIG. 1. (A) Organization of the rabbit nephron. The dark portions indicate the localization of the different segments isolated by microdissection. (B) Specific binding of [^{3}H]aldosterone (10^{-18}mol/cm) along the rabbit nephron was measured at the saturating concentration of labeled ligand. (C) Concentration dependence of total (closed circles) and nonspecific binding of [^{3}H]aldosterone (open circles) in cortical collecting tubules from rabbits. At each concentration of [^{3}H]aldosterone, nonspecific binding was determined in the presence of a 2000-fold excess of unlabeled aldosterone. (Redrawn in part from Doucet and Katz, 1981b.)

content per millimeter of length (such as the thin segments of the loop of Henle) or which are short (such as the connecting tubules).

Because of the small size of the samples, it is also obviously not possible to distinguish between cytoplasmic and nuclear receptors. Specific binding of [^{3}H]aldosterone found exclusively in the rabbit cortical and medullary collecting tubules (Fig. 1B) might therefore represent the sum of the hormone binding to these two classes of receptors. In fact, autoradiographic studies of [^{3}H]aldosterone binding performed on microdissected rabbit collecting tubules reveal that most of the specific binding sites are located in the nucleus (Vandewalle *et al.*, 1981a). Thus, it is likely that, using these binding assay procedures, the aldosterone–receptor complexes migrate from the cytoplasm to the nucleus of target cells, and that further binding to the chromatin displaces all of the receptors to the nuclear pool.

Despite the technical limitations mentioned above, the dissociation constant determined on the cortical collecting tubule (apparent K_D = 2.2 n*M* at 25°C; see Fig. 1C) is similar to that reported for mineralocorticoid receptors on acellular preparations (Edelman, 1981). That [^{3}H]aldosterone binding sites in the collecting tubule are indeed mineralocorticoid (and not glucocorticoid) receptors is confirmed by displacement studies that reveal the highest affinity for aldosterone, followed by deoxycorticosterone acetate (DOCA) and the mineralocorticoid antagonist spironolactone. The affinity for the synthetic glucocorticoid dexamethasone is intermediate, and that for 5α-dihydrotestosterone, progesterone, and 17β-estradiol is negligible (Doucet and Katz, 1981b). This rank order of receptor affinity for various steroids is substantially similar to that reported for mineralocorticoid receptors in other systems (Marver, 1980).

This localization of mineralocorticoid receptors is in agreement with a large body of evidence which indicates that the collecting tubule is the major site of action of aldosterone. Studies in rabbit cortical collecting tubules obtained from animals receiving mineralocorticoids *in vivo* or subjected to experimental manipulations aimed at increasing endogenous aldosterone levels, have shown stimulation of sodium transport and/or enhancement of transepithelial voltage in this segment of nephron (Gross *et al.*, 1975; O'Neil and Helman, 1977; Schwartz and Burg, 1978; Imai, 1979). Although autoradiographic studies reveal a cellular heterogeneity of the nuclear labeling of rabbit collecting tubules (Vandewalle *et al.*, 1981a), aldosterone receptors are likely to be present in both principal and intercalated cells which constitute this segment. Indeed, mineralocorticoids control both the reabsorption of sodium and the secretion of protons, which likely originate in these two types of cells, respectively (Morel and Doucet, 1986).

In fact, it is widely accepted that the collecting tubule is a target site for mineralocorticoids, and the controversy regarding the localization of aldosterone receptors in the kidney is whether this hormone also acts in other parts of the nephron. The main dispute concerns the connecting tubule, which was not analyzed in our study. Since this segment contains intercalated cells similar to those present in the cortical collecting tubule, it is presumed to respond to mineralocorticoids. And indeed, autoradiography reveals the presence of corticosteroid receptors with a higher affinity for aldosterone than dexamethasone in the rabbit connecting tubule (Farman *et al.*, 1982). However, long-term administration of DOCA does not alter the transepithelial voltage in the connecting tubule, whereas it does in the collecting tubule (Imai, 1979). This apparent unresponsiveness of the connecting tubule as compared to the collecting tubule is likely due to the fact that the electrophysiologically measured response of the cortical collecting tubule originates in the principal cells that are absent in the

connecting tubule. In addition, there are likely species differences in the localization of mineralocorticoid receptors along the nephron. For example, the rat thick ascending limb displays both receptors (Farman and Bonvalet, 1983) and a physiological response (Stanton, 1986) to mineralocorticoids, whereas the rabbit thick ascending limb is insensitive to aldosterone.

III. ALDOSTERONE CONTROL OF Na^+,K^+-ATPase ACTIVITY IN SPECIFIC NEPHRON SEGMENTS

A. Determination of Na^+,K^+-ATPase Activity in Single Nephron Segments

The method developed to determine Na^+,K^+-ATPase activity in single segments of nephron is based on the direct measurement of inorganic phosphate released from adenosine triphosphate (ATP) labeled with ^{32}P in its γ position (Doucet *et al.*, 1979). Quantitative separation of [^{32}P] P_i from unhydrolyzed ATP is easily accomplished by filtration after absorption of the nucleotides on activated charcoal. Due to the very high level of activity of Na^+,K^+-ATPase in kidney tubules and to the reduction of the volume of incubation to 1 μl, this activity can be determined in a single nephron segment, even though [^{32}P]ATP is used in tracer doses. Because ATPase activity is linearly related to tubular length within a wide range (Fig. 2A), tubular length may be used as a reference for ATPase activity.

To allow access of exogenous ATP to its intracellular sites of action and to stimulate fully ATPase activity, tubular cell membranes are permeabilized by submitting each nephron segment to a hypoosmotic medium and to rapid freezing. Despite this procedure, however, ATPase activity did not reach its apparent maximum until ATP concentration was raised to above 6–8 m*M*, a value considerably higher than those reported on homogenates and membrane fractions (Jørgensen, 1980). This difference is likely related to the presence of unstirred layers, since the tubules are not agitated during incubation. Consequently, the concentration of ATP in the immediate vicinity of the catalytic site is probably lower than that in the incubation medium as a whole. This concept is supported by kinetic experiments carried out at low ATP concentrations, showing that the enzyme activity decreases as the incubation time increases above 1–3 min, whereas, at high ATP concentrations, ATPase activity is linearly related to incubation time up to 30 min (Fig. 2B). This technical limitation precludes determination of the apparent affinity of Na^+,K^+-ATPase for ATP

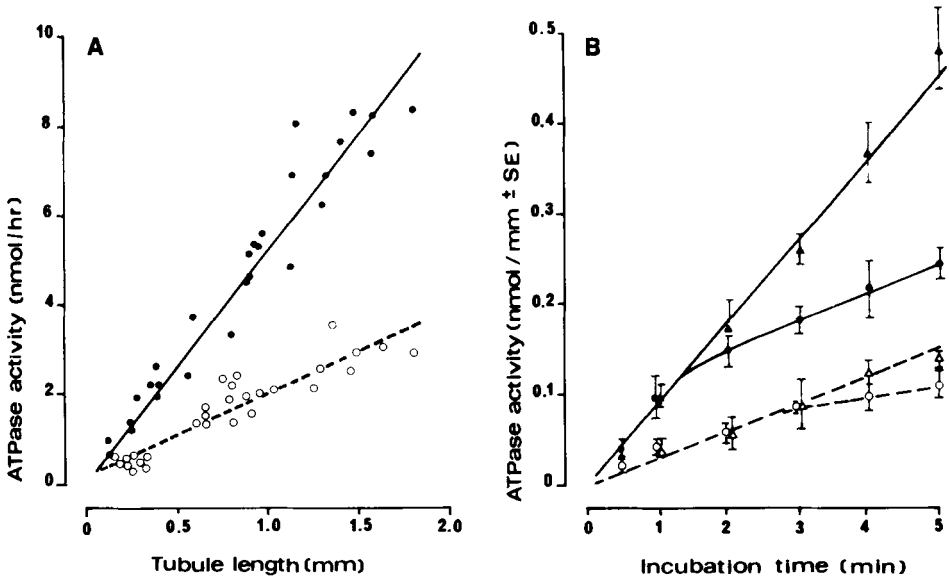

FIG. 2. (A) Effect of tubular length on total (solid circles) and Mg^{2+}-ATPase (open circles) of rabbit medullary thick ascending limb. (B) Effect of incubation time on total (solid symbols) and Mg^{2+}-ATPase (open symbols) of mouse medullary thick ascending limb. Kinetics were studied in the presence of either 10 m*M* ATP (triangles) or 3 m*M* ATP (circles). In the presence of 10 m*M* ATP, both total and Mg^{2+}-ATPase activity increased linearly with incubation time up to 30 min. (Redrawn in part from Doucet *et al.*, 1979.)

and its possible variations in response to aldosterone at the level of single nephron segments. Conversely, the affinities of tubular Na^+,K^+-ATPase for sodium and potassium (Doucet *et al.*, 1979) and its sensitivity to ouabain (Doucet and Barlet, 1986) can be determined and are consistent with data on acellular preparations, probably because these reactants are not consumed during the reaction, or they diffuse more freely, or, in the case of ouabain, because it binds to the extracellular side of the membrane. The permeabilization procedure may also unmask the activity of intracellular Na^+,K^+-ATPase units that are not functionally coupled to transmembrane sodium transport *in vivo*, and therefore lead to overestimation of the physiologically active pump.

The existence of several ATPases in kidney cells (Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, H^+-ATPase, F_0,F_1-ATPase, etc.) requires that Na^+,K^+-ATPase activity be determined by the difference between a total ATPase activity (measured under conditions allowing full stimulation of Na^+,K^+-ATPase as well as partial stimulation of other ATPases), and a

basal ATPase activity measured after specific and complete inhibition of Na^+,K^+-ATPase (by removal of both sodium and potassium ions and addition of 1 m*M* ouabain). Due to the biological and methodological variability of these two ATPase activities, each must be determined on four or five replicate samples, so that the difference of their mean values (i.e., Na^+,K^+-ATPase activity) has a statistical significance (Doucet *et al.*, 1979). Thus, each determination of Na^+,K^+-ATPase activity requires microdissection and processing of eight to ten individual nephron segments. This prompted us to automate several steps of the assay, allowing us to handle up to 150 samples in a 1-day experiment.

B. Effects of Adrenalectomy and Steroid Replacement on Tubular Na^+,K^+-ATPase Activity

The profile of Na^+,K^+-ATPase activity along the successive portions of the rabbit nephron exhibits a pattern similar to that of the sodium transport capacity (Fig. 3A): A high activity is observed in the segments reabsorbing large amounts of sodium against a concentration gradient (in the diluting segment and the distal convoluted tubule), intermediate activity is present where sodium is reabsorbed from an isosmotic solution (in the proximal convoluted tubule), and a relatively low Na^+,K^+-ATPase activity is found in the last nephron segments where final adjustments of sodium reabsorption occur (in the collecting tubule).

Within 1 week, bilateral adrenalectomy decreases Na^+,K^+-ATPase activity in all nephron segments except the proximal convoluted tubule (El Mernissi and Doucet, 1982), a special feature of the rabbit nephron, since adrenalectomy inhibits the pump activity in the proximal convoluted tubule of both mice (Doucet and Katz, 1981a) and rats (El Mernissi and Doucet, 1983a). In the rabbit, the decrement in Na^+,K^+-ATPase activity is over 75% in the cortical and medullary portions of the collecting tubule, whereas it averages only 20–40% in more proximal segments (Fig. 3A). This maximal decrease is reached 4–5 days after adrenalectomy (Doucet and Katz, 1981a), in agreement with the biological half-life of Na^+,K^+-ATPase (Jørgensen, 1968). In the collecting tubule, however, Na^+,K^+-ATPase activity apparently decreases only after a 24- to 36-hr period of latency, during which a latent pool of Na^+,K^+-ATPase is recruited (A. Doucet and C. Barlet-Bas, unpublished observations).

Within 3 hr, injection of aldosterone (10 μg/kg body wt) to adrenalectomized (ADX) rabbits fully restores Na^+,K^+-ATPase activity up to control values in the collecting tubule exclusively (El Mernissi and Doucet, 1982), whereas injection of dexamethasone (100 μg/kg body wt) restores Na^+,K^+-ATPase activity in all nephron segments (El Mernissi and

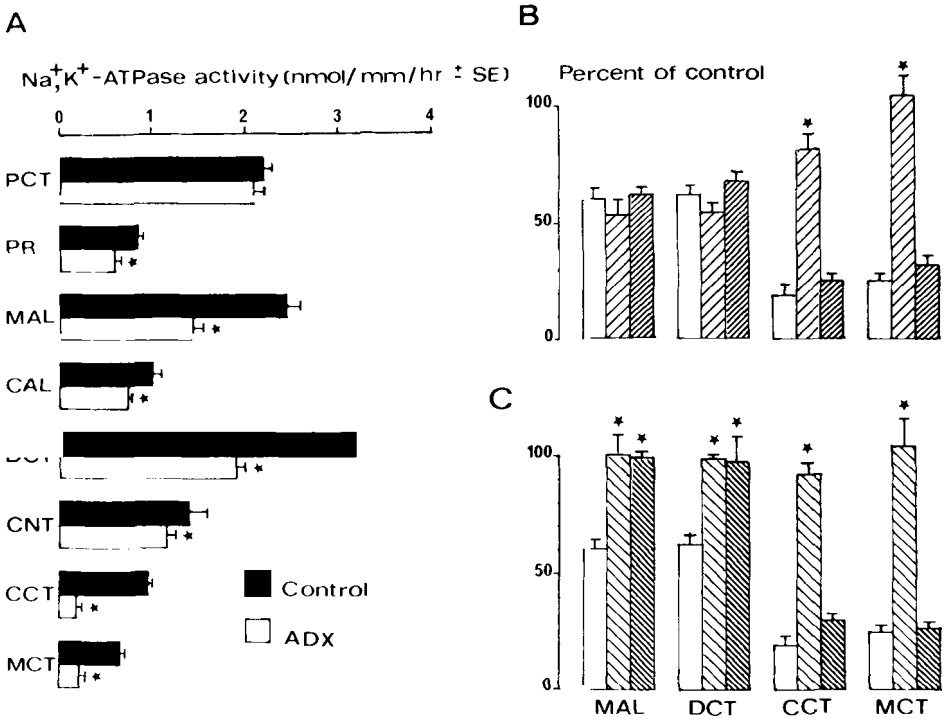

FIG. 3. (A) Na^+,K^+-ATPase activity along the nephrons of normal (control) and adrenalectomized (ADX) rabbits. ADX rabbits were studied 5–8 days after surgery. *, Statistically different normal rabbits. (B) Na^+,K^+-ATPase activity in the medullary thick ascending limb (MAL), the distal convoluted tubule (DCT) and the cortical and medullary collecting tubules (CCT and MCT) of ADX rabbits (open columns), ADX rabbits injected with aldosterone (10 μg/kg body wt) 3 hr before study (hatched columns), and ADX rabbits injected with spironolactone (100 μg/kg body wt) 1 hr before aldosterone (thin hatched columns). Results are expressed as the percentage of activity determined in corresponding segments of normal rabbits. *, Statistically different from ADX animals. (C) Same as in (B) except that ADX rabbits received dexamethasone (100 μg/kg body wt) instead of aldosterone. [Redrawn from El Mernissi and Doucet (1983b) and Doucet and El Mernissi (1985).]

Doucet, 1983b). Stimulation of Na^+,K^+-ATPase by aldosterone is related to the occupancy of mineralocorticoid receptors since (1) it is specific of the nephron segments in which mineralocorticoid receptors are present and (2) it is suppressed by prior administration of the mineralocorticoid antagonist spironolactone (Fig. 3B). In the collecting tubule, stimulation of Na^+,K^+-ATPase by dexamethasone also involves receptors to mineralocorticoids, since it is abolished by spironolactone, whereas on more

proximal segments it is not altered by the antimineralocorticoid (Fig. 3C), suggesting the implication of receptors to glucocorticoids (El Mernissi and Doucet, 1983b).

Although not demonstrated in these studies, aldosterone action on Na^+,K^+-ATPase likely originates in the principal cells rather than in intercalated cells of the collecting tubule. Indeed, principal cells are responsible for the transepithelial sodium reabsorption and contain more Na^+,K^+-ATPase units than do intercalated cells (Kashgarian *et al.*, 1985). This does not preclude that aldosterone may also control the function of the intercalated cells, which are mainly involved in proton secretion. In fact, it has been shown that aldosterone controls the activity of the proton pump present in these cells (Khadouri *et al.*, 1987; Mujais, 1987).

Thus, tubular Na^+,K^+-ATPase is controlled by both mineralocorticoids and glucocorticoids, but on distinct nephron segments. This spatial organization of corticosteroid sensitivity along the nephron has greatly facilitated assessment of the mineralocorticoid specificity of aldosterone in the further characterization of its mechanism of action on Na^+,K^+-ATPase in the collecting tubule.

IV. METHODOLOGY FOR STUDYING THE MECHANISM OF ALDOSTERONE ACTION ON TUBULAR Na^+,K^+-ATPase

Stimulation of the V_{max} of an enzyme results from increasing either the number of active catalytic units of the enzyme or the activity of each of its catalytic units, or both. Thus, understanding the mechanism responsible for the stimulation of Na^+,K^+-ATPase activity by aldosterone required quantifying not only the activity of the enzyme but also the number of active pump units present in the collecting tubule. This can be achieved by measuring the binding capacity for [^{3}H]ouabain. Another prerequisite to further evaluating the molecular events underlying aldosterone action was to develop an *in vitro* system which reproduced the stimulation of Na^+,K^+-ATPase by adding the hormone to isolated segments of collecting tubule. Such a system is absolutely necessary in order to test the action on aldosterone response of pharmacological agents that cannot be administered *in vivo* (because of their toxicity or the difficulty to control their concentration), and also to discriminate between aldosterone actions primarily originating in the collecting tubule from those resulting from alterations originating elsewhere—for example, in the nephron or in the whole organism—in response to aldosterone. Before analyzing results obtained by such approaches (see Section IV), this section will focus on the problems raised by these methodologies.

A. Determination of [^{3}H]Ouabain Binding in Single Nephron Segments

Ouabain inhibits specifically Na^+,K^+-ATPase by binding stoichiometrically to an extracellular site of the α subunit of the enzyme (Jørgensen, 1986). Thus, at saturating concentrations of ligand, the binding of [^{3}H]ouabain quantifies the number of Na^+,K^+-ATPase units. Due to the great number of Na^+,K^+-ATPase units in kidney cells, [^{3}H]ouabain binding can be evaluated at the level of a single nephron segment (El Mernissi and Doucet, 1984a) despite the low specific activity of available [^{3}H]ouabain (10–20 Ci/mmol). Furthermore, binding of [^{3}H]ouabain is linearly dependent on tubular length (0.2–2.0 mm), which can serve as a reference.

Since ouabain binds to an extracellular site, it is not necessary to permeabilize the tubules to measure its binding. In fact, ouabain binding is similar in nonpermeabilized tubules and in tubules permeabilized by the same procedure as that used for measuring Na^+,K^+-ATPase activity (Fig. 4A). This indicates that if there were any intracellular masked Na^+,K^+-ATPase units (as discussed in Section II,A), they would not be

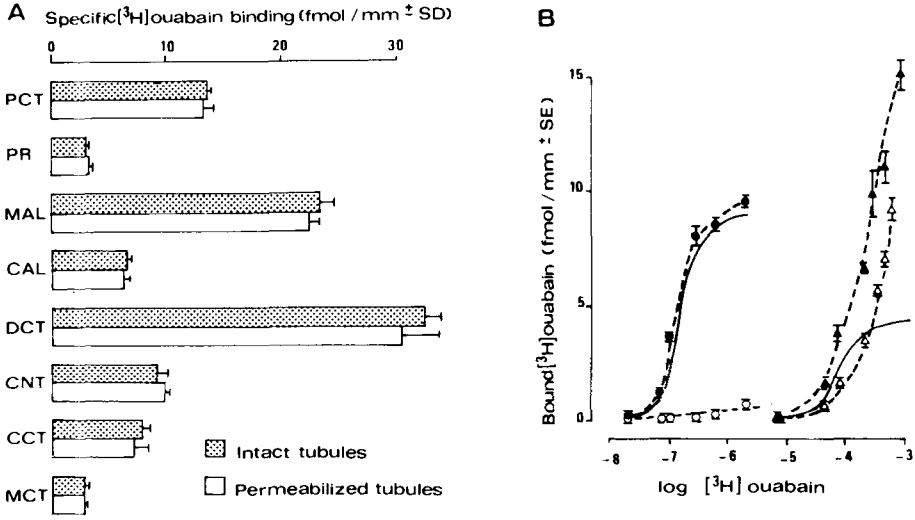

FIG. 4. (A) Specific [^{3}H]ouabain binding along the rabbit nephron. Ouabain binding was measured either in intact tubules or in tubules that were successively submitted to a hyposmotic medium and to freeze-thawing (permeabilized tubules). (B) Concentration dependency of total (solid symbols) and nonspecific [^{3}H]ouabain binding (open symbols) in the rabbit cortical collecting tubule (circles) and in the rat medullary collecting tubule (triangles). Solid lines represent the specific binding. (Redrawn in part from El Mernissi and Doucet, 1984a.)

active, since they cannot bind ouabain. The finding that saturation of [^{3}H]ouabain-binding sites on nonpermeabilized tubules is obtained with monophasic kinetics and within the same delay as in acellular preparations (El Mernissi and Doucet, 1984a) also indicates that there is a single pool of active Na^+,K^+-ATPase.

It is well established that ouabain binds to the phosphorylated Na^+,K^+-ATPase (the E_2 form) and blocks its potassium-induced dephosphorylation. Thus, vanadate, which binds with high affinity to the phosphorylation site of Na^+,K^+-ATPase, can be used instead of ATP to displace all enzyme units to their E_2 form (Hansen, 1979) and thereby to facilitate the saturation of Na^+,K^+-ATPase from tubular segments by [^{3}H]ouabain.

The very slow dissociation rate of specific ouabain binding at low temperatures allows the separation of bound from unbound [^{3}H]ouabain by successive washings of tubular samples. Conversely, nonspecific binding of ouabain is rather reversible at low temperatures, and this property is used to decrease the nonspecific binding by postincubating the samples for 60–90 min at 0°C in the absence of free ligand. This reduces the nonspecific fraction of [^{3}H]ouabain binding by over 90% without altering its specific one.

Saturation of Na^+,K^+-ATPase by ouabain is easily achieved on the rabbit collecting tubule, since the rabbit is among the species which display a high sensitivity to ouabain, and the collecting tubule has a higher affinity for ouabain than do other nephron segments (Doucet and Barlet, 1986). Thus, [^{3}H]ouabain binds to the rabbit collecting tubule with an apparent K_D of 0.12 μM and saturation occurs in the micromolar range of ouabain concentration. At these concentrations, nonspecific binding of [^{3}H]ouabain (determined in the presence of a 100-fold excess of unlabeled ligand) accounts for less than 5% of the total binding (Fig. 4B). Conversely, the rat has a very low sensitivity to ouabain, and despite the higher affinity of the collecting tubule as compared to more proximal nephron segments, the apparent K_D for [^{3}H]ouabain is $\sim 10^{-4}$ M. At saturating concentration of ligand ($\sim$300 μM), the nonspecific binding of [^{3}H]ouabain is over 50% of its total binding (Fig. 4B), which compromises the accuracy of determination of the specific binding of [^{3}H]ouabain. Another limitation of the high concentration of [^{3}H]ouabain necessary to saturate the specific binding sites of the rat collecting tubule is that total displacement of the specific binding would require concentration of unlabeled ouabain beyond its limit of solubility. Since potassium and ouabain compete for binding to Na^+,K^+-ATPase, this problem can be circumvented by using a combination of 5 mM ouabain and 30 mM potassium to displace specific [^{3}H]ouabain binding.

This method permits quantitation of the number of active (phosphory-

lated) Na^+,K^+-ATPase units and its variations in all of the segments of the rabbit nephron and, with some limitations mentioned above, in the rat collecting tubule. However, estimation of [^{3}H]ouabain binding under saturation conditions in more proximal segments of the rat nephron is beyond its sensitivity limit. In normal rats and rabbits, the number of catalytic sites of Na^+,K^+-ATPase is proportional to the pump activity in all segments tested. This indicates that the rate of activity of each catalytic unit (~2000 molecules of ATP split per ouabain binding site per minute) is similar in different nephron segments (El Mernissi and Doucet, 1984a; Barlet-Bas *et al.*, 1988).

B. *In vitro* Stimulation of Tubular Na^+,K^+-ATPase by Aldosterone

Although *in vivo* administration of aldosterone to ADX rabbits or rats reproducibly restores Na^+,K^+-ATPase activity of the collecting tubule (see Section II,B) in 3 hr, preincubation of collecting tubules from ADX animals for 3 hr in the presence of physiological concentrations of aldosterone fails to alter Na^+,K^+-ATPase. Similarly, although it is clearly established that transepithelial potential difference and sodium reabsorption are higher in cortical collecting tubules (CCTs) isolated from mineralocorticoid-treated rabbits than in those from normal animals (Gross *et al.*, 1975; O'Neil and Helman, 1977; Schwartz and Burg, 1978; Imai, 1979), *in vitro* addition of aldosterone fails to alter, or irreproducibly modifies, sodium transport and/or transepithelial voltage of CCTs from mineralocorticoid-deficient rabbits (Gross and Kokko, 1977; Schwartz and Burg, 1978; Wingo *et al.*, 1985). Thus, factor(s) present *in vivo* which are necessary to trigger both the stimulation of Na^+,K^+-ATPase and the final physiological effect in response to aldosterone might be absent in the *in vitro* assay.

Based on the similarities of sites and kinetics of stimulation of tubular Na^+,K^+-ATPase by aldosterone and by thyroid hormones (El Mernissi and Doucet, 1983a; Barlet and Doucet, 1986), triiodothyronine (T_3) has been implicated in the genesis of aldosterone response. In fact, T_3 increases tubular sensitivity to aldosterone (Barlet and Doucet, 1987), since administration of doses of aldosterone sufficient to increase Na^+,K^+-ATPase activity in CCTs from ADX rabbits has no effect on CCTs from rabbits which are both ADX and thyroidectomized (Fig. 5A). This is also consistent with the finding that the antinatriuretic action of mineralocorticoids requires higher doses of DOCA in hypothyroid rats than in euthyroid rats (Taylor and Fregly, 1964).

Addition of aldosterone plus T_3 on CCTs from ADX rats restores Na^+,K^+-ATPase activity in 3 hr, whereas either hormone separately has

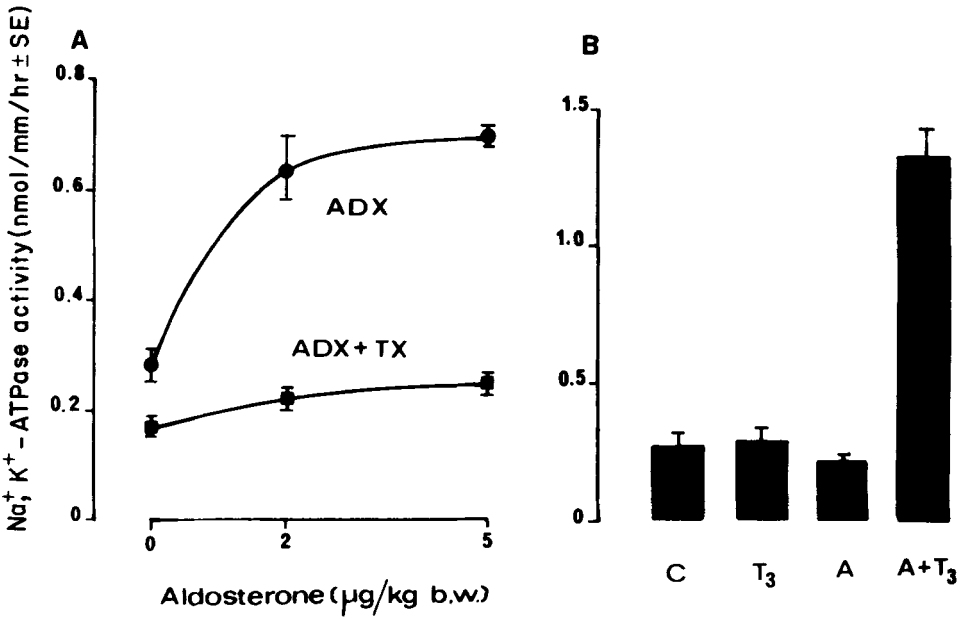

FIG. 5. (A) Na^+,K^+-ATPase activity in the cortical collecting tubule of 1-week adrenalectomized (ADX) and thyroadrenalectomized rabbits (ADX + TX) 3 hr after injection of doses of aldosterone (2–5 μg/kg body wt). (B) Na^+,K^+-ATPase activity in cortical collecting tubules from 7- to 8-day adrenalectomized rats preincubated *in vitro* for 3 hr at 37°C. Tubules were preincubated either in the absence of hormone (C) or in the presence of triiodothyronine $10^{-9}M$ (T_3), aldosterone $10^{-7}M$ (A), or both hormones together (A + T_3). [Redrawn from Barlet and Doucet (1987) and Barlet-Bas *et al.* (1988).]

no effect at all (Fig. 5B). These *in vitro* experiments are run on rat rather than rabbit CCTs because, in the absence of hormone, Na^+,K^+-ATPase activity remains constant throughout a 4-hr preincubation at 37°C in the rat, whereas it progressively and variably decreases in the rabbit. In the presence of saturating concentrations of either hormone, stimulation of Na^+,K^+-ATPase activity is dose dependent, with apparent affinities in the range of physiological concentrations: $K_{1/2}$ for aldosterone, 10^{-9} *M*; $K_{1/2}$ for T_3, 10^{-10} *M* (Barlet-Bas *et al.*, 1988). The molecular mechanism of the "permissive" action of T_3 is not yet known. Although both hormones promote their physiological effects by modulating the expression of specific genes, it is uncertain whether their interaction involved in the stimulation of Na^+,K^+-ATPase occurs at the genomic level or on posttranscriptional steps of mRNA and/or protein processing. In any case, the putative factor induced by T_3 has a short biological half-life, since, in

the absence of added T_3, it disappears during the time course of tubule microdissection and *in vitro* preincubation (a 6- to 7-hr procedure).

At this point, it should be stressed that the requirement of T_3 to induce antinatriuresis (Taylor and Fregly, 1964) and stimulation of Na^+,K^+-ATPase in the collecting tubule in response to aldosterone is specific for mammalian tissues. Indeed, *in vitro* addition of aldosterone alone does stimulate sodium transport in amphibian epithelia (Crabbé, 1963). Moreover, T_3 even curtails the late stimulation by aldosterone of sodium transport (that occurring without change in transepithelial resistance) in the toad bladder (Rossier *et al.*, 1979).

In the following section, *in vitro* action of aldosterone on tubular Na^+,K^+-ATPase will refer to the effect promoted by both aldosterone and T_3.

V. MECHANISM OF ALDOSTERONE ACTION ON Na^+,K^+-ATPase IN THE COLLECTING TUBULE

Aldosterone induces its physiological effects by binding to intracellular receptors which, in turn, interact with specific sites of the genome and thereby modulate the expression of specific genes. Changes in the rate of synthesis of the proteins encoded by these genes (called aldosterone-induced proteins) are responsible for the early, primary physiological effects of the hormone. Besides these actions, aldosterone may also elicit secondary effects which are delayed. These late effects are not supported by proteins directly induced by the interaction of the aldosterone receptor–aldosterone complexes with the genome, but are related to alterations in the expression of other proteins (secondary aldosterone-induced proteins) in response to the primary aldosterone actions. Whether the stimulation of Na^+,K^+-ATPase activity in response to aldosterone is due to synthesis of new pump units and whether this is a primary or a secondary effect of the hormone is discussed in the following section.

A. Does Aldosterone Induce the Synthesis of Na^+,K^+-ATPase?

When aldosterone is administered to ADX rabbits, not only the activity but also the number of units of Na^+,K^+-ATPase increases in the CCT (Fig. 6A): The two parameters rise in parallel 0.5 hr after hormone administration and are fully restored within 3 hr (El Mernissi and Doucet, 1984b). Similarly, *in vitro* addition of aldosterone to rat CCTs increases Na^+,K^+-ATPase activity and [^{3}H]ouabain binding to the same extent, so that the maximal turnover rate of each catalytic unit is not altered (Barlet-

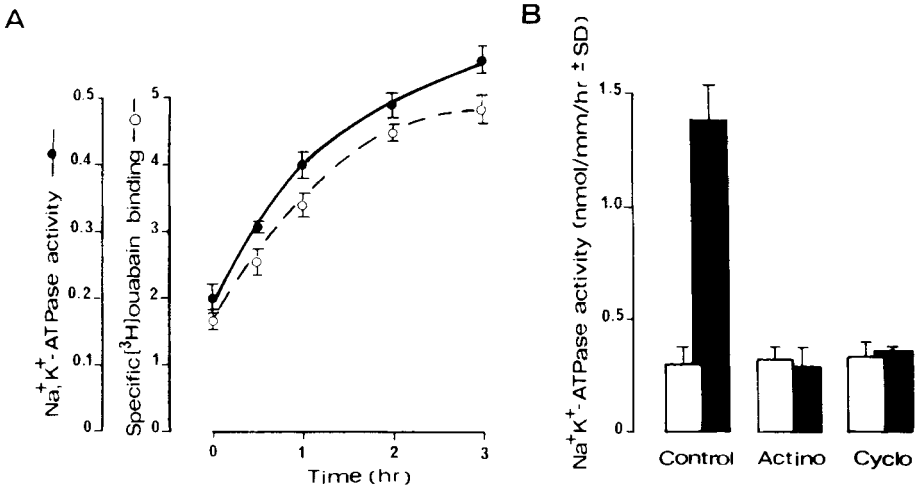

FIG. 6. (A) Time dependency of Na^+,K^+-ATPase activity (nmol/mm/hr ± SE) and of specific [^{3}H]ouabain binding (fmol/mm ± SE) in cortical collecting tubules from adrenalectomized rabbits after injection of aldosterone (10 μg/kg body wt). (B) Na^+,K^+-ATPase activity in cortical collecting tubules of adrenalectomized rats preincubated for 3 hr at 37°C in the absence of hormone (open columns) or in the presence of $10^{-8}M$ aldosterone plus $10^{-8}M$ triiodothyronine (solid columns), under normal conditions (Control) or in the presence of 5 μ*M* actinomycin D (Actino) or 20 μ*M* cycloheximide (Cyclo). [Redrawn from El Mernissi and Doucet (1984b) and Barlet-Bas *et al.* (1988).]

Bas *et al.*, 1988). These results may indicate that aldosterone induces either the synthesis of new Na^+,K^+-ATPase units or the unmasking of latent sites.

The fact that actinomycin D and cycloheximide abolish the stimulation of Na^+,K^+-ATPase activity induced by aldosterone *in vitro* (Fig. 6B) indicates that control of the pump activity is related to *de novo* protein synthesis. However, this does not demonstrate that aldosterone induces the synthesis of Na^+,K^+-ATPase itself. Indeed, aldosterone might induce the synthesis of proteins which, in turn, would control the recruitment of preexisting, inactive Na^+,K^+-ATPase units. At the present time, it is not unequivocally demonstrated that synthesis of Na^+,K^+-ATPase is induced by aldosterone in the mammalian kidney, although further pieces of evidence support this hypothesis (see Section IV,B).

Conversely, it is now clearly established that in amphibian cells Na^+,K^+-ATPase is indeed an aldosterone-induced protein. On one hand, Geering *et al.* (1982) reported that in toad bladder the rate of biosynthesis of immunoprecipitable α and β subunits of Na^+,K^+-ATPase increases

two- to threefold in response to aldosterone after a lag of 3–6 hr. This effect is abolished in the presence of actinomycin D (Rossier, 1984). On the other hand, Verrey *et al.* (1987) have shown that on A6 cells, aldosterone increases the rate of synthesis of Na^+,K^+-ATPase and the number of cytoplasmic mRNAs encoding for its α and β subunits in parallel.

It should be noted, however, that although aldosterone increases the rate of synthesis of Na^+,K^+-ATPase in A6 cells, the size of the total immunoprecipitable cellular pool of the pump is apparently not altered by the hormone (Paccolat *et al.*, 1987), in opposition to our observations in the mammalian collecting tubule. Thus, the authors postulated the presence of two distinct pools of Na^+,K^+-ATPase in amphibian kidney cells: a large constitutive aldosterone-independent pool and a small pool that is controlled by aldosterone. With regard to this hypothesis, results from our laboratory would suggest the presence of a preponderant aldosterone-sensitive pool of Na^+,K^+-ATPase in the mammalian collecting tubule. This does not preclude the existence of a small aldosterone-insensitive pool of Na^+,K^+-ATPase, which could be responsible for the residual pump activity measured in the collecting tubule of ADX mammals. These two pools of Na^+,K^+-ATPase might correspond to several isoforms of Na^+,K^+-ATPase expressed in different proportions in amphibian and mammalian aldosterone target cells.

B. Is Stimulation of Na^+,K^+-ATPase a Primary Aldosterone Effect?

In ADX rats, administration of aldosterone decreases urinary sodium excretion and increases Na^+,K^+-ATPase activity in the CCT with similar dose dependency (Fig. 7A) and time course (Fig. 7B). This suggests that changes in pump activity are primary hormone effects responsible in part for the antinatriuretic action of aldosterone. It should be stressed, however, that the kinetics of stimulation of Na^+,K^+-ATPase in the collecting tubule vary with the aldosterone status of the animal prior to the administration of aldosterone. Thus, chronic administration of DOCA to adrenal-intact rabbits increases Na^+,K^+-ATPase activity in their CCTs (Garg *et al.*, 1981), but the stimulation appears only after a 24-hr lag period (El Mernissi *et al.*, 1983; O'Neil and Hayhurst, 1986). In fact, there is a linear relationship between the basal Na^+,K^+-ATPase activity in the collecting tubule and the latency necessary to observe its threshold stimulation in response to aldosterone injection. The higher the basal activity (normal versus mineralocorticoid-deficient animals), the longer the lag (Hayhurst and O'Neil, 1988). This observation suggests that the response to aldosterone depends on the differentiation state of the cells. Such a hypothesis is also supported by studies of A6 cells (Paccolat *et al.*, 1987) indicating

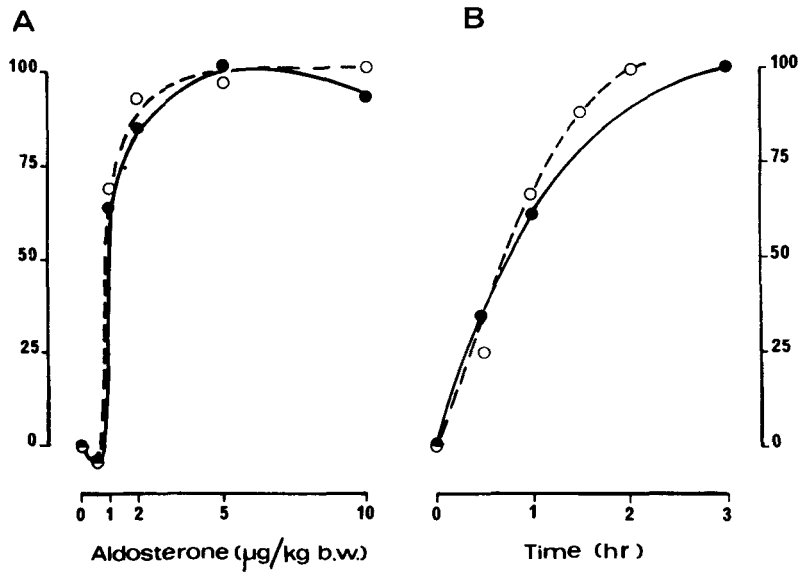

FIG. 7. Changes in Na^+,K^+-ATPase activity in cortical collecting tubule (solid symbols) and urinary sodium excretion (open symbols) after administration of aldosterone to rats adrenalectomized 5–8 days before study. Sodium excretion was determined by clearance technique on anesthetized animals. Values are expressed as the percentage of the maximal changes of both parameters (which correspond to their restoration to levels in normal rats). (A) Dose dependency was performed 2.5 hr after administration of various intravenous doses of aldosterone (0.5–10 µg/kg body wt). (B) Time dependency was studied after injecting a single intravenous dose of aldosterone (5 µg/kg body wt). (Redrawn from El Mernissi and Doucet, 1983a.)

that the delay observed between changes in the rate of synthesis of Na^+,K^+-ATPase subunits, on one hand, and changes in sodium transport as measured by the short-circuit current, on the other hand, varies with cell culture conditions, and thus with the differentiation of the cells. Nevertheless, it should be noted that *in vitro* rapid addition of aldosterone (with a 30-min latency) stimulates Na^+,K^+-ATPase in CCTs isolated from normal rats (Barlet-Bas *et al.*, 1988), conversely to what is observed *in vivo*. This difference in time course may stem from the loss of some negative regulatory parameters in the *in vitro* system.

The kinetic evidence for a primary effect of aldosterone on Na^+,K^+-ATPase in the mammalian collecting tubule is not absolute, since hormone action on the enzyme was determined on target nephron segments exclusively (whereas that on sodium handling was evaluated on the whole

animal) and since aldosterone elicits extrarenal actions. Thus, this issue was investigated in the *in vitro* system, in particular with regard to the possible role of increased intracellular sodium as a second messenger for the induction of Na^+,K^+-ATPase in response to aldosterone. This hypothesis was proposed by Petty *et al.* (1981), who reported that the stimulatory action of aldosterone on Na^+,K^+-ATPase of the rabbit CCTs is suppressed by administration of the luminal sodium channel blocker amiloride (Palmer and Frindt, 1986) prior to aldosterone. Thus, they proposed that one of the primary actions of aldosterone is to enhance luminal sodium entry, and thereby to increase intracellular sodium concentration. Since it is usually admitted that the sodium pump works below its V_{max}, increased intracellular sodium concentration would immediately activate the pump (a phenomenon not measurable by the authors, since enzymatic assay is always run under V_{max} conditions) and later would induce the synthesis of new pump units and thereby increase its V_{max}. However, *in vitro* addition of concentrations of amiloride sufficient to block selectively the apical sodium channels of the CCT fails to alter the aldosterone-induced stimulation of Na^+,K^+-ATPase in the CCTs of ADX rats (Fig. 8A). Furthermore, aldosterone elicits a normal stimulation of the pump when CCTs are incubated in a medium containing choline chloride instead of sodium choride (Fig. 8B). Conversely, *in vitro* addition of high concentrations of amiloride (200 μM), which might have been attained in the *in vivo* study mentioned above, does abolish the induction of Na^+,K^+-ATPase by aldosterone (Barlet-Bas *et al.*, 1988). This action of amiloride, which is observed even in a sodium-deprived medium, is likely related to other inhibitory actions of this drug on either the Na^+–H^+ exchanger (Kinsella and Aronson, 1980), which has been involved in aldosterone action (Oberleithner *et al.*, 1987; Weigt *et al.*, 1987), or on protein synthesis (Leffert *et al.*, 1982), or even on Na^+,K^+-ATPase itself (Soltoff and Mandel, 1983).

Thus, the rapid induction of Na^+,K^+-ATPase in response to aldosterone is independent of alterations in intracellular sodium content. This conclusion is also supported by the following evidence: (1) in various types of cultured cells, induction of Na^+,K^+-ATPase synthesis by increased intracellular sodium level is observed only after a 24-hr latent period (Boardman *et al.*, 1975; Wolitzky and Fambrough, 1986) and (2) decreasing intracellular sodium concentration by chronic administration of amiloride or furosemide, which block luminal sodium entry in specific nephron segments, does not reduce Na^+,K^+-ATPase activity in their targets, i.e., the collecting tubule and the thick ascending limb, respectively (El Mernissi and Doucet, 1984c; Scherzer *et al.*, 1987).

These results constitute a body of evidence indicating that induction of Na^+,K^+-ATPase in the mammalian collecting tubule is a primary aldoste-

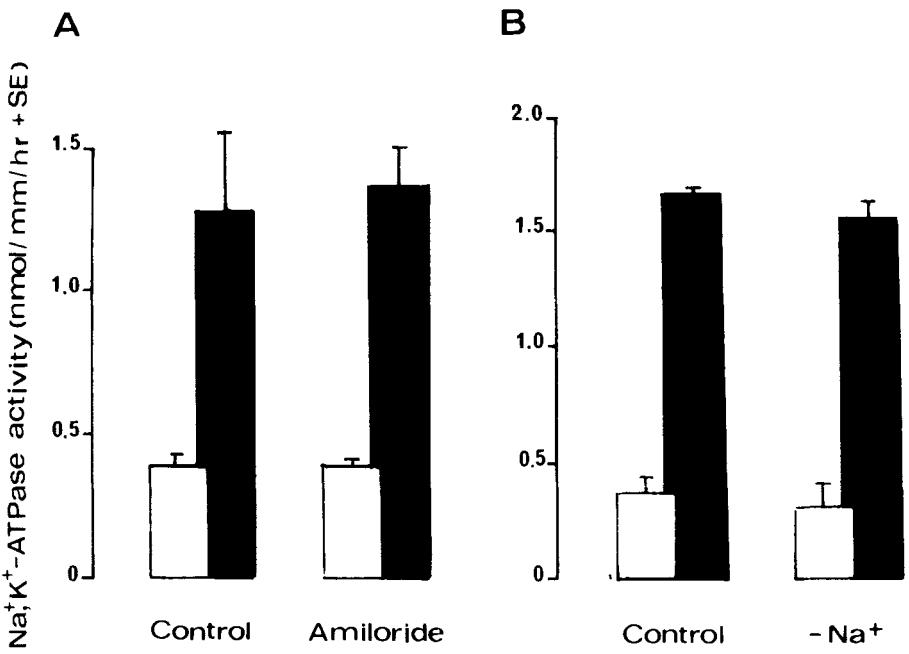

FIG. 8. Na^+,K^+-ATPase activity in cortical collecting tubules from adrenalectomized rats preincubated for 3 hr at 37°C in the absence of hormone (open columns) or in the presence of 10^{-8} *M* aldosterone plus 10^{-8} *M* triiodothyronine (solid columns). (A) Tubules were incubated either under normal conditions (control) or in the presence of 5–50 μ*M* amiloride (amiloride). (B) Incubation was carried out either under normal conditions (Control) or in a medium in which choline chloride was substituted for NaCl (-Na^+). (Redrawn from Barlet-Bas *et al.*, 1988.)

rone action. Definitive demonstration of this issue was recently obtained in A6 cells, in which inhibition of mRNA translation by cycloheximide curtailed by over 90% the aldosterone-induced stimulation of the short-circuit current across the cellular monolayer but did not prevent the aldosterone-dependent increase of synthesis of the mRNAs encoding for the two subunits of Na^+,K^+-ATPase (Verrey *et al.*, 1987).

That induction of Na^+,K^+-ATPase synthesis is a primary action of aldosterone does not preclude that other primarily induced proteins are also necessary to elicit the final physiological effect of mineralocorticoids in collecting tubule principal cells. Indeed, aldosterone has a pleiotropic action at the level of the genome. In particular, it must control in a coordinate fashion the activity of the proteins involved in sodium translocation at the apical and basolateral poles of the cells, since it increases transcel-

lular sodium transport without altering intracellular sodium concentration.

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Chapter 11

Modulation of Na^+,K^+-ATPase Expression in Renal Collecting Duct

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I. INTRODUCTION

The renal cortical collecting duct (CCD) plays a central role in maintaining Na^+ and K^+ balance in the mammal by regulating the rates of Na^+ reabsorption from, and K^+ secretion into, the tubular lumen of this early collecting duct segment. Numerous humoral, neural, and physical factors are known to modulate the rates of ion transport in these cells, although the interrelationships among these factors is only partially understood. Perhaps the most important and well-characterized modulator of Na^+ and K^+ transport in this segment is the mineralocorticoid hormone aldosterone. Elevation of plasma aldosterone levels is known to bring about an increased capacity of the CCD cells (principal cells) to reabsorb Na^+ and to secrete K^+ (Koeppen and Giebisch, 1985; O'Neil,

1987; O'Neil and Helman, 1977; Sansom *et al.*, 1987; Sansom and O'Neil, 1985, 1986; Schwartz and Burg, 1978; Stokes, 1981, 1985; Wingo *et al.*, 1985). Underlying this action of aldosterone is an up-regulation in the Na^+ and K^+ conductances of the luminal (apical) membrane and in the K^+ conductance and Na^+,K^+-ATPase activity of the basolateral (antiluminal) membrane (El Mernissi and Doucet, 1983a,b; Garg *et al.*, 1981; Horster *et al.*, 1980; Koeppen and Giebisch, 1985; O'Neil, 1987; O'Neil and Hayhurst, 1985b; Petty *et al.*, 1981; Sansom *et al.*, 1987; Sansom and O'Neil, 1985, 1986; Stokes, 1985).

The actions of aldosterone on Na^+ and K^+ transport in the CCD may in part be modulated by other factors such as Na^+ entry into the cells. It has long been known that the Na^+ load presented to renal tubular cells can modulate the Na^+,K^+-ATPase levels and the actions of aldosterone on this enzyme (Charney *et al.*, 1974; Hendler *et al.*, 1972; Jørgensen, 1968; O'Neil and Hayhurst, 1985b; Westenfelder *et al.*, 1977). More recently, it has been demonstrated that alterations in Na^+ entry into and/or Na^+ accumulation within a variety of cell types can modulate the expression of the Na^+,K^+-ATPase at the plasma membrane and the rate of synthesis of the enzyme (Boardman *et al.*, 1974; Bowen and McDonough, 1987; Kim and Smith, 1986; Pressley *et al.*, 1986; Rayson and Gupta, 1985; Wolitzky and Fambrough, 1986). In the rabbit CCD, it has been observed that conditions that alter Na^+ entry into the cells may modulate the actions of aldosterone on Na^+,K^+-ATPase (Hayhurst and O'Neil, 1988; O'Neil and Hayhurst, 1985b; Petty *et al.*, 1981) and on apical membrane K^+ conductance (Sansom *et al.*, 1987), although this view is not without controversy in *in vitro* studies of nonperfused rat CCD (see Chapter 10 by Doucet and Barlet-Bas, this volume). Nonetheless, Na^+ entry, or a related factor, such as cell volume, may play a pivotal role in modulating certain actions of aldosterone.

The purpose of this manuscript is to present work from the author's laboratory on the aldosterone-induced expression of the Na^+,K^+-ATPase activity in the rabbit CCD and the role of Na^+ entry in modulating this expression. Emphasis is on the interrelationship of early and late phases of aldosterone actions, the possible modes of up-regulation of Na^+,K^+-ATPase activity, and the signal transduction paths by which Na^+ entry could act as a modulator.

II. CELLULAR MODEL FOR SODIUM AND POTASSIUM TRANSPORT IN CORTICAL COLLECTING DUCT

The cell ultrastructure and transport properties of the rabbit CCD have been studied in detail by several laboratories. The CCD is composed of two cell types—the intercalated cell, accounting for ~30% of all cells,

and the principal cell, accounting for the remaining 70% of all cells (Kaissling and Kriz, 1979; O'Neil and Hayhurst, 1985a; Wade *et al.*, 1979; Welling *et al.*, 1981). The principal cell is responsible for Na^+ and K^+ transport, while the intercalated cell appears to be primarily responsible for H^+ ion and/or bicarbonate transport (see O'Neil, 1987; O'Neil and Hayhurst 1985a). It is now known that the principal cell of the CCD is a major target site for aldosterone and other factors that modulate Na^+ and K^+ excretion (Katz, 1982; Marver and Kokko, 1983; Morel and Doucet, 1986; O'Neil, 1987).

The basic model of Na^+ and K^+ transport in the principal cell of the CCD is shown in Fig. 1. The initial step in Na^+ absorption is passive entry across the luminal (apical) cell membrane via an amiloride-sensitive Na^+-conductive pathway (O'Neil, 1987). Sodium is then actively extruded into the peritubular interstitial space (blood side) across the basolateral cell membrane via the ouabain-sensitive Na^+–K^+ exchange pump. The reabsorption of Na^+ is paralleled by secretion of K^+, beginning with active uptake of K^+ into the cell in exchange for Na^+ via the Na^+–K^+ exchange pump (normally with a Na^+–K^+ coupling ratio of 3 : 2; see Sansom and O'Neil, 1986). From the cell, potassium moves passively down its electrochemical gradient across the apical cell membrane via a major

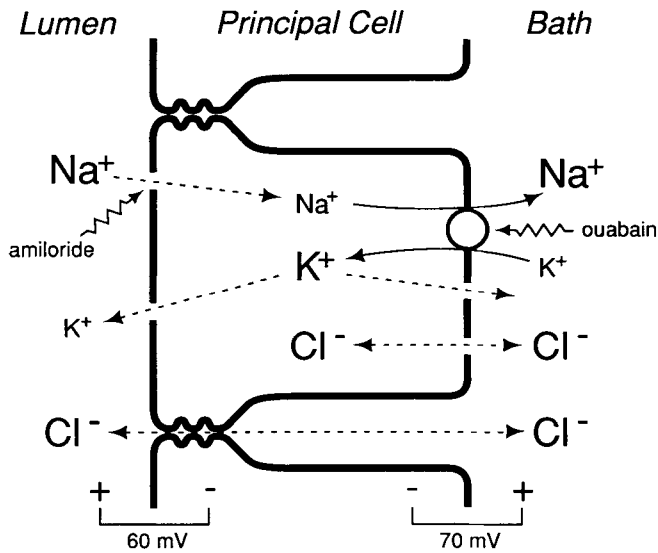

FIG. 1. Cellular model for Na^+ and K^+ transport for the rabbit cortical collecting duct principal cell. Solid lines indicate active transport and dashed lines indicate passive transport. The sites of amiloride inhibition of the Na^+ conductance and ouabain inhibition of the Na^+–K^+ exchange pump (Na^+,K^+-ATPase) are indicated.

K^+-conductive pathway, giving rise to K^+ secretion. This parallel pathway for K^+ secretion, like the Na^+-absorptive pathway, is modulated by aldosterone and, hence, is an important component of aldosterone response in the mammalian kidney. It has recently been shown, as discussed below, that aldosterone brings about an up-regulation of the transport processes for Na^+ and K^+ at both the apical and basolateral membranes. The rate of up-regulation for some processes, such as the Na^+,K^+-ATPase activity, displays a remarkable degree of variation, depending upon initial conditions, as might be expected for a process that may have to adapt to a wide range of acute and chronic Na^+ and K^+ loads.

III. CELLULAR ACTIONS OF ALDOSTERONE

A. Sodium- and Potassium-Conductive Pathways

Once it had been established that the principal cell of the CCD was responsible for Na^+ absorption and K^+ secretion, studies on the action of aldosterone focused on modulation of the principal cell conductive pathways. Early studies utilized adrenal intact animals to assess the influence of elevating plasma aldosterone levels, or an aldosterone analog, deoxycorticosterone acetate (DOCA), on the properties of the principal cells in CCDs isolated from these mineralocorticoid-treated animals. In experiments in which the isolated tubules were perfused *in vitro* after treatment and studied using microelectrode techniques, it was demonstrated that mineralocorticoid treatment for several days caused an increase of both the Na^+ and K^+ conductances of the apical cell membrane (Sansom and O'Neil, 1985). The up-regulation of these conductive pathways was, in large part, responsible for the increase in Na^+ absorption and K^+ secretion estimated for these cells (Sansom and O'Neil, 1985).

In more recent microelectrode studies on adrenalectomized (ADX), aldosterone-depleted rabbits, it was also demonstrated that DOCA treatment caused an increase in the apical membrane Na^+ and K^+ conductances (Sansom *et al.*, 1987). The increase differs from that observed in the adrenal-intact animals in that it is very rapid. In the ADX animals not treated with aldosterone, the Na^+ and K^+ apical cell membrane conductances are markedly suppressed compared to normal conditions, but within a few hours of DOCA treatment, the ionic conductances have increased to, or above, the normal values (Sansom *et al.*, 1987). This short latent period of hormone action contrasts, at least for the K^+ conductance, with the long latent period of several days observed for adrenal-

intact animals as noted above (Sansom and O'Neil, 1985). The reason for these differences is not known, but may be related to other secondary factors as discussed below.

B. Na^+,K^+-ATPase Activity

A second action of mineralocorticoids on the CCD is on the Na^+–K^+ exchange pump. This is readily observed as an increase in the Na^+,K^+-ATPase activity measured under V_{max} conditions for the enzyme. The enzyme activity is again measured in tubules isolated from mineralocorticoid-treated animals. The isolated tubules are permeabilized and the ouabain-sensitive activity is measured for the whole tubule, using a microenzymatic coupling assay as described previously (O'Neil and Dubinsky, 1984; O'Neil and Hayhurst, 1985b). The V_{max} Na^+,K^+-ATPase activity measured under these conditions closely parallels both the number of ouabain binding sites of cell-intact tubules (El Mernissi and Doucet, 1984; Mujais *et al.*, 1985) and the absolute area of the principal cell basolateral membrane (Kaissling and Le Hir, 1982; Le Hir *et al.*, 1982; O'Neil, 1987). It has also been shown, using an antibody to the α subunit of the Na^+,K^+-ATPase, that the basolateral cell membrane of the principal cell contains most of the Na^+,K^+-ATPase enzyme, with nearly undetectable levels associated with the basolateral membrane of the intercalated cell (Kashgarian *et al.*, 1985). Hence, the V_{max} Na^+,K^+-ATPase activity in large part reflects the activity at the basolateral membrane of the principal cell and can be used as an index of the number of enzyme units or sodium pump sites at that membrane.

Using adrenal-intact animals, treatment with DOCA for 3–4 days was observed to cause a doubling in the V_{max} Na^+,K^+-ATPase activity of CCD (O'Neil and Hayhurst, 1985b). Similar treatment with the glucocorticoid dexamethasone had no influence on the Na^+,K^+-ATPase activity, confirming that the actions of DOCA were due to its mineralocorticoid, and not glucocorticoid, activity. Furthermore, we have shown that if adrenal-intact animals are first modestly aldosterone-depleted by maintaining the animals on a high-Na^+, low-K^+ diet for 2 weeks, then treated with physiological levels of exogenous aldosterone (osmotic minipump infusion) for 1–2 days, the Na^+,K^+-ATPase activity is up-regulated (Hayhurst and O'Neil, 1988). Numerous other laboratories have demonstrated that in ADX animals, aldosterone treatment produces an increase in the Na^+,K^+-ATPase activity of CCD within a few hours. It is evident that in all cases aldosterone does stimulate the enzyme activity, but this stimulation is associated with a highly variable latent period (see Section IV).

C. Early and Late Phases of Aldosterone Actions

It was originally proposed, for the toad urinary bladder, that the action of aldosterone on Na^+ transport involved two phases. There was an early phase in which the aldosterone-induced stimulation of Na^+ transport was associated with an increase in tissue conductance due to activation of apical membrane Na^+ channels (Garty, 1986; Spooner and Edelman, 1975). This was followed by a late phase in which the rate of Na^+ transport continued to increase, but without an increase in tissue conductance (Spooner and Edelman, 1975). This late phase has subsequently been shown to be associated with increased synthesis of the α and β subunits of the Na^+,K^+-ATPase and, hence, is thought to reflect increased expression of active pump sites (Geering *et al.*, 1982, 1985; Paccolat *et al.*, 1987).

The actions of aldosterone on Na^+ and K^+ transport processes of the cortical collecting duct likewise appear to be associated with two distinct phases. This is evident by comparing the time-dependent actions of aldosterone on the apical membrane Na^+ and K^+ conductances of the principal cells with the V_{max} Na^+,K^+-ATPase activity. This laboratory has shown that treatment of adrenal-intact rabbits with mineralocorticoid for 1 day causes a doubling in the apical cell membrane Na^+ conductance without influencing either the apical membrane K^+ conductance or the V_{max} Na^+,K^+-ATPase activity (O'Neil, 1987; O'Neil and Hayhurst, 1985b; Sansom and O'Neil, 1985, 1986). However, by 2–3 days of treatment, both the apical membrane K^+ conductance and the Na^+,K^+-ATP-

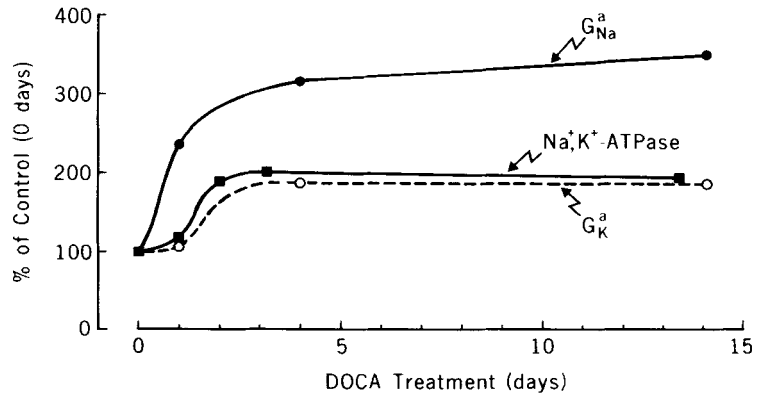

FIG. 2. Time-dependent actions of mineralocorticoids on the cortical collecting duct apical cell membrane Na^+ conductance (G^a_{Na}), apical cell membrane K^+ conductance (G^a_K), and V_{max} Na^+,K^+-ATPase activity. [Modified from O'Neil (1987), O'Neil and Hayhurst (1985b), and Sansom and O'Neil (1985).]

ase activity have likewise doubled. This is depicted in Fig. 2, where it is evident that the early phase of aldosterone actions is associated with an up-regulation of the apical membrane Na^+ conductance, while the late phase is associated with an up-regulation of the K^+ conductance and the Na^+,K^+-ATPase activity. In animals which were initially aldosterone-depleted, this same two-phase response appears to be evident, but with a much more rapid late phase (Petty *et al.*, 1981; Sansom *et al.*, 1987). This may account for the up-regulation of the Na^+,K^+-ATPase activity under these conditions within a few hours, as discussed in the next section.

IV. MODULATION OF ALDOSTERONE ACTIONS

A. Effects of Amiloride

The initial action of aldosterone in the CCD is an up-regulation of the apical membrane Na^+ conductance, causing a stimulation of Na^+ influx during the early phase (Sansom and O'Neil, 1985). The stimulation in Na^+ influx could, in part, be responsible for the actions of aldosterone treatment on the K^+ conductance and the Na^+,K^+-ATPase activity during the late phase; i.e., the early phase may be a primary event, while the late phase may be a secondary event and not a direct action of aldosterone.

To evaluate the role of Na^+ entry on the Na^+,K^+-ATPase activity, the Na^+ channel blocker, amiloride, has often been employed as a probe. It was first shown by Petty *et al.* (1981) that if ADX rabbits were treated with amiloride, the aldosterone-induced increase in the Na^+,K^+-ATPase activity of the CCD was abolished. In adrenal-intact rabbits, studies from the author's laboratory have shown also that treatment with amiloride (10 mg/kg, twice daily by subcutaneous injection) markedly reduces the aldosterone-induced increase in Na^+,K^+-ATPase activity of the CCD (O'Neil and Hayhurst, 1985b; Hayhurst and O'Neil, 1988), as shown in Fig. 3. The entire late phase of aldosterone actions may be influenced by Na^+ entry, as it has been shown that, in CCDs from adrenalectomized rabbits studied with microelectrode techniques, amiloride treatment reduced both the DOCA-induced increase in the apical membrane K^+ conductance and the V_{max} of the sodium pump current, but not the up-regulation of the apical membrane Na conductance (Sansom *et al.*, 1987).

The use of amiloride as a probe for Na^+ entry in the above studies must be viewed with some caution, as it has been shown that, with high concentrations of amiloride near 1 m*M* or more, numerous cellular functions can be impaired, such as Na^+–H^+ exchange activity (Benos, 1982) and protein synthesis (e.g., Besterman *et al.*, 1984; Lubin *et al.*, 1982). In studies from this laboratory employing amiloride injections of 10 mg/

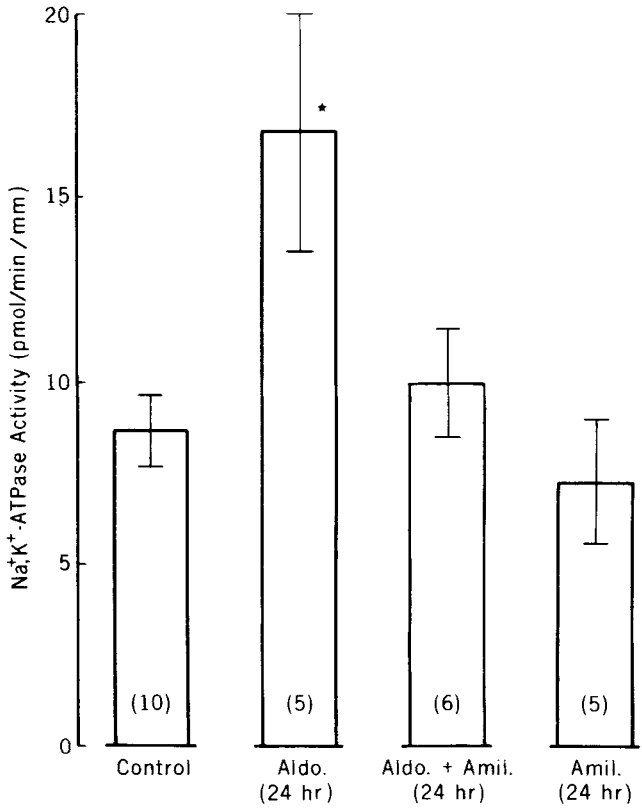

FIG. 3. Effect of aldosterone (aldo.) and amiloride (amil.) on the cortical collecting duct V_{max} Na^+,K^+-ATPase activity. Note that amiloride treatment abolishes the actions of aldosterone. See text for details. *, $p < 0.01$ versus control. The number in each treatment group is shown in parentheses. (From Hayhurst and O'Neil, 1988.)

kg (twice daily, subcutaneously), the extracellular fluid concentration of amiloride should be well below 1 m*M*. Indeed, even assuming that all of the injected amiloride immediately distributed in the total body water, the peak concentration of amiloride could approach 60 μ*M* maximum (assuming total body water of 60% of the total body weight). The amiloride concentration, which is likely much lower than this calculated maximum peak value, is well below the level in which other actions of amiloride are likely to be observed, but is still within a range which would block Na^+ entry into collecting duct cells [K_i of amiloride block of Na^+ entry via the apical membrane Na^+ conductance = 0.07 μ*M* (O'Neil and Boulpaep, 1979)]. It is most probable, therefore, that the influence of amiloride on

the aldosterone-induced upregulation of the Na^+,K^+-ATPase in the rabbit CCD reflects a secondary effect of reducing Na^+ entry across the apical cell membrane into the cells.

B. Sodium-Dependent Modulation of Na^+,K^+-ATPase Activity

It has long been recognized that delivery of Na^+ to the renal tubule can play a major role in modulating the Na^+,K^+-ATPase activity of kidney cell microsomal preparations (Jørgensen, 1968; Westenfelder *et al.*, 1977) and could markedly influence the actions of mineralocorticoids on this enzyme (Charney *et al.*, 1974; Hendler *et al.*, 1972; O'Neil and Hayhurst, 1985b). As an alternative method to evaluate the role of Na^+ entry in modulating the actions of aldosterone on the CCD Na^+,K^+-ATPase activity, studies were performed to evaluate aldosterone-induced up-regulation of the Na^+,K^+-ATPase activity of CCD under conditions in which Na^+ delivery to the CCD, and hence Na^+ entry into the cells across the apical border, was markedly reduced (O'Neil and Hayhurst, 1985b). Rabbits with intact adrenal glands were initially maintained on a high-Na^+ diet to moderately aldosterone-deplete the animals. After 1 week or more on this diet, the chow was changed to a low-Na^+ diet and the animal was injected with a diuretic (1 mg/kg Lasix, intravenously, on days 1 and 4) to rapidly Na^+-deplete the animals. Since this treatment stimulates endogenous aldosterone secretion due to Na^+ and volume depletion, exogenous aldosterone is not required. This treatment reduces the amount of Na^+ delivered to the collecting duct in the ensuing days. An index of the concentration of Na^+ in the CCD tubular lumen can be obtained from the urinary Na^+ concentration, since urinary Na^+ excretion will closely mirror the delivery of Na^+ from the CCD under these conditions.

The effects of the low-Na^+ diet on serum aldosterone levels, V_{max} Na^+,K^+-ATPase activity of CCD, and urinary Na^+ concentration are given in Fig. 4. As evident, the urinary Na^+ concentration, and hence the Na^+ concentration in the CCD tubular lumen, fell to 20% of the control value (to ~18 m*M*) within 3–4 days of treatment, and approached a minimal sustained value of 2% of the control value (to ~3 m*M*) after 6–7 days and beyond. The serum aldosterone levels increased by nearly fourfold at 3–4 days of treatment, and then continued to increase slowly over the remaining treatment period. Interestingly, the Na^+,K^+-ATPase activity was initially elevated at 3–4 days of treatment, supposedly due to continued Na^+ entry into the cells as a result of the moderate Na^+ concentration still present in the CCD tubular fluid. However, as the luminal Na^+ concentration was depressed to very low levels near 3 m*M*, well below the apparent K_m for Na^+ entry of 25–30 m*M* (Stokes, 1981), the Na^+,K^+-

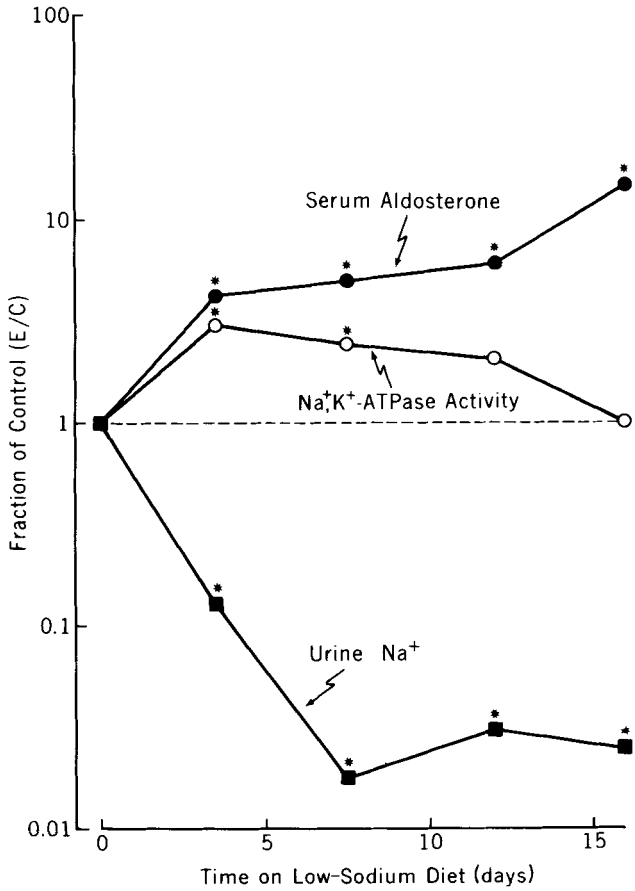

FIG. 4. Time-dependent effects of a low-Na^+ diet on rabbit serum aldosterone levels, urine Na^+ concentration, and V_{max} Na^+,K^+-ATPase activity of cortical collecting duct. The animals were initially maintained on a high-Na^+ diet for 1 week during a control period. On day 0, the diet was changed to a low-Na^+ diet and the changes in the indicated parameters were followed for 2 weeks. All results are expressed as experimental values (E) divided by those for the initial control period (C). During the control period, the serum aldosterone concentration was 16 ng/dl, the urine Na^+ concentration was 151 mM, and the V_{max} Na^+,K^+-ATPase activity of the cortical collecting duct was 13 pmol ADP/min/mm. The urine Na^+ concentration provides an index of the Na^+ concentration of the tubular fluid leaving the cortical collecting duct *in situ* under the conditions of the experiment. It is apparent that early after switching to the low-Na^+ diet, the serum aldosterone levels and Na^+,K^+-ATPase activity are both elevated. However, when urine Na^+ concentration is reduced to minimal values near 2% of the control (3 mM), reflecting reduced Na^+ concentrations in the cortical collecting duct tubular fluid and hence reduced Na^+ entry into the cells, the Na^+,K^+-ATPase activity decreases toward control values despite elevated serum aldosterone levels. See text for additional details. *, $p < 0.05$ versus day 0. (Modified from O'Neil and Hayhurst, 1985b.)

ATPase activity declined, returning to the initial control values within 2 weeks. The half-time of the down-regulation of the Na^+,K^+-ATPase activity was approximately 6–7 days. Since the serum aldosterone was elevated to very high levels during the down-regulation of the Na^+,K^+-ATPase activity, the decline in activity likely occurs as a result of reduced Na^+ entry across the apical border of the principal cells.

Over the past few years, it has become increasingly apparent that Na^+ entry into cells may play a central role in modulating the number of sodium pump sites. It has been shown in a variety of cell types, including HeLa cells (Boardman *et al.*, 1974), chick skeletal muscle cells (Wolitzky and Fambrough, 1986), chick cultured ventricular cells (Kim and Smith, 1986), cultured rat liver cells (Pressley *et al.*, 1986), renal medullary cells (Rayson and Gupta, 1985), and renal cultured Madin–Darby canine kidney (MDCK) cells (Bowen and McDonough, 1987), that increased Na^+ entry into cells or elevation of intracellular Na^+ levels can bring about up-regulation of the synthesis and expression of the Na^+,K^+-ATPase molecule. In renal epithelial cells, such as the MDCK cell line (Bowen and McDonough, 1987) or medullary collecting duct cells (Rayson and Gupta, 1985), it has also been observed that exposure of the cells to either a low-K^+ bathing medium or a low concentration of ouabain causes an increase in intracellular Na^+ levels and an up-regulation of the synthesis and expression of the Na^+,K^+-ATPase in these cells.

The heretofore-mentioned effects of Na^+ on the aldosterone-induced modulation of the Na^+,K^+-ATPase of the rabbit cortical collecting duct would be consistent with the view that it is Na^+ entry that modulates the Na^+,K^+-ATPase activity of these cells as well. These studies are in contrast with the actions of aldosterone reported for the toad urinary bladder (Geering *et al.*, 1982) and for the A6 renal cell line (Paccolat *et al.*, 1987). Using a polyclonal antibody to the α and β subunits of the Na^+,K^+-ATPase, these investigators observed that aldosterone stimulated the rate of synthesis of the two subunits—although an increased expression of active enzyme in the form of Na^+,K^+-ATPase activity was not observed.

Exposure of the toad urinary bladder cells to sufficient concentrations of amiloride to block Na^+ entry via the Na^+ channel, but perhaps not via other modes of entry, such as Na^+–H^+ exchange, had no influence on the aldosterone-induced up-regulation of the immunoprecipitated subunits (Geering *et al.*, 1982). Why this tissue does not display Na^+-dependent regulation of the enzyme subunits is not known. It may be that Na^+-dependent modulation involves a posttranslational mechanism. Alternatively, other modes of Na^+ entry may be relevant, since it has been shown that a major fraction of Na^+ entry into toad urinary bladder cells is not blocked by low concentrations of amiloride (Wong and Chase, 1986)—as opposed to the rabbit CCD (Sansom and O'Neil, 1986)—and

that aldosterone can activate Na^+–H^+ exchange processes in some cells (Oberleithner *et al.*, 1987).

C. Time-Dependent Modulation of Na^+,K^+-ATPase Activity

The actions of aldosterone on the Na^+,K^+-ATPase activity of mammalian CCD have been studied in several laboratories. The latent period (response time) for aldosterone-induced up-regulation of the enzyme activity has been reported to be highly variable among laboratories, making it particularly difficult to compare results from the various investigators. Numerous investigators, utilizing ADX animals, have observed short latent periods of from 1–2 hr to as long as 12 hr prior to being able to detect an increase in Na^+,K^+-ATPase activity (El Mernissi and Doucet, 1983a,b; Horster *et al.*, 1980; Mujais *et al.*, 1984, 1985; Petty *et al.*, 1981). In adrenal-intact animals, the latent period for aldosterone actions has been observed to be between 24 and 48 hr (O'Neil and Hayhurst, 1985b). The reason for this wide range of latent periods from a few hours to days is not known, but it does demonstrate the tremendous latitude for regulation underlying this response.

To begin to elucidate the basis of the variable latent period for aldosterone-induced up-regulation of the Na^+,K^+-ATPase activity, studies were initiated to relate the initial state of aldosterone depletion/repletion of rabbits to the latent period of the aldosterone response (Hayhurst and O'Neil, 1988). It was observed that the greater the initial state of aldosterone depletion, the lower the initial Na^+,K^+-ATPase activity and the shorter the latent period. A close correlation was observed between the initial Na^+,K^+-ATPase activity of the CCD prior to aldosterone treatment and the latent period of the up-regulation of Na^+,K^+-ATPase activity after aldosterone treatment. This is depicted in Fig. 5 for three groups of tubules from rabbits with differing initial states of aldosterone depletion/repletion. In normal adrenal-intact, aldosterone-replete animals, the initial Na^+,K^+-ATPase activity was highest (100% of normal) and the latent period was between 24 and 48 hr (midpoint of 36 hr) (O'Neil and Hayhurst, 1985b). In adrenal-intact, moderately aldosterone-depleted rabbits, the initial Na^+,K^+-ATPase activity was reduced to 55% of normal and the latent period decreased to 5–7 hr (Hayhurst and O'Neil, 1988). Finally, in adrenalectomized, severely aldosterone-depleted rabbits, the initial Na^+,K^+-ATPase activity was reduced to 19% of normal and the latent period decreased to 1–3 hr (El Mernissi and Doucet, 1983a; Horster *et al.*, 1980; Petty *et al.*, 1981). In other species, e.g., the mouse (Doucet and Katz, 1981) and the rat (El Mernissi and Doucet, 1983a; Mujais *et al.*, 1984, 1985), a variable latent period has also been observed which, when the initial Na^+,K^+-ATPase activity is considered, is identi-

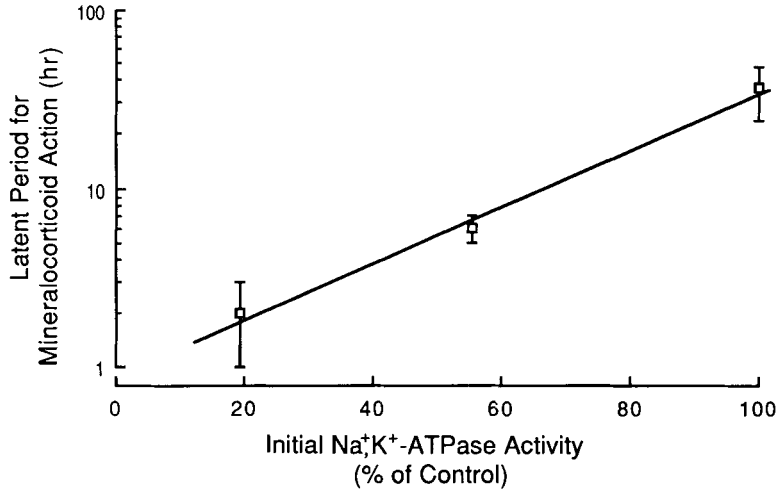

FIG. 5. Relationships between the initial Na^+,K^+-ATPase activity (as a percentage of the normal control values) and the latent period (response time) for mineralocorticoid actions on rabbit cortical collecting duct V_{max} Na^+,K^+-ATPase activity ($r = 0.997$). The bars represent approximate ranges for the latent period and the open squares are midpoints of these ranges. See text for details and references. (From Hayhurst and O'Neil, 1988.)

cal to that predicted from the relationship shown in Fig. 5. Hence, it appears that the latent period is directly related to the initial Na^+,K^+-ATPase activity (or aldosterone status) of the CCD.

The mechanism of regulation responsible for the variations of the latent period for aldosterone actions is not known. In all cases, however, the aldosterone-induced up-regulation of the Na^+,K^+-ATPase activity is blocked by amiloride, and may indicate a role for Na^+ entry (Hayhurst and O'Neil, 1988). As discussed below, differing initial levels of sodium pump sites in the face of aldosterone-induced Na^+ entry across the luminal border could lead to variations in Na^+ loading of cells and/or cell volumes which could, in part, underlie the variations in latent period.

V. CELL VOLUME CHANGES AS PART OF ALDOSTERONE ACTIONS

A. *In Vitro* Actions of Aldosterone on Sodium Transport and Cell Volume

To define more clearly the actions of aldosterone on Na^+ transport in the rabbit CCD, *in vitro* perfusion conditions were established to maintain isolated tubules for 6 hours or more in an artificial medium (R. G. O'Neil

and J. M. Reid, unpublished observations). In the past, most laboratories utilizing *in vitro* perfusion techniques perfused and bathed tubules in a simple Ringer's solution. Tissue viability under these conditions could normally be maintained for only 1–3 hr at 37°C, which precluded studying the long-term affects of some hormones, such as aldosterone. To circumvent the tissue viability problem, tubules were perfused and bathed with a tissue culture medium [Dulbecco's modified Eagle's medium (DMEM)] at 37°C. Under these conditions, CCDs remained viable for 6–12 hr or more (G. Schwartz, personal communication), as confirmed in studies from the author's laboratory (unpublished observations).

Cortical collecting ducts were isolated either from adrenalectomized rabbits or from animals on a high-Na^+, low-K^+ diet for 2 weeks, and perfused and bathed *in vitro* with DMEM at 37°C. The tubules were observed in a study chamber on an inverted Diaphot Nikon microscope using Hoffman Modulation Contrast optics as before (O'Neil and Hayhurst, 1985a). Tubule images were continuously recorded with a video camera (Cohu Vidicon) and stored on videotape (Sony U-matic) for later stop-frame playback and measurement of cell areas from which relative cell volumes (V/V_o) were calculated. The transepithelial voltage was monitored continuously, as described previously (O'Neil, 1983). The perfusion solution was periodically (30-min intervals) changed to DMEM containing 50 μM amiloride for 2–4 min and the amiloride was then flushed from the perfusate. These short pulses of amiloride permitted the amiloride-sensitive voltage to be measured periodically as an index of the active Na^+ transport rate.

To assess the actions of aldosterone, the hormone was added to the bathing medium (1 μM) after a 1- to 2-hr equilibration period, during which cell volumes and membrane voltages reached stable values. After a subsequent latent period of ~2 hr from the time of aldosterone addition, the amiloride-sensitive voltage and cell volume began to increase. Three to five hours after aldosterone addition, the amiloride-sensitive voltage had doubled, increasing from 2.6 to 4.9 mV (mean change = 2.3 ± 0.6 mV, $n = 3$). This would be consistent with an apparent doubling of Na^+ reabsorption. The increase in amiloride-sensitive voltage was accompanied by a significant, although modest, increase in cell volume of 24 ± 8% ($n = 3$). Hence, aldosterone stimulation of Na^+ transport during the apparent early phase was accompanied by significant increases in cell volume (R. G. O'Neil and J. M. Reid, unpublished observations).

In a parallel series of studies, adrenal-intact, moderately aldosterone-depleted rabbits (high-Na^+, low-K^+ diet) were studied as done previously (Hayhurst and O'Neil, 1988) to assess whether, under these conditions, aldosterone actions involved changes in cell volume during the early

phase (R. G. O'Neil, S. T. Vu, and L. T. Garretson, unpublished observations). Aldosterone was continuously infused at physiological levels via a subcutaneously implanted minipump as done before (Hayhurst and O'Neil, 1988). After 8 or 24 hr of aldosterone infusion (corresponding to the latter part of the early phase and late phase, respectively, under these conditions), a kidney was perfusion-fixed *in situ* via infusion of the left renal artery with quarter-strength Karnovsky's fixative diluted with 0.2 *M* sucrose for 5 min, followed by slicing of the cortex into small blocks and bathing of the tissue for an additional 90 minutes in fixative. The tissue was washed in 0.16 *M* sodium cacodylate buffer, postfixed in 1% OsO_4 plus 1.5% $K_4Fe(CN)_6$, washed in deionized water, dehydrated with increasing ethanol solutions, and exchanged gradually for 100% Spurrs medium for embedding. Thick sections were employed at the light microscopic level to measure inner and outer CCD diameters for calculation of total cell volume (intercalated cells were not subtracted from the volumes, which may result in an underestimate of the principal cell volume changes). It was observed that after 8 hr of aldosterone treatment the cell volumes had increased by 44 ± 8% (n = 3), but by 24 hr the volume had returned to initial control values (change from control = 3 ± 3%, n = 3). Hence, even under these conditions, there was an initial transient increase in cell volume during the early phase of aldosterone actions, supposedly due to stimulated Na^+ entry (along with accompanying anion), with a subsequent return of cell volume to near normal values during the late phase, when more Na^+ pumps had been added to the basolateral membrane.

B. Role for Cell Volume in Modulating Aldosterone Actions

Variable changes in cell volume of the CCD during the early phase of aldosterone actions could, in part, underlie the variable latent period of aldosterone on the Na^+,K^+-ATPase. It has been shown that mineralocorticoid treatment of either adrenal-intact, aldosterone-replete rabbits (Sansom and O'Neil, 1985) or ADX, aldosterone-depleted animals (Sansom *et al.*, 1987) causes an up-regulation in the apical membrane Na^+ conductance of the CCD to about the same level in both groups (to ~2 mS cm^{-2}). This occurs despite the fact, as discussed above, that the V_{max} Na^+,K^+-ATPase activity in the adrenalectomized group is initially only about 20% of that in CCDs from adrenal intact animals. It is likely, therefore, that Na^+ influx is similar in all groups during the peak of the early phase, except that aldosterone-depleted animals have fewer active pumps to handle the Na^+ load. The more pronounced the initial degree of aldosterone depletion, the fewer sodium pumps initially present. This would re-

sult in a greater accumulation of Na^+ within the cell, which, in turn, would lead to a greater degree of cell swelling. It is hypothesized that the cell swelling may stimulate signaling pathways to activate the late phase of aldosterone actions. The greater the degree of cell swelling, the greater the degree or rate of stimulation of signaling pathways, which may result in a more rapid activation of the late phase. Hence, variations in cell swelling during the early phase of aldosterone actions could underlie the variations in the latent period of the late phase of aldosterone actions in the CCD, including up-regulation of the Na^+,K^+-ATPase activity.

While the role of cell volume changes in modulating the actions of aldosterone in the CCD is presently a working hypothesis, cell volume changes have been shown to activate a myriad of transport processes and signaling pathways in numerous cell types. It has been shown routinely that swelling of cells can activate various ion transport processes at the plasma membrane, most notably K^+ and Cl^- transport processes (Eveloff and Warnock, 1987), and may include acute activation of sodium pumps (Lewis *et al.*, 1985; Hudson and Schultz, 1984). Levels of some second messengers, such as intracellular calcium, are also elevated during cell swelling (Grinstein *et al.*, 1982; Hoffman *et al.*, 1986; Wong and Chase, 1986). Investigations in the author's laboratory have shown that hypotonic swelling of either the renal proximal straight tubule (McCarty *et al.*, 1988) or the CCD (unpublished observations) causes a transient elevation in the intracellular calcium levels. Furthermore, swelling of the proximal straight tubule has been shown to stimulate turnover of the luminal and basolateral plasma membranes (O'Neil and Reid, 1988). It is becoming increasingly evident that cell volume changes can activate or modulate numerous cellular functions. Since aldosterone actions include an increase in cell volume, volume-dependent pathways/parameters likely play a role in the response of the cell to this steroid hormone. Studies are presently under way to begin to address the critical role of cell volume changes in the actions of aldosterone in the CCD.

VI. SUMMARY

The principal cells of the CCD are a primary target site for aldosterone in the mammalian kidney. This steroid hormone plays a critical role in regulating the rates of Na^+ reabsorption and K^+ secretion by this nephron segment. Elevated aldosterone levels bring about an increased capacity of the principal cells to transport both Na^+ and K^+. The increased capacity for transport arises from an up-regulation of the apical (luminal) cell membrane Na^+ and K^+ conductances and the basolateral cell mem-

brane V_{max} Na^+,K^+-ATPase activity. This up-regulation occurs in two phases: an early phase, associated with an increase in the apical cell membrane Na^+ conductance, and a late phase, associated with an increase in the apical cell membrane K^+ conductance (and supposedly and basolateral cell membrane K^+ conductance) and the basolateral cell membrane V_{max} Na^+,K^+-ATPase activity.

The late phase of aldosterone actions, at least for the up-regulation of the Na^+,K^+-ATPase activity, appears to be modulated by Na^+ entry into the cell across the luminal border during the early phase. Reducing Na^+ entry into the cells, either by treatment with the Na^+ channel blocker amiloride or by reduction of the luminal tubular fluid Na^+ concentration to low levels, abolishes the aldosterone-induced increase in the Na^+,K^+-ATPase activity of the rabbit CCD. The mechanism by which Na^+ entry modulates this late phase may be secondary to changes in cell volume. During the early phase of aldosterone actions, the cells are observed to swell, supposedly due to increased Na^+ entry and accumulation within the cell (along with accompanying anion) prior to up-regulation of the Na^+,K^+-ATPase activity during the late phase. Since cell swelling is known to activate numerous renal cellular functions and parameters, including activation of plasma membrane transport processes, stimulation of membrane turnover, and elevation of certain second messengers such as intracellular calcium activity, the aldosterone-induced cell swelling associated with Na^+ entry during the early phase likely plays a critical role in modulating the actions of aldosterone during the late phase. Indeed, variations in cell swelling during the early phase of aldosterone actions may, in part, underlie the highly variable nature of the latent period for the up-regulation of the Na^+,K^+-ATPase activity observed in the CCD of the rabbit. The signaling pathways by which Na^+ entry and/or cell volume may modulate the actions of aldosterone remain to be elucidated in future studies.

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Chapter 12

Electrogenic Properties of the Na/K Pump: Voltage Dependence and Kinetics of Charge Translocation

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I. INTRODUCTION

The Na/K pump in the plasma membrane of mammalian cells carries out uphill transport of Na^+ and K^+ ions at the expense of free energy of ATP hydrolysis (Skou, 1975; Robinson and Flashner, 1979; Cantley, 1981; Schuurmans Stekhoven and Bonting, 1981; Jørgensen, 1982; Glynn, 1985; Kaplan, 1985). Since (under normal conditions) in a single turnover three Na^+ are moved outward and two K^+ inward, the transport process is associated with the translocation of net charge. The electrogenic nature of the Na/K pump has interesting consequences. The pump acts as a current generator and contributes to the membrane potential of cells. Furthermore, the transport rate becomes a function of transmembrane voltage. Of particular interest is this question: In which step(s) of the transport cycle is charge translocated? This problem may be studied by

recording transient, pump-generated currents after an ATP concentration jump. Further information on the nature of the electrogenic reactions in the pumping cycle may be obtained by investigating the current–voltage behavior of the pump. Both methods will be discussed in the following section.

II. FAST CHARGE TRANSLOCATIONS ASSOCIATED WITH PARTIAL REACTIONS OF THE Na/K PUMP

A. Current Transients after Photochemical Release of ATP

Information on the nature of charge-carrying steps and on the kinetic parameters of the pumping cycle may be obtained from experiments in which nonstationary pump currents are induced by a sudden change of ATP concentration (Fendler *et al.*, 1985; Borlinghaus *et al.*, 1987; Apell *et al.*, 1987; Nagel *et al.*, 1987; Borlinghaus and Apell, 1988). For the measurement of the transient current signals, flat membrane fragments rich in Na^+,K^+-ATPase are bound to a planar lipid bilayer acting as a capacitive electrode (Figs. 1 and 2). The membrane preparation which is

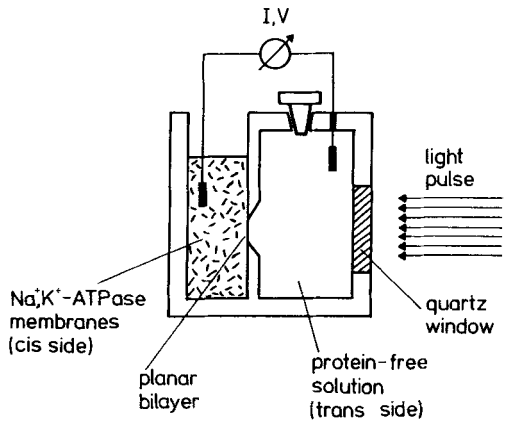

FIG. 1. Cell for the measurement of current and voltage signals from optically black lipid films with adsorbed Na^+,K^+-ATPase membrane fragments. The *trans* compartment was closed after filling with the solution. The solution volumes of the open and closed compartments were 5 and 0.3 ml, respectively; the diameter of the circular membrane was 1.3 mm. A magnetic stirrer (not shown) was present in the *cis* compartment. The solutions were connected to the external measuring circuit via agar bridges and silver/silver chloride electrodes. In order to minimize stray-light artifacts from the electrodes, a suspension of carbon black was added to the agar.

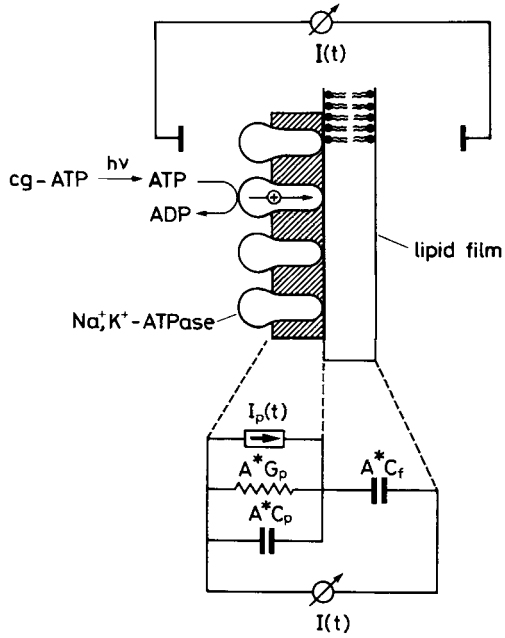

FIG. 2. Equivalent circuit of the compound membrane system consisting of a black lipid film with adsorbed Na^+,K^+-ATPase membrane fragments. Photochemical release of ATP leads to a transient pump current $I_p(t)$. In the external measuring circuit, a time-dependent current $I(t)$ is recorded. G_p and C_p are the specific conductance and the specific capacitance, respectively, per unit area of the membrane fragments; C_f is the specific capacitance of the black film. A^* is the area of the covered part of the black film.

obtained by dodecyl sulfate extraction of kidney microsomes (Jørgensen, 1974) consists of flat membrane sheets 0.2–1 μm in diameter containing oriented Na^+,K^+-ATPase molecules with a density of several thousand per μm^2 (Deguchi *et al.*, 1977; Skriver *et al.*, 1981; Hebert *et al.*, 1982; Zampighi *et al.*, 1986). In the aqueous phase, which is in contact with the bound membrane sheets, ATP is released within milliseconds from an inactive, photolabile derivative ("caged" ATP) by an intense flash of light (Kaplan *et al.*, 1978; McCray *et al.*, 1980). After the ATP concentration jump, which leads to a (nearly) simultaneous activation of many pump molecules, transient current and voltage signals can be recorded in the electrical circuit connecting the aqueous phases adjacent to the lipid bilayer (Fig. 2).

At pH 7.0, ATP is liberated from caged ATP with a time constant of 4.6 msec (McCray *et al.*, 1980). With a starting concentration of caged

ATP of 0.5 m*M*, the concentration of released ATP after a single flash is typically ~50 μ*M*.

1. Transient Current Signals in Absence of K^+

In the experiment represented in Fig. 3, the aqueous solutions contained 150 m*M* Na^+ and 2 m*M* Mg^{2+}, but no K^+. When 0.5 m*M* caged ATP and 40 μg/ml Na^+,K^+-ATPase in the form of membrane fragments

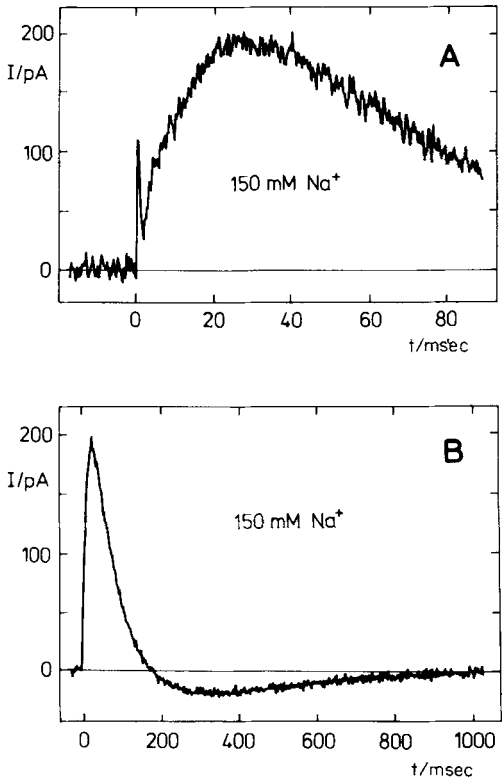

Fig. 3. Current transients in the absence of K^+ after a 40-μsec light flash given at time $t = 0$, recorded at two different time scales. The light pulse from a xenon flash tube passed through a bandpass filter with >50% transparency between 260 and 380 nm. The aqueous solutions contained 150 m*M* NaCl, 50 m*M* Tris–Cl, pH 7.0, 20 m*M* dithiothreitol, and 2 m*M* $MgCl_2$. The temperature was 20°C. Caged ATP (0.5 m*M*) and 40 μg/ml Na^+,K^+-ATPase in the form of membrane fragments were added to the *cis* compartment 20 min prior to the flash experiment. The positive sign of the current corresponds to a translocation of positive charge from the membrane fragment toward the black film (Fig. 2). The area of the black film was 0.64 mm^2. The current spike at time $t = 0$ is also seen in the presence of caged ATP alone, without the addition of protein. (from Borlinghaus *et al.*, 1987.)

were added to the *cis* side, transient current signals could be recorded after a 40 μsec light flash given at time $t = 0$ (Borlinghaus *et al.*, 1987). The current rose within about 30 msec toward a maximum and thereafter declined, reversing its sign at $t \approx 180$ msec. The direction of the current during the first 180 msec corresponds to a translocation of positive charge from the membrane fragment toward the black film (Fig. 2). As will be discussed later, the negative phase of the signal is likely to result from a backflow of charge through the membrane fragments.

The current response to the light flash developed within 20–30 min after addition of the membrane fragments. This slow increase of photosensitivity is likely to reflect the time course of the adsorption of membrane fragments to the black film.

To examine the origin of the observed current signals, the following control experiments were carried out: (1) In the absence of caged ATP, no photoresponse was observed (Fig. 4). (2) In the presence of caged ATP, but without addition of protein, only a brief current spike (which is also seen in Fig. 3) was recorded. This current spike, which was always observed when caged ATP was present in the open compartment, may result from the creation of an interfacial potential by the photochemical reaction of caged ATP adsorbed to the lipid membrane. (3) In the absence of Mg^{2+} (which is required as a cofactor in the phosphorylation of the protein) no flash-induced signal was observed apart from the initial current spike; subsequent addition of 10 m*M* Mg^{2+} to the open compartment restored the normal photoresponse (Fig. 4). (4) Addition of 100 μ*M* ATP prior to flash excitation completely inhibited the photoresponse. A likely explanation of this finding exists in the assumption that, in the presence of ATP, the pump spends most of its time in phosphorylated states and thus is unavailable for phosphorylation following photorelease of ATP. (5) When the membrane fragments were preincubated for 10 min with 4 m*M* ouabain prior to addition to the membrane cell, neither the first nor subsequent light flashes elicited a photoresponse. Addition of ouabain to the open compartment after binding of the membrane fragments resulted in an inhibition that developed over a period of ~10–20 min. (6) In the presence of 50 m*M* K^+ on both sides, but in the absence of Na^+, no flash-induced photosignal was detected. Experiments in which Na^+ and K^+ were simultaneously present will be described later. (7) Orthovanadate (250 μ*M*) in the presence of 150 m*M* Na^+ and 50 m*M* K^+ reduced the photosignal to less than 10% of the control value within 20 min. In the absence of K^+, vanadate had virtually no effect, as expected from the known effect of K^+ on vanadate inhibition (Glynn, 1985).

These results are consistent with the notion that membrane fragments containing Na^+,K^+-ATPase bind with their extracellular side to the lipid film, as schematically depicted in Fig. 2. Photochemically released ATP

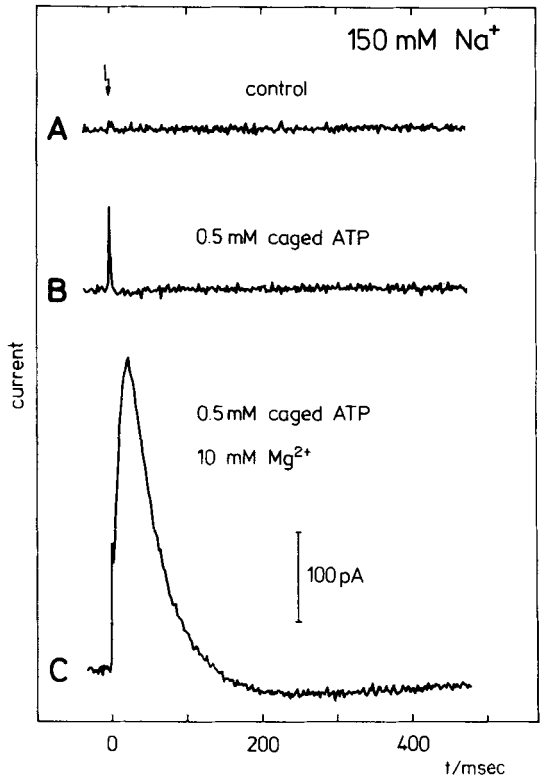

FIG. 4. Current signals in the absence and in the presence of Mg^{2+} and caged ATP after a light-flash of 40 μsec duration at time $t = 0$. (A) Recorded in the absence of caged ATP and of Mg^{2+}. (B) After addition of 0.5 m*M* caged ATP to the *cis* compartment. (C) After additon of 0.5 m*M* caged ATP and 10 m*M* $MgCl_2$ to the *cis* compartment. Both aqueous solutions contained 150 m*M* NaCl, 50 m*M* Tris–Cl, pH 7.0, and 20 m*M* dithiothreitol; 40 μg/ml Na^+,K^+-ATPase in form of membrane fragments was added to the *cis* compartment prior to the first experiment (A). $T = 20°C$. The area of the black film was 0.72 mm^2. All signals were recorded from the same membrane. (From Borlinghaus *et al.*, 1987.)

activates the pump from the cytoplasmic side, leading to a movement of Na^+ toward the extracellular side.

2. CHYMOTRYPSIN-MODIFIED ENZYME

Treatment of Na^+,K^+-ATPase with α-chymotrypsin in the presence of Na^+ at low ionic strength leads to cleavage of a single peptide bond in the α subunit; the split is located in the cytoplasmic portion of the protein between Leu-266 and Ala-267 (Jørgensen and Collins, 1986). Under suit-

able reaction conditions, secondary cleavage is negligible. In the α-chymotrypsin-treated enzyme, phosphorylation by ATP, occlusion of Na^+, and ADP–ATP exchange are preserved, while Na/K pumping and Na^+–Na^+ exchange are abolished (Glynn *et al.*, 1984; Jørgensen and Petersen, 1985). These findings indicate that modification by chymotrypsin stabilizes the enzyme in the E_1 conformation by preventing the $E_1 \rightarrow E_2$ transition.

Experiments with α-chymotrypsin-treated membrane fragments may thus provide information on the nature of the charge-translocating steps. In a first series of experiments, Na^+,K^+-ATPase membranes were preincubated for 10–30 min at 30°C in a medium containing 160 μg/ml α-chymotrypsin, 2 μg/ml Na^+,K^+-ATPase, 10 m*M* NaCl, and 15 m*M* Tris–Cl, pH 8.1. Under these conditions, the proteolytic reaction may be expected to be selective, consisting in the cleavage of a single peptide bond, as discussed above. After addition of the modified membrane fragments to the *cis* compartment, no photoresponse was observed in the usual flash experiment.

In a second series of experiments, bound Na^+,K^+-ATPase membranes were treated with α-chymotrypsin *in situ*. Membrane fragments were added to the *cis* compartment in the presence of 10 m*M* Na^+ and 50 m*M* Tris, pH 7.0 (total ionic strength ~260 m*M*). After a waiting time of ~30 min, the photoresponse was measured as usual (curve labeled "0 min" in Fig. 5). Thereafter, a high concentration of α-chymotrypsin (5 mg/ml) was added to the *cis* compartment, and the photoresponse was recorded again after certain time intervals. As seen in Fig. 5, the amplitude of the current signal progressively decreased within ~30 min, whereas the time course of the signal remained essentially unchanged.

The observation that α-chymotrypsin treatment abolishes the transient current which is normally seen after the adenosine triphosphate (ATP) concentration jump indicates that phosphorylation by ATP and occlusion of Na^+ are electrically silent reaction steps. The mechanistic implications of this finding will be discussed later.

3. Evaluation of Intrinsic Pump Current $I_p(t)$

The compound membrane system, consisting of a black lipid film with adsorbed Na^+,K^+-ATPase membrane fragments may be represented by the equivalent circuit shown in Fig. 2. G_p and C_p are the conductance and the capacitance, respectively, per unit area of the membrane fragments; G_f and C_f refer to the black film (G_p also accounts for conductive pathways parallel to the bilayer in the space between bilayer and membrane fragment). A^* is the area of the covered part of the film.

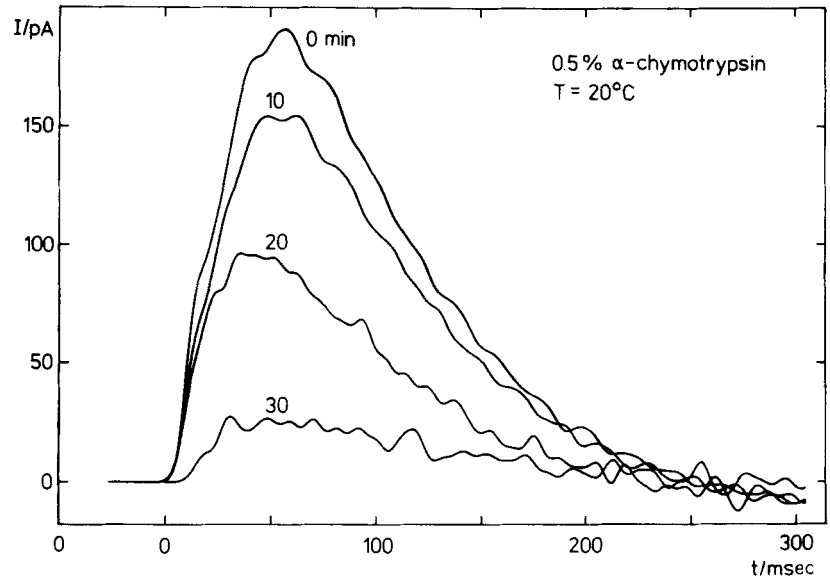

FIG. 5. Modification of bound Na^+,K^+-ATPase membranes by α-chymotrypsin *in situ*. Membrane fragments were added to the *cis* compartment in the presence of 10 m*M* NaCl, 50 m*M* Tris–Cl, 5 m*M* $MgCl_2$, 20 m*M* dithiothreitol, and 0.5 m*M* caged ATP, pH 7.0. After a waiting time of 30 min, the short-circuit current signal was measured after a 40-μsec light flash (curve labeled "0 min"). Thereafter, 5 mg/ml α-chymotrypsin was added to the *cis* compartment,and the photoresponse was recorded again after 10, 20, and 30 min. In control experiments without α-chymotrypsin, the amplitude of the current signal was found to remain nearly constant in the same period.

Flash excitation at time $t = 0$ elicits a time-dependent pump current $I_p(t)$; $I_p(t)$ is defined as the short-circuit current that would be recorded in the external measuring circuit if a continuous layer of oriented membrane fragments, but no lipid film, were present. Under the conditions of a real experiment (Fig. 2), the measurable short-circuit current $I(t)$ is influenced by the electric circuit parameters of the membrane system. If the conductance of the black film is negligible, $I(t)$ and $I_p(t)$ are related in the following way (Borlinghaus *et al.*, 1987):

$$I(t) = \frac{C_f}{C_f + C_p}\left[I_p(t) - \exp(-t/\tau_I)\frac{1}{\tau_I}\int_0^t I_p(t)\exp(t/\tau_I)\,dt\right] \quad (1)$$

$$I_p(t) = \left(1 + \frac{C_p}{C_f}\right)\left[I(t) + \frac{1}{\tau_I}\int_0^t I(t)\,dt\right] \quad (2)$$

$$\tau_I = \frac{C_f + C_p}{G_p} \quad (3)$$

Using Eq. (2), the pump current $I_p(t)$ may be computed from the current signal $I(t)$.

From the decay time of voltage signals measured under open-circuit conditions, a value of $G_p \approx 3$ μS/cm^2 may be estimated for the specific conductance of the membrane fragments (Borlinghaus *et al.*, 1987). Assuming that the membrane fragments have a specific capacitance similar to that of a cell membrane ($C_p \approx 1$ μF/cm^2) and using a value of $C_f \approx 0.37$ μF/cm^2 for the specific capacitance of the diphytanoylphosphatidylcholine bilayer (Benz and Janko, 1976), the quantity ($C_f + C_p$) may be estimated to be ~1–2 μF/cm^2; this yields a value of τ_1 of the order of 0.5 sec.

An example for the evaluation of $I_p(t)$ from $I(t)$ is shown in Fig. 6. It is seen that the time courses of I_p and I are identical at short times, corresponding to ideal capacitive coupling. The negative phase of $I(t)$ at large t, however, is no longer present in $I_p(t)$, which monotonously decreases to a small, quasistationary current $I^{\infty}_p \equiv I_p(\infty)$. The biphasic shape of $I_p(t)$ can be approximately represented by

$$I_p(t) = I_1 \exp(-k_1 t) + I_2 \exp(-k_2 t) + I^{\infty}_p \tag{4}$$

I_1, I_2, and I^{∞}_p are time-independent constants. For the I_p curve of Fig. 6 an optimum fit was obtained with $k_1 = 67.3$ sec^{-1} and $k_2 = 24.2$ sec^{-1}. From 22 experiments with different membranes, carried out under the conditions of the experiment of Fig. 6, the following average values and standard deviations were evaluated:

$$k_1 = (58.4 \pm 13.1)\text{sec}^{-1}$$

$$k_2 = (23.8 \pm 8.2)\text{sec}^{-1}$$

4. Interpretation of Shape of $I_p(t)$

The intrinsic pump current I_p (Fig. 6) exhibits a biphasic time course with a fast rising period, followed by a slower decline toward a small quasistationary current I^{∞}_p. The rate constant k_1 of the rising phase depends on the concentration of released ATP and on the release rate, which is a function of pH (Borlinghaus *et al.*, 1987). As discussed in detail elsewhere (Apell *et al.*, 1987), the rising phase of $I_p(t)$ is likely to be governed by electrically silent reactions (release of ATP and binding to the protein) preceding charge translocation. Accordingly, the main information on pump kinetics is contained in the declining phase of $I_p(t)$, which can be described, to a first approximation, by a single exponential process with a rate constant $k_2 \approx 20$ sec^{-1}. This value is much larger than the maximum steady-state rate of ATP hydrolysis in the absence of extracellular K^+, which may be estimated to be ~3 sec^{-1} at 25°C (Mårdh and

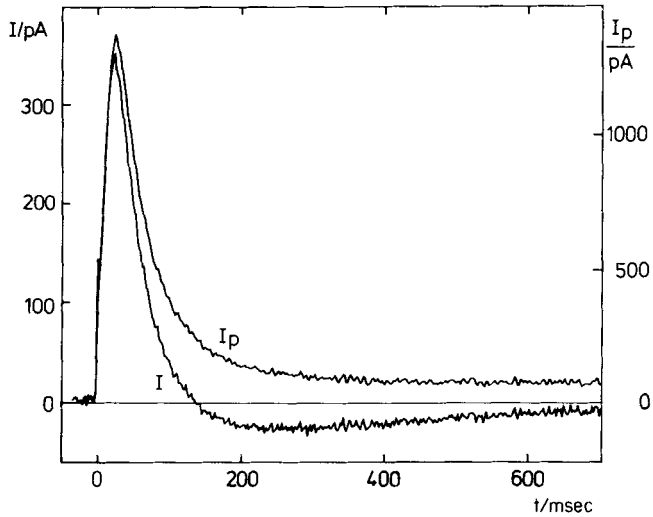

FIG. 6. Intrinsic pump current $I_p(t)$, calculated from the experimental current signal $I(t)$ according to Eq. (2). The left ordinate scale refers to $I(t)$ and the right ordinate scale refers to $I_p(t)$. The time constant $\tau_I \approx \tau_V$ in Eq. (2) was estimated from the decay time of the open-circuit voltage (measured on the same membrane) to be ~400 msec. For the scaling factor $(1 + C_p/C_t)$, a value of 3.7 was used (see text). $I(t)$ was measured under the experimental conditions described in the legend of Fig. 3; the membrane area was 0.75 mm^2. $I_p(t)$ can be approximately fitted by Eq. (4), with $k_1 = 67.3$ sec^{-1}, $k_2 = 24.2$ sec^{-1}, $I_1 = -2.54$ nA, $I_2 = 2.86$ nA, and $I_p^\infty = 81$ pA. (From Borlinghaus *et al.*, 1987.)

Post, 1977). This means that I_p decays to nearly 0 before the pump has completed a full transport cycle.

For the origin of the decline of I_p, at least two possibilities exist. Translocation of Na^+ may create gradients of electric potential and Na^+ concentration across the membrane sheet which tend to inhibit the pump. Information on possible effects of potential and ion concentration gradients is obtained from the α-chymotrypsin experiments. From previous studies, it is known that, at the level of the single pump molecule, inactivation by α-chymotrypsin is an all-or-none process (Jørgensen and Collins, 1986); one may therefore assume that partially inactivated membranes represent a mixture of completely blocked and fully active ATPase molecules. The magnitude of the voltage built up in a given time after ATP release depends on the density of functionally active pumps in the membrane. If the decline of pumping current results from the inhibitory action of transmembrane voltage (or of a Na^+ concentration gradient), the decay of the current signal should become slower when part of the pump molecules are inactivated by α-chymotrypsin. Since such a depen-

dence of decay time on the degree of proteolytic inactivation is not found in the experiment, it is unlikely that the decline of the current results from concentration or potential gradients. While Na^+ ions are likely to be accumulated in the gap as a result of pump activity, the effect on the current transient may be small, since the affinity of the extracellular sodium-release sites is very low ($K_D \approx 0.1$ *M;* see Glynn, 1985).

A likely interpretation of the time course of pumping current I_p consists in the assumption that the decline of $I_p(t)$ reflects an intrinsic property of the transport cycle. After activation by the ATP concentration jump, the pump goes through the first steps of the cycle and then enters a long-lived state from which return to the initial state is slow. As will be discussed below, the rate-limiting step for the completion of the cycle is likely to be the dephosphorylation of the protein, which is extremely slow in the absence of potassium. As long as ATP is present in the solution, pump molecules reentering the initial state may become phosphorylated again, which gives rise to the small, quasistationary current I^{∞}_p.

B. Microscopic Analysis of Transient Pump Currents

For the analysis of transient pump currents, we assume that sudden release of ATP initiates a sequence of transitions between discrete states $P_1, P_2, \ldots, P_n$ of the pump molecule:

$$P_1 \underset{k_b^1}{\overset{k_f^1}{\rightleftharpoons}} P_2 \underset{k_b^2}{\overset{k_f^2}{\rightleftharpoons}} P_3 \rightleftharpoons \cdots \rightleftharpoons P_n \tag{5}$$

k_f^i and k_b^i are the rate constants of the ith elementary reaction step in forward and backward direction. When the pump performs a closed cycle, state P_n is identical with the initial state P_1. Charge translocations associated with the individual transitions may be described by a set of (dimensionless) dielectric coefficients α_i (Läuger *et al.*, 1981). In the transition $P_i \rightarrow P_{i+1}$ the electric charge $\alpha_i e_0$ is translocated in the external measuring circuit (e_0 is the elementary charge). The coefficients α_i are microscopic parameters describing the electrogenic properties of the ion pump in a hypothetical system in which the Na^+,K^+-ATPase membrane is on both sides in direct contact with an aqueous electrolyte solution. If in the transition $P_i \rightarrow P_{i+1}$ a charge $\gamma_i e_0$ moves over a distance a_i in a homogeneous dielectric layer of thickness d, the coefficient α_i has the form

$$\alpha_i = \gamma_i a_i / d \tag{6}$$

Since in reality the protein layer is likely to be inhomogeneous with respect to its dielectric properties, the quantity a_i/d in Eq. (6) has to be replaced by an effective dielectric distance.

When x_i is the fraction of pump molecules in state P_i, the net rate ϕ_i of transitions $P_i \rightarrow P_{i+1}$ (referred to a single pump molecule) is given by

$$\phi_i = x_i k_f^i - x_{i+1} k_b^i \tag{7}$$

This equation may be applied to the case that the system is perturbed at time $t = 0$ by a sudden change of an external parameter (such as ATP concentration) and thereafter relaxes toward a stationary state. Since $e_0 N \alpha_i \phi_i$ is the contribution of transitions $P_i \rightarrow P_{i+1}$ to the total charge translocation (N is the number of pump molecules in the membrane), the pump current may be represented by

$$I_p(t) = e_0 N \sum_i \alpha_i \phi_i(t) \tag{8}$$

Equation (8) connects the experimental quantity $I_p(t)$ with microscopic parameters (α_i, k_f^i, k_b^i) of the pump. When the pump goes through a closed cycle ($P_1 \equiv P_n$) in which ν elementary charges are translocated from one to the other aqueous solution, the relation

$$\sum_i \alpha_i = \nu \tag{9}$$

holds. This relation is obtained by applying Eq. (8) to the steady state ($t \rightarrow \infty$) in which all rates ϕ_i become identical and equal to the stationary transport rate $\phi = I_p(\infty)/\nu e_0$.

The analysis of transient pump currents may be based on the Albers–Post reaction scheme (Fig. 7) of the Na/K pump (Glynn, 1985). In K^+-free media, the enzyme is predominantly in conformation E_1, which has a high affinity for ATP. After binding of Na^+, the enzyme becomes phosphorylated, forming an "occluded" state $(Na_3)E_1$-P in which the bound Na^+ ions are unable to exchange with the aqueous phases. After transition to state P-E_2·Na_3 in which the ion-binding sites are facing outward, Na^+ ions are released to the extracellular side. In the absence of K^+, the transition back to the original state involving dephosphorylation of the enzyme (P-$E_2 \rightarrow E_1$) is extremely slow (Post *et al.*, 1972; Mårdh and Post, 1977; Karlish *et al.*, 1978; Kapakos and Steinberg, 1986; Schuurmans Stekhoven *et al.*, 1986). Thus, after ATP release in the absence of K^+, the pump moves through the upper part of the Albers–Post cycle, and slowly returns to the original state via the following reaction steps:

$$P\text{-}E_2 \underset{r_b c_P}{\overset{r_f}{\rightleftharpoons}} E_1 \underset{a_b}{\overset{a_f c_T}{\rightleftharpoons}} E_1{\cdot}ATP \tag{10}$$

r_f, r_b, a_f, and a_b are rate constants, and c_P and c_T are the concentrations of P_i and ATP, respectively.

In principle, any of the transitions in the reaction scheme of Fig. 7 can be associated with charge translocation, corresponding to a nonzero value of α_i. Obvious candidates for charge displacements are the reactions $Na_3{\cdot}E_1{\cdot}ATP \leftrightarrow (Na_3)E_1\text{-}P \leftrightarrow P\text{-}E_2{\cdot}Na_3$ involving transitions of Na^+ binding sites between an inward-facing (E_1) and outward-facing (E_2) configuration. Since it is unlikely that binding sites move over the entire transmembrane length of the protein in the E_1/E_2 transition, it is usually assumed that the protein contains access channels connecting the binding sites with the respective aqueous phases. Part of the access channel may consist of a wide, water-filled pore having a low electric resistance (Fig. 8). Ion movement within the low-resistance pore does not result in charge displacement in the external circuit. The gating part of the pump molecule may be represented by a narrow, high-resistance pathway consisting of a series of energy barriers and wells (Fig. 9). In state $Na_3{\cdot}E_1{\cdot}ATP$, the ion

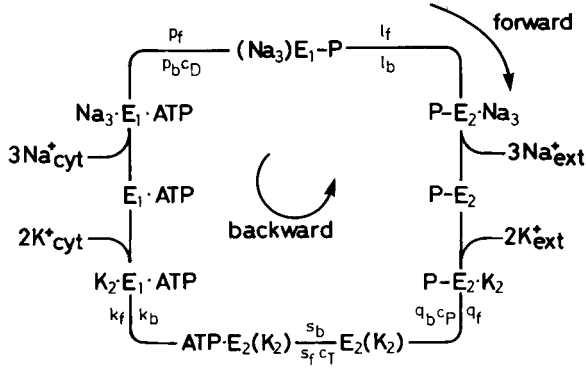

FIG. 7. Albers–Post scheme for the pumping cycle of Na^+,K^+-ATPase (adapted from Cantley *et al.*, 1984). E_1 and E_2 are conformations of the enzyme with ion binding sites exposed to the cytoplasm and the extracellular medium, respectively. In the occluded states $(Na_3)E_1$ and $E_2(K_2)$, the bound ions are unable to exchange with the aqueous phase. Dashes indicate covalent bonds, and dots indicate noncovalent bonds. p_f, l_f, q_f, $s_f c_T$, k_f and $p_b c_D$, k_b, s_b, $q_b c_P$, l_b are rate constants for transitions in forward and backward direction, respectively. c_T, c_D, and c_P are the cytoplasmic concentrations of ATP, ADP, and P_i (inorganic phosphate), respectively.

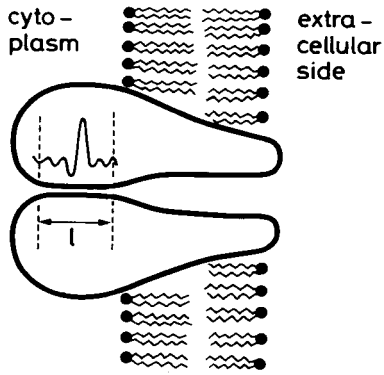

FIG. 8. Na^+,K^+-ATPase molecule represented as a transmembrane protein with a water-filled access channel (or vestibule) having a low electrical resistance. The inset depicts a hypothetical potential-energy profile for Na^+ along the high-resistance part of the ion pathway in conformation E_1 of the enzyme.

binding site is connected with the cytoplasmic phase via a series of low barriers but separated from the extracellular side by a high barrier. In the occluded state $(NaV_3)E_1$-P the energy barriers on either side are high. In state $P\text{-}E_2\cdot Na_3$, the binding sites are easily accessible from the extracellular phase.

Since Na^+ migrating from the cytoplasm to the binding site has, in general, to traverse part of the dielectric (Figs. 8 and 9), the binding step may be associated with charge displacement in the external circuit. The corresponding dielectric distance, α', is assumed to be the same for the three Na^+ binding sites. In a completely analogous way, the release of Na^+ to the extracellular side is described by a dielectric distance α'' (Fig. 9).

In the transition $Na_3\cdot E_1\cdot ATP \rightarrow (Na_3)E_1$-P, the loaded binding sites move over a dielectric distance β' (Fig. 9). If $z_L e_0$ is the charge of the ligand groups, the dielectric coefficient associated with $Na_3\cdot E_1\cdot ATP \rightarrow (Na_3)E_1$-P (rate constant p_f) is given by

$$\alpha_p = (3 + z_L)\beta' + \eta' \tag{11}$$

The parameter η' accounts for rotation of dipolar groups and translocation of intrinsic charges of the protein other than charged ligands (Läuger and Apell, 1986). In an analogous way, the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2\cdot Na_3$ (rate constant l_f) is described by a dielectric coefficient α_l:

$$\alpha_l = (3 + z_L)\beta'' + \eta'' \tag{12}$$

The possibility that the transition $Na_3\cdot E_1\cdot ATP \rightarrow (Na_3)E_1$-P is an electrogenic step may be excluded on the basis of the experiments in the presence of α-chymotrypsin. In the α-chymotrypsin-modified enzyme, the phosphorylation reaction and the formation of the occluded state $(Na_3)E_1$-

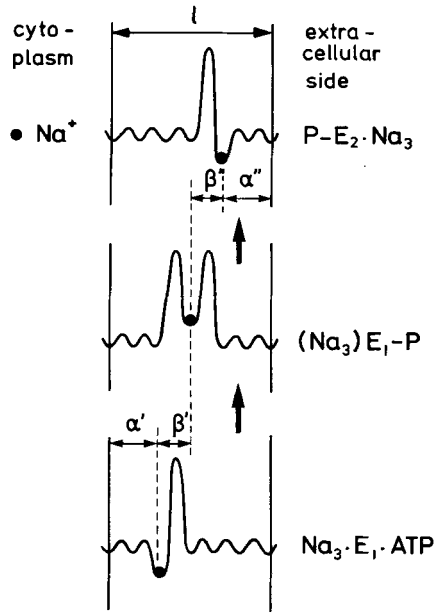

FIG. 9. Hypothetical energy profile of a sodium ion along the transport pathway. The ion binding sites in state $Na_3 \cdot E_1 \cdot ATP$ are connected with the cytoplasmic side by a series of low barriers, but are separated from the extracellular medium by a high barrier. In the occluded state $(Na_3)E_1$-P, the energy barriers on either side are high. In state P-$E_2 \cdot Na_3$, the binding sites are easily accessible from the extracellular phase. α', α'', β', and β'' are dielectric distances depending on the location of the ion binding site in the protein and on the dielectric properties of the protein and the surrounding medium.

P are preserved, but the transition $(Na_3)E_1$-P $\rightarrow$ P-$E_2 \cdot Na_3$ is blocked (Jørgensen and Petersen, 1985; Glynn *et al.*, 1984). The finding that in α-chymotrypsin-treated Na^+,K^+-ATPase membranes the photoresponse was abolished therefore indicates that the reactions preceding the transition $(Na_3)E_1$-P $\rightarrow$ P-$E_2 \cdot Na_3$ are electrically silent.

The rate constant p_f of the phosphorylation reaction [$Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-P] was found to be $\sim$220 sec^{-1} for enzyme from guinea pig kidney at 25°C (Mårdh and Post, 1977) and $\sim$180 sec^{-1} for bovine brain enzyme at 21°C (Mårdh and Zetterquist, 1974; Mårdh and Lindahl, 1977). Thus, phosphorylation and formation of the occluded state are fast processes within the time scale of the current transient (Fig. 5).

A possible candidate for the reaction governing the slow decay of the current signal (rate constant $k_2 \approx$ 20–30 sec^{-1}) is the E_1/E_2 transition, which is associated with the deocclusion of sodium [$(Na_3)E_1$-P $\rightarrow$ P-

$E_2 \cdot Na_3$]. From experiments with bovine brain enzyme, Mårdh (1975) reported a value of $l_f \approx 80$ sec^{-1} at 21°C for the rate constant of the E_1/E_2 transition. Nakao and Gadsby (1986) recently described voltage-jump studies of ouabain-dependent Na^+ currents in heart cells under K^+-free conditions and proposed that the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 \cdot Na_3$ is a rate-limiting electrogenic step. Similarly, Karlish and Kaplan (1985) concluded that Na^+ deocclusion is a slow step that limits the rate of the transient (pre-steady state) Na^+ flux in uncoupled ATP-driven Na^+ transport.

The following reaction step, the release of Na^+ from the outside-facing binding sites ($P\text{-}E_2 \cdot Na_3 \rightarrow P\text{-}E_2 + 3Na^+_{ext}$) is likely to be much faster. This assumption is based on the low affinity of the extracellular sites for sodium binding ($K_D \approx 0.1$ *M*) and on the fact that association–dissociation reactions of alkali ions with ligands are generally very fast. It is feasible, however, that a fraction of the observed charge displacement is associated with the release of Na^+, if Na^+ ions have to migrate through part of the membrane dielectric on their way from the binding sites to the extracellular medium (Fig. 9; $\alpha'' > 0$).

The mechanistic interpretation of the pump current $I_p(t)$ discussed here may be substantiated by numerical simulation of the transport cycle of Fig. 7 (Apell *et al.*, 1987), using estimated values of the kinetic parameters taken from the literature. An example is shown in Fig. 10, in which the case $z_L = -2$ is analyzed. A model with a ligand system bearing two negative charges ($z_L = -2$) has been recently proposed by Karlish *et al.* (1985) as an explanation for the effect of transmembrane voltage on pumping rates. As seen in Fig. 10, the amplitude of the current transient decreases with increasing values of the dielectric coefficient β''. This results from the fact that for $z_L = -2$ the E_1/E_2 transition is associated with translocation of a single net (positive) charge, whereas in the process $P\text{-}E_2 \cdot Na_3 \rightarrow P\text{-}E_2 + 3Na^+_{ext}$, which is described by the coefficient $\alpha'' = 1 - \beta''$, three charges are translocated. When β'' is increased, the relative contribution of the second process (release of three Na^+) decreases. The results presented in Fig. 10 show that the observed time course of I_p is consistent with the proposal (Karlish *et al.*, 1985; Nakao and Gadsby, 1986) that the ligand system for Na^+ bears two negative charges ($z_L = -2$).

In conclusion, the observation that the transient pump current requires sodium but not potassium indicates that charge movement is associated with Na^+ translocation and is thus in agreement with the Albers–Post reaction scheme, in which translocation of Na^+ precedes translocation of K^+. A "simultaneous model," in which Na^+ and K^+ are translocated in the same step, would be difficult to reconcile with the experimental find-

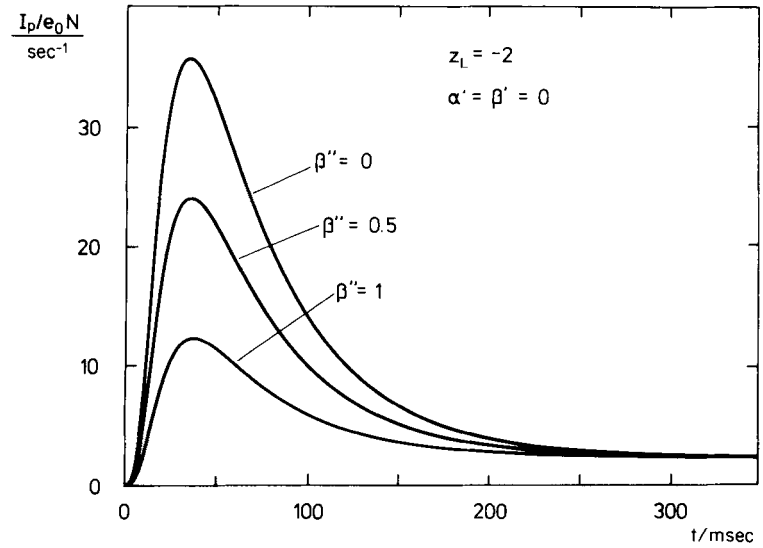

FIG. 10. Numerical simulation of the transport cycle represented in Fig. 7. The transient pump current I_p is plotted as a function of time t. I_p is referred to a single pump molecule and is given in sec^{-1}; e_0 is the elementary charge and N is the number of pump molecules which are activated at $t = 0$. $z_L e_0$ is the charge of the ligand system, and α', β', and β'' are dielectric coefficients defined by Fig. 9. The simulation has been carried out using the following values of the kinetic parameters: $a_f c_T = 100$ sec^{-1}, $a_b = 20$ sec^{-1}, $p_f = 200$ sec^{-1}, $p_b c_D = 0$, $l_f = 19$ sec^{-1}, $l_b = 1.6$ sec^{-1}, $r_f = 0.9$ sec^{-1}, $r_b c_P = 0$, $K'_N = 4$ mM, $c''_N = 100$ mM, $c''_N << K''_N$, $\alpha_a = \eta' = \eta'' = 0$. c'_N and c''_N are the Na^+ concentrations on the cytoplasmic and extracellular sides, respectively, and K'_N and K''_N are the corresponding equilibrium dissociation constants.

ings. A similar conclusion, that Na^+ translocation is an early step in the pumping cycle, has been reached by Forbush (1984, 1985) on the basis of measurements of transient $^{22}Na^+$ release from kidney membrane vesicles after an ATP concentration jump.

The most likely interpretation of the observed pump currents exists in the assumption that the principal electrogenic step is the transition $(Na_3)E_1$-P $\rightarrow$ P-$E_2 \cdot Na_3$, followed by release of Na^+ to the extracellular side. This interpretation agrees with recent proposals based on work with reconstituted vesicles (Karlish *et al.*, 1985; Rephaeli *et al.*, 1986a,b; Goldschlegger *et al.*, 1987) and with intact heart cells (Nakao and Gadsby, 1986). Since Na^+ migrating from the binding sites to the extracellular medium probably has to traverse part of the membrane dielectric, the release process may contribute to the overall charge translocation.

III. VOLTAGE DEPENDENCE OF TRANSPORT RATES

By investigating the voltage dependence of the pump current, information on the nature of the electrogenic steps in the transport cycle may be obtained (Chapman *et al.*, 1983; De Weer, 1984; Läuger and Apell, 1986). The $I(V)$ method is based on the fact that the rate of a charge-translocating step necessarily depends on the electic field strength in the membrane. While previous experimental studies have been restricted to a narrow voltage interval, more recently it has become possible, using the whole-cell recording technique with patch pipettes, to measure the current–voltage characteristic of the Na/K pump in a more extended voltage range (Gadsby *et al.*, 1985).

A. Predictions from Kinetic Models

The current–voltage characteristic of the pump is determined by the voltage dependence of the rate constants of the single transport steps. The voltage dependence of the kinetic parameters may be described introducing the energy profile of the ion along the transport pathway (Fig. 9). In Fig. 9, it is assumed that the ion binding sites are connected with the aqueous medium by a series of low barriers, meaning that ion binding and release are equilibrium reactions. Since the potential energy of an ion in the binding site is modified by the presence of a transmembrane voltage V, the equilibrium dissociation constants become voltage dependent. If $\alpha' V$ is the fraction of voltage which drops between the sodium site and water in state E_1 (Fig. 9), the dissociation constant of Na^+ at the cytoplasmic side is given by

$$K'_N = \tilde{K}'_N \, exp(-\alpha' u) \tag{13}$$

$$u \equiv e_0 V/kT \tag{14}$$

k is the Boltzmann constant, T is the absolute temperature, and K'_N is the value of K'_N at zero voltage. If the potential of the cytoplasm is positive with respect to the external medium ($u > 0$), the equilibrium constant $1/K'_N$ of sodium binding is increased by the Boltzmann factor $\exp(\alpha' u)$. The binding site acts as an "ion well" (Mitchell and Moyle, 1974), i.e., a change of electrical potential has a similar effect on the occupancy of the site as a change of external concentration.

According to the theory of absolute rates, the forward and backward rate constants are given by

$$p_f = \tilde{p}_f \exp(\alpha_p u/2) \tag{15}$$

$$p_b = \tilde{p}_b \exp(-\alpha_p u/2) \tag{16}$$

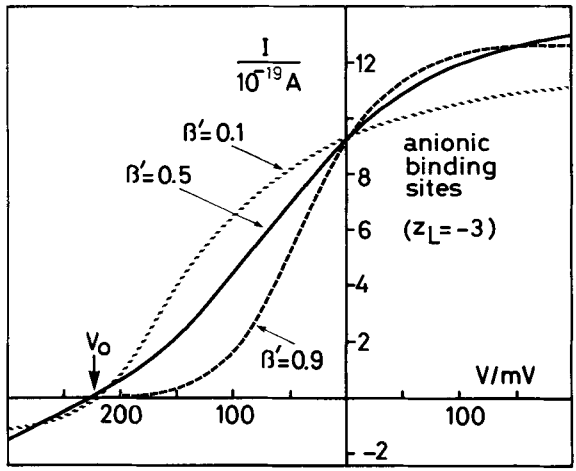

FIG. 11. Current–voltage curve of a pump molecule with negatively charged ion binding sites (z_L = -3). The net charge of the binding site is zero in state $Na_3 \cdot E_1 \cdot ATP$ and -1 in state $P\text{-}E_2 \cdot K_2$. I is the pump current (referred to a single pump molecule), V is the transmembrane voltage, and V_0 is the reversal potential. The coefficient β' represents the dielectric distance over which sodium ions move in the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ (Fig 9). For the calculation of $I(V)$, the following values of the kinetic parameters have been used (Läuger and Apell, 1986): $\alpha' = \alpha'' = \eta' = \eta'' = \phi' = \phi'' = 0$, $\gamma' = -\beta'$, $\gamma'' = -\beta'' = 1$; $\tilde{p}_f = 270$ sec^{-1}, $\tilde{p}_b c_D = 2$ sec^{-1}, $\tilde{l}_f = 120$ sec^{-1}, $\tilde{l}_b = 10$ sec^{-1}, $\tilde{q}_f = 500$ sec^{-1}, $\tilde{q}_b c_P = 5000$ sec^{-1}, $\tilde{s}_f c_T = 5000$ sec^{-1}, $\tilde{s}_b = 110$ sec^{-1}, $\tilde{k}_f = 80$ sec^{-1}, $\tilde{k}_b = 10$ sec^{-1}, $\tilde{K}'_N = 4\text{m}M$, $\tilde{K}''_N = 100\text{m}M$, $\tilde{K}'_K = 8\text{m}M$, $\tilde{K}''_K = 2\text{m}M$, $c'_N = 10\text{m}M$, $c'_N = 140\text{m}M$, $c'_K = 150\text{m}M$, $c''_K = 5$ mM, $\Delta G = 58$ kJ/mol. ΔG is the free energy of ATP hydrolysis. Quantities marked by ′ and ″ refer to the intracellular and extracellular sides, respectively.

(Läuger and Apell, 1986). α_p is defined by Eq. (11). The voltage dependence of the other rate constants, l_f, l_b, q_f, q_b, k_f, and k_b, is obtained by replacing α_p by the quantities

$$\alpha_l = (3 + z_L)\beta'' + \eta'' \tag{17}$$

$$\alpha_q = (2 + z_L)\gamma'' + \varphi'' \tag{18}$$

$$\alpha_k = (2 + z_L)\gamma'' + \varphi' \tag{19}$$

β'', η'', φ'', φ', and φ'' are dielectric coefficients defined in an analogous ways as β' and η'.

Examples of a current–voltage curve obtained by numerical simulation of the reaction cycle of Fig. 7 are represented in Fig. 11. The ligand system is assumed to bear three negative charges ($z_L = -3$), so that the net charge of the alkali ion binding sites is zero in state $Na_3 \cdot E_1 \cdot ATP$ and -1 in state $P\text{-}E_2 \cdot E_2$. Current–voltage curves are given for three different values of parameter $\beta' = -\gamma'$, representing the dielectric distance over

which Na^+ moves in the transition $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$. The current monotonically increases with voltage V and reaches asymptotic values at $V \rightarrow \infty$ and $V \rightarrow -\infty$. For $\beta' = 0.5$, the limiting currents (referred to a single pump molecule) are $I(\infty) = 13.5 \times 10^{-19}$ A and $I(-\infty) = -3.1 \times 10^{-19}$ A. The saturating behaviour of the current–voltage curve may be qualitatively understood in the following way. Under the conditions considered here ($\alpha' = \alpha'' = \gamma' = \gamma'' = \varphi' = \varphi'' = 0$; $z_L = -3$), the only voltage-dependent steps are the transitions $P\text{-}E_2 \cdot K_2 \rightarrow E_2(K_2) \rightarrow K_2 \cdot E_1 \cdot ATP$, which involve a movement of the K^+-loaded binding sites (total charge $-e_0$) within the membrane dielectric. Thus, for increasing positive voltages ($V > 0$) the voltage-dependent transition rates in forward direction increase, until eventually the voltage-insensitive steps of the cycle become rate limiting. This means that for $V \rightarrow \infty$ (and also, by the same argument, for $V \rightarrow -\infty$) the current approaches an asymptotic value.

B. Comparison with Experimental Results

Gadsby *et al.* (1985) described whole-cell patch-clamp experiments with isolated heart cells in which the pump current was determined as the difference of total membrane current in the presence and in the absence of ouabain. In the experimental voltage range (−140 to +60 mV), the current increased monotonically from very small positive values at −140 mV, exhibiting a saturation behavior above −50 mV (Fig. 12). Under the conditions of the experiment, the reversal potential V_0 is estimated to be about −340 mV. This value of V_0 is much larger (in absolute magnitude) than the reversal potential estimated by extrapolation of the experimental $I(V)$ curve to $I = 0$ ($V_0 \approx -160$ mV). This discrepancy may result from a $Na^+ : K^+$ coupling ratio which is different from 3 : 2 (Anner and Moosmayer, 1982; Glynn, 1984; Blostein, 1985) and/or from incomplete coupling between ATP hydrolysis and transport (Navarro and Essig, 1984). Another possible origin is the shape of the $I(V)$ curve itself. For certain parameter values, the pump current approaches the reversal potential with a very small slope, as seen, for instance, from the curve with $\beta' = 0.9$ in Fig. 11. This means that linear extrapolation to $I = 0$ may lead to a large error in V_0.

Voltage effects on the Na/K pump reconstituted in artificial lipid vesicles have been described by Karlish *et al.* (1985). They observed that the rate of ATP-driven sodium flux was increased when the potential of the cytoplasmic side was made positive. The voltage effect disappeared at low ATP concentrations at which the $E_2(K_2) \rightarrow E_1 \cdot K_2$ transition becomes rate limiting. As an explanation of this finding, the authors proposed that the ligand system bears two negative charges ($z_L = -2$). This would

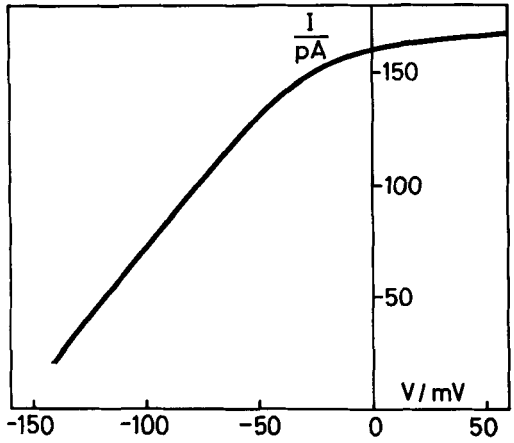

FIG. 12. Current–voltage characteristics of the Na/K pump of isolated heart cells (Gadsby *et al.*, 1985). The cell interior was perfused with 34 m*M* Na^+, 10 m*M* K^+, 100 m*M* Cs^+, and 10 m*M* ATP; the extracellular medium contained 144 m*M* Na^+, 5.4 m*M* K^+, and 1 m*M* Cs^+. *I* is the ouabain-sensitive component of the membrane current of a single cell. (Redrawn from Fig. 2 of Gadsby *et al.*, 1985.)

mean (if intrinsic charge translocations are excluded) that the $E_2(K_2) \rightarrow E_1 \cdot K_2$ transition is voltage independent.

More recently, the *I(V)* characteristic of the Na/K pump in reconstituted vesicles has been studied using potential-sensitive fluorescent dyes (Apell and Bersch, 1988). In these experiments, the transmembrane voltage *V* generated by the pump is estimated by calibrating the fluorescence signal with known values of the K^+ equilibrium potential (Apell *et al.*, 1985). The pump current *I* is obtained as the product of the membrane capacitance and the time derivative of transmembrane voltage. The *I(V)* characteristic of the pump determined in this way qualitatively agrees with the current–voltage curve obtained from electrophysiological experiments (Fig. 12).

IV. CONCLUSION

The analysis of electrogenic properties of the Na/K pump described above had been based on the notion that operation of the pump involves a sequence of conformational transitions and ion-binding and -release steps. The electrogenic behavior of the pump is determined by a set of dielectric coefficients α', α'', α_p, α_i, . . ., describing the contribution of a given reaction step to the overall charge translocation. The dielectric coefficients are fundamental for the description of nonstationary pump currents [Eq. (8)] as well as for the analysis of current–voltage behavior [Eqs. (13)–(16)].

The observation that the transient pump current after an ATP concentration jump requires sodium but not potassium is consistent with a consecutive (Albers–Post) reaction scheme in which translocation of Na^+ precedes translocation of K^+. Experiments with α-chymotrypsin-modified enzyme, in which the pumping cycle is arrested after the phosphorylation step, indicate that deocclusion of Na^+ followed by release to the extracellular side is a major electrogenic reaction step. The conclusion is consistent with the results of voltage-jump, current-relaxation experiments recently described by Nakao and Gadsby (1986).

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Chapter 13

Sodium Pump Current in Renal Tubular Cells

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I. INTRODUCTION

The membrane-bound enzyme Na^+,K^+-ATPase is responsible both for the maintenance of a low intracellular Na^+ activity and for the accumulation of K^+ in the cell (Sweadner and Goldin, 1980). In a wide variety of cell types, the ratio of $Na^+ : K^+$ transported by the Na^+,K^+-ATPase is 3 : 2 (De Weer, 1985). Accordingly, the Na/K pump must carry one net electrical charge out of the cell during each cycle, which therefore produces a current across the membrane. Although the presence of such a pump current has been well demonstrated in several cell types, the physiological effects of this current, the relevance of the membrane potential changes that it may create, and its possible role in the regulation of transport processes are still open to investigation.

The first question is: Does this current generated by the activity of the

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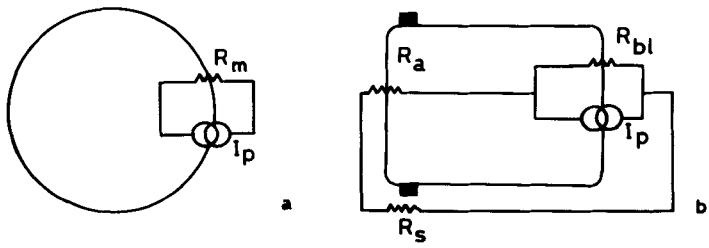

FIG. 1. Equivalent electrical circuits used to calculate the effect of the pump current I_p on the membrane potential, in a nonpolarized cell (a) and in an epithelial cell (b).

pump have any significant effect on the membrane potential? The pump hyperpolarizes the cell membrane by the current (I_p) that circulates through the resistance (R_m) of the membrane, according to Ohm's law: $E_p = I_p R_m$ (Fig. 1a). The cell membrane contains, in parallel, Na^+ and K^+ permeabilities *(P)* and a Na^+–K^+ exchange pump with a fixed coupling ratio *(r)*. With this in mind, Mullins and Noda (1963) derived an equation that allows an estimate of the membrane potential (E_m) under steady-state conditions:

$$E_m = \frac{RT}{F} \ln \frac{P_{Na}[Na^+]_o + rP_K[K^+]_o}{P_{Na}[Na^+]_i + rP_K[K^+]_i} \tag{1}$$

Thomas (1972) used this equation to show that the pump-generated membrane potential could not exceed a maximum value (E_p) equal to

$$E_p = \frac{RT}{F} \ln \frac{1}{r} \tag{2}$$

This value amounts to ~10 mV for a coupling ratio (r) of 1.5 (or a 3 : 2 Na^+ : K^+ flux ratio).

Accordingly, in a steady-state situation, the ion fluxes carried by the pump (and thus the pump current, I_p) cannot increase without a parallel increase in the ionic permeabilities, that is, without a reduction of the membrane resistance (R_m). Thus, the product $I_p R_m$ has a maximum value. However, this analysis concerns only nonpolarized cells in which the ionic conductances and the electrogenic pump are in parallel in the same membrane. In polarized cells, the pump of most epithelial cells is located specifically in the basolateral membrane, whereas part of the ionic conductances are in the apical membrane. These two domains are separated by tight junctions. Accordingly, the current generated by the pump circulates along two loops: one containing the tight junctions and the apical membrane resistances in series, the other containing the basolateral mem-

brane resistance (Fig. 1b). The basolateral membrane potential (E_p) generated by the circulation of that fraction of the pump current (I_p) that crosses the basolateral membrane (Boulpaep and Sackin, 1979) can be calculated by the following equation:

$$E_p = I_p \frac{R_{bl}(R_s + R_s)}{R_a + R_{bl} + R_s} \tag{3}$$

where R is the resistance and the subscripts a, bl, and s indicate the apical membrane, the basolateral membrane, and the paracellular shunt pathway, respectively. In this circuit, the pump-generated potential has no theoretical upper limit: By increasing the values of the in parallel resistances of the basolateral membrane and of the tight junctions, the resistance term can increase without reducing the ion fluxes flowing through the apical membrane. For instance, in a tight epithelium (i.e., an epithelium with a large shunt resistance), the basolateral membrane potential directly generated by the rheogenic activity of the pump mostly depends on the value of the basolateral membrane resistance. In steady-state situations, it could be large and potentially of physiological relevance.

Another aspect of the electrogenicity of the Na/K pump is the voltage dependence of the pump activity. In general, the work to move electrical charge against an electrical field increases proportionally with the electrical potential difference; and as the energy provided by the hydrolysis of ATP is finite, the activity of the electrogenic pump must somehow be voltage dependent (De Weer, 1984). Indeed, voltage dependence of the pump current has been demonstrated in numerous nonepithelial cell types: myocardial cells (Gadsby *et al.*, 1985; Nakao and Gadsby, 1986), oocytes (Lafaire and Schwarz, 1986; Rakowski, 1987), the squid giant axon (De Weer *et al.*, 1986), and several types of neurons (Thomas, 1972). This subject is discussed in more detail in Chapter 14 by Gadsby *et al.* in this volume. The Na/K pump is also a major component of transepithelial ion transport, but the precise role of the basolateral membrane potential in the regulation of the Na/K pump activity of an epithelium is still to be defined.

De Weer (1985) recently reviewed data supporting the electrogenic nature of the Na^+–K^+ exchange process in several cell types, especially excitable tissue such as nerve fibers of numerous animal species, and skeletal and heart muscle cells. Electrogenicity of pump activity or a $Na^+ : K^+$ coupling ratio different from unity has also been demonstrated in nonexcitable cells such as red blood cells, macrophages, and neutrophils (Hoffman *et al.*, 1979; Gallin and Livengood, 1983; Simchowitz *et al.*, 1982). Much less quantitative information is available concerning the

pump current in epithelia, and this is somewhat surprising since, for instance, most renal epithelial cells have a number of pump units three or four orders of magnitude larger than red blood cells. Also, these pumps are present in a membrane that does not contain the highly voltage-sensitive Na^+ and K^+ conductances that make the measurements of the pump current so difficult in excitable tissue.

There are, however, reports supporting the hypothesis of an electrogenic Na/K pump in epithelial cells. The principles of the methods used to estimate the electrogenicity of the Na/K pump will be briefly summarized in the next section.

II. METHODS USED TO STUDY PUMP CURRENT IN EPITHELIAL CELLS

A. Sodium-to-Potassium Coupling Ratio

One approach to studying pump current in epithelial cells is to measure the Na^+ and K^+ fluxes, linked to the pump activity, across the basolateral membrane. If the ratio of these fluxes is different from 1, the activity of the pump must be electrogenic, provided that there is no other ion transported by this mechanism. Such studies measured the ouabain-sensitive Na^+ and K^+ fluxes in liposomes containing reconstituted, purified Na^+,K^+-ATPase prepared from shark rectal gland, and lamb and dog kidney. Most of these studies have yielded results consistent with a $Na^+ : K^+$ coupling ratio of 3 : 2 (Anner *et al.*, 1977; Goldin, 1977; Hilden and Hokin, 1975), although different observations have also been reported (Anner *et al.*, 1977; Goldin and Tong, 1974). Electrophysiological studies (see Section II, B) have also indicated that the stoichiometry of the pump could be variable, depending on the pump activity (Sackin and Boulpaep, 1983) or on the hormonal status of the animal (Sansom and O'Neil, 1986).

Recently, Avison and colleagues (1987) have used an elegant combination of a K^+-sensitive electrode to monitor the extracellular K^+ activity and nuclear magnetic resonance methods to measure the intracellular Na^+ content in a suspension of rabbit proximal tubules. They recorded the time course of the extrusion of K^+ from the cells and the accumulation of Na^+ in the cells after suddenly activating the pump by addition of K^+ to a K^+-free bath and found a 3 : 2 $Na^+ : K^+$ coupling ratio of the pump. This ratio was constant over a wide range of Na^+ gradients, extracellular K^+ concentrations, and absolute rates of Na^+–K^+ exchange (see Fig. 2).

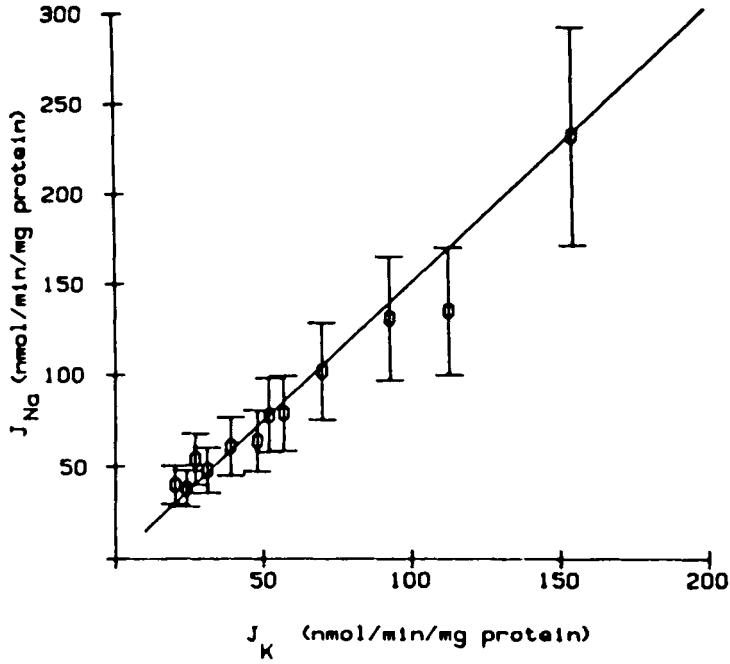

FIG. 2. Plot of the absolute rate of Na^+ extrusion (J_{Na}) versus K^+ uptake (J_K) in the initial period after pump stimulation by addition of K^+ to the bath solution. Intracellular Na^+ content was measured by nuclear magnetic resonance and extracellular K^+ activity was measured by an ion-sensitive electrode, in a suspension of rabbit proximal tubules. Thus, over a large range of absolute pump rate, the coupling ratio of 1.5 was well conserved. (Data from Avison *et al.*, 1987.)

B. Basolateral Membrane Potential

A second approach estimates the current of the pump from its effects on the basolateral membrane potential (V_{bl}). The current carried by an electrogenic pump, present in the basolateral membrane of an intact epithelium, generates a potential across this membrane according to Ohm's law applied to the equivalent electrical circuit shown in Fig. 3; this potential can be calculated from Eq. (3) (Boulpaep and Sackin, 1979). By solving this equation for the pump current I_p, it is theoretically possible to estimate the pump current from that part of the membrane potential which is due to the pump (V_p), if all of the resistances are known. V_p can be calculated as the difference between the measured membrane potential and the estimated membrane electromotive force (E.M.F.) due to the diffusion potentials. However, the precision of the membrane potential mea-

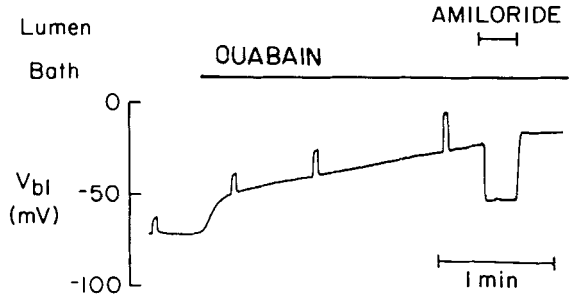

FIG. 3. Effect of ouabain (100μM) on the basolateral membrane potential (V_{bl}) of an isolated perfused collecting tubule of *Amphiuma*. Ouabain induced a fast depolarization (within 10 sec) of about 15 mV, followed by a continuous slow depolarization, due to the changes of the intracellular K^+ concentration and the modification of the basolateral membrane conductance. The upward deflections are due to positive current injections in the lumen. Amiloride (10 μM) hyperpolarized V_{bl} by blocking the apical membrane sodium conductance.

surements and of the estimates of the potential due to the conductances is generally not sufficient to calculate V_p under control conditions. The pump activity has to be increased either by loading the cells with sodium by a previous exposure to a K+-free solution (Sackin and Boulpaep, 1983) or by increasing the permeability of the apical membrane, using an ionophore (Lewis and Wills, 1983; Wills *et al.*, 1979).

The pump-generated potential can also be measured as the change in the basolateral membrane potential when the pump is suddenly blocked or activated *if none of the other determinants of the membrane potential are modified.* Indeed, several investigators have measured the effect on the basolateral membrane potential when the Na/K pump is blocked by ouabain (Sansom and O'Neil, 1986; Sackin and Boulpaep, 1983; Lewis *et al.*, 1978) or when the bath temperature is suddenly lowered (Rose *et al.*, 1977). Although, in some epithelia, ouabain had no measurable immediate effects on V_{bl} (Lewis *et al.*, 1978; Nagel, 1980; Reuss *et al.*, 1979), fast depolarizations have been reported (Horisberger and Giebisch, 1987; Sackin and Boulpaep, 1983; Rose *et al.*, 1977; Novak and Greger, 1988). Figure 3 shows an example of the depolarizing effect of ouabain on the basolateral membrane potential of the collecting tubule of *Amphiuma*. In the choroid plexus, an epithelium in which the Na/K pump is situated in the apical membrane, addition of ouabain to the ventricular side also resulted in a fast depolarization (Zeuthen and Wright, 1981). In the rabbit cortical collecting tubule, simultaneous measurements of the membrane potentials and conductances gave an estimated pump current ranging

from 32 to 195 $\mu A/cm^2$ (tubular surface), in control and DOCA-treated animals, respectively (Sansom and O'Neil, 1986).

There are, however, several problems with this approach. First, it is often difficult to distinguish clearly the fast depolarization due directly to inhibition of the pump current, from the slower one due to changes in intracellular K^+ concentration and/or changes in K^+ conductance. Second, the activity of the pump may create a K^+ gradient in the unstirred layers on both sides of the basolateral membrane. Blocking the pump may then produce changes in the K^+ concentration in these unstirred layers. The complicated geometry of the basolateral membrane in Na^+-transporting epithelia (Stanton *et al.*, 1984) makes it very difficult to obtain reliable estimates of these effects, and it is possible to argue that part or even all of the fast potential changes after the addition of ouabain are due to K^+ concentration changes in the unstirred layers. A third problem with this approach is the need to know the precise membrane and tight junction conductances in order to calculate the pump current according to Eq. (3). Because the measurements are done during a phase in which the membrane potentials are rapidly changing, the conductances may not be constant. Hence, an inward-rectifying basolateral membrane conductance would tend to exaggerate the estimates of the pump current, while outward-rectifying properties of this membrane would have the opposite effect.

Another technique is to measure the basolateral membrane potential when the pump is suddenly activated, by readmitting K^+ into the peritubular solution after a period of exposure to a K^+-free bath. Increasing the K^+ in the bath solution should produce a depolarization, if the membrane contains a K^+-selective conductive pathway like the basolateral membranes of most of the epithelial cells studied until now. The activation by K^+ of an electrogenic process carrying current out of the cell would have the opposite effect: The addition of K^+ to the bath solution would result in a net hyperpolarization that is sensitive to ouabain. This provides a very strong argument in favor of the activation by K^+ of an electrogenic Na/K pump. Further, such a hyperpolarization cannot be explained by a change in the K^+ concentration of the unstirred layers secondary to changes in the pump activity.

The first definite evidence by this method of an electrogenic Na/K pump in epithelial cells was obtained in liver cells (Haylet and Jenkinson, 1972). A basolateral membrane hyperpolarization after K^+ readmission has also been observed in several other epithelial preparations, e.g., mouse pancreas cells (Petersen, 1973), liver cells from mouse and guinea pig (Graf and Petersen, 1974; Haylet and Jenkinson, 1972), rabbit urinary bladder (Lewis and Wills, 1983; Wills and Lewis, 1980), rabbit and guinea pig proximal tubule (Biagi *et al.*, 1981; Proverbio and Whittembury,

1975), and *Amphiuma* collecting tubule (Horisberger and Giebisch, 1987). However, the potential change which follows the addition of K^+ to the bath solution depends not only on the pump current but also on the change of membrane EMF due to the K^+ conductance, an effect often difficult to evaluate precisely. Furthermore, this hyperpolarization is measured during a transient situation in which the conductances may be changing rapidly. For these reasons, it is difficult to obtain a precise calculation of the pump current by this method.

C. Transepithelial Current

Instead of measuring voltage changes and membrane conductances to calculate a current, the direct measurement of a current under voltage-clamp conditions should provide a more precise evaluation of the pump current. In an intact epithelium, the problem lies in the facts that the resistance of the apical cell membrane is in series with that of the basolateral membrane, and that is not possible to voltage clamp the basolateral membrane directly.

To get around this difficulty, one possibility is to physiologically eliminate the apical membrane resistance. Lewis and collaborators (Lewis *et al.*, 1977; Lewis and Wills, 1982) have shown that it is possible to increase, considerably, the ionic permeability of the apical membrane of a tight epithelium by adding ionophore antibiotics to the mucosal solution, without modifying the electrical properties of the basolateral membrane or the tight junctions. In a tight epithelium, after permeabilization of the apical membrane, the paracellular current becomes negligible and the transepithelial current is then predominantly composed of a K^+ current and a pump current across the basolateral membrane. These two components can be distinguished by sensitivity to ouabain or barium (Nielsen, 1979a,b; Lewis *et al.*, 1978). Wills *et al.* (1979) have demonstrated how the pump current depends on the intracellular Na^+ activity, using a similar method. In another study by Kirk *et al.* (1980), comparison of the ouabain- and barium-sensitive currents with the Na^+ and K^+ fluxes, measured under the same conditions, indicated that active Na^+ transport is entirely attributable to an electrogenic Na/K pump with a stoichiometry of $3:2\ Na^+:K^+$.

D. Na/K Pump in Artificial Membranes

Recently, purified Na^+,K^+-ATPase isolated from kidney medulla homogenate has been reconstituted in artificial phospholipid vesicles. This technique allows complete control of the chemical compositions on both

sides of the membrane. The vesicles containing reconstituted Na^+,K^+-ATPase have been used with a voltage-sensitive dye to evaluate the electrogenic nature of the Na/K pump, and these studies have confirmed both the electrogenic nature of the Na^+–K^+ exchange and the voltage dependency of the pump activity (Apell *et al.*, 1985; Apell and Bersch, 1987). The reconstitution of kidney Na^+,K^+-ATPase in black lipid membranes permits current measurement with a very high time resolution. Several investigators have studied the current transients (in the millisecond range) induced by the photolytic release of adenosine triphosphate (ATP) from caged ATP (Borlinghaus *et al.*, 1987; Apell *et al.*, 1987; Fendler *et al.*, 1985). In Chapter 12 of this volume, Apell *et al.* describe in greater detail how such measurements clearly confirm the electrogenic nature of the pump activity and are useful in understanding the mechanistic details of ion transport by the Na^+,K^+-ATPase.

III. PUMP CURRENT IN COLLECTING TUBULE OF *AMPHIUMA*

We have used the principle of transcellular current measurement under basolateral membrane voltage-clamp conditions to measure the strophanthidin-sensitive current in the isolated perfused collecting tubule of *Amphiuma*.

The collecting tubule of the *Amphiuma* is a moderately tight epithelium. The electrophysiological properties of its principal cells have been published (Hunter *et al.*, 1987; Horisberger *et al.*, 1987). Briefly, the apical membrane is Na^+ selective, while the basolateral membrane conductance is 70–80% K^+ selective, as estimated by transference number measurement. Also, there is an ouabain-sensitive Na/K pump in the basolateral membrane. If *Amphiuma* are exposed to a high-K^+ environment for several days preceding the experiments, they increase their aldosterone blood levels (Oberleithner *et al.*, 1983). The effects on the collecting tubule are a larger apical membrane Na^+ conductance, a higher rate of Na^+ transport, and a lower shunt conductance (Horisberger *et al.*, 1987), conditions which are favorable for measurements of the transcellular current. For these reasons we have used K^+-adapted *Amphiuma* for the experiments presented in this chapter.

Segments of *Amphiuma* collecting tubule were isolated and perfused according to a technique similar to that described in previous papers (Hunter *et al.*, 1987; Horisberger *et al.*, 1987). These tubules were short (~200 μm) and were selected for high transepithelial resistance. In this case, the voltage decrease along the tubule when current is injected at one end is negligible and the whole tubule can be considered clamped at

the same potential. By means of a data acquisition system consisting of a microcomputer (IBM-XT), an analog-digital converter (Analog Device RTI-815), and a software package (UnkelScope, Unkel Software, Lexington, Massachusetts), we measured, simultaneously, the transepithelial potential (V_{te}) recorded at both ends of the tubule, the basolateral membrane potential (V_{bl}), and the transepithelial current (I_{te}). The basolateral membrane potential, recorded from an intracellular microelectrode, was used to drive the transepithelial current injection device and thus allow for voltage clamping of the basolateral membrane potential. The transcellular current (I_{cell}) was calculated as the difference between the measured transepithelial current (I_{te}) and the current flowing through the shunt pathway (I_s). I_s was estimated from the transepithelial potential and the shunt conductance measured after the addition of amiloride to the luminal perfusate (Hunter *et al.*, 1987; Horisberger *et al.*, 1987).

In the first series of experiments, the basolateral membrane potential (V_{bl}) was clamped at the value measured under control and open-circuit conditions. Strophanthidin (20 μM) uniformly induced a fast reduction of the current flowing across the basolateral membrane. In ten experiments, the transcellular current was reduced from a mean value of some 104 to 66 $\mu A/cm^2$, the difference (equal to the pump current) amounting to 38 $\mu A/cm^2$. The ratio of the pump current to the total transcellular current averaged 0.37, a value compatible with the hypothesis that the pump carried one third of the current across the basolateral membrane and, thus, a $Na^+ : K^+$ coupling ratio of 3 : 2.

In order to reduce the possible errors due to currents flowing through a basolateral membrane potassium conductance, we did a second series of experiments in which the pump current was large and the basolateral membrane K^+ conductance was minimal. As in our earlier experiments, this situation was designed to study the basolateral membrane conductance in relation to the intracellular ionic content (Horisberger and Giebisch, 1987). In these experiments, the Na/K pump was blocked for 4 min by lowering the bath K^+ concentration to 0.1 mM. As expected, the intracellular Na^+ activity increased from ~8 to 40 mM, while that of K^+ decreased from ~60 to 15 mM. Within the same time course, there was a very large reduction of the basolateral membrane conductance from 5 to 0.5 mS/cm^2. Thus, the cells were first loaded with sodium by a 4-min exposure to a 0.1 mM K^+ bath solution. Then, barium (2 mM) and K^+ (3.0, 1.0, or 0.1 mM) were added to the bath solution and two basolateral membrane I–V curves were obtained under voltage-clamp conditions, one before and one 20 sec after the addition of strophanthidin (20 μM) to the bath solution.

Examples of basolateral membrane *I–V* curves obtained before and after the addition of strophanthidin, with bath K^+ concentrations of 3.0, 1.0, and 0.1 m*M* are presented in Fig. 4a, b, and c, respectively. The difference of transcellular current with and without strophanthidin can be taken as a measure of the pump current. In Fig. 4a, with a 3.0 m*M* K^+ concentration in the bath, the pump current was ~60 $\mu A/cm^2$ at low membrane potential and decreased to a negligible value at −180 mV. Thus, there was a clear voltage dependency of the pump current. The slope of this *I–V* curve, i.e., the phenomenological conductance of the pump, could be estimated to be ~0.5 $mS.cm^{-2}$ of tubular surface, a conductance of the same magnitude as the remaining basolateral conductance under the conditions of the measurements. With a bath K^+ concentration of 1.0 m*M* (Fig. 4b), the pump current was similarly voltage dependent but had a lower absolute value (~20 $\mu A\ cm^{-2}$ at low membrane potential). With

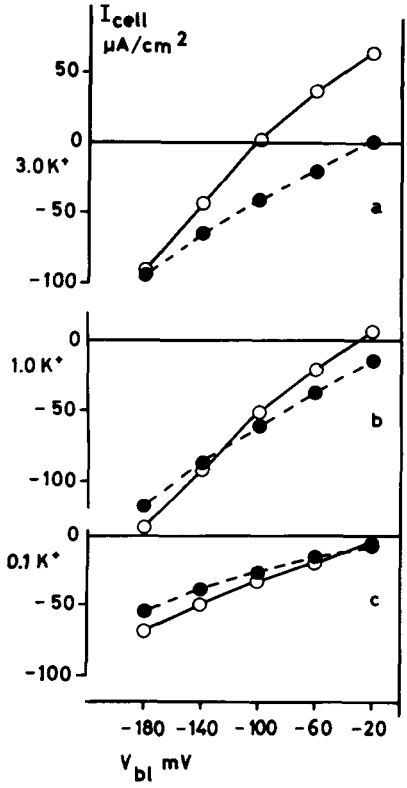

FIG. 4. Current (I_{cell})–voltage (V_{bl}) curves of the basolateral membrane of an isolated perfused collecting tubule of *Amphiuma*. The open circles and continuous lines indicate the values recorded before the addition of strophanthidin; the filled circles and the dotted lines indicate the values recorded 10–20 sec after the addition of 10 μM strophanthidin. The difference between transcellular current before and after strophanthidin is attributed to the current carried by the Na/K pump.

the 0.1 m*M* K^+ bath (Fig. 4c), there was no measurable ouabain-inhibitable current at low membrane potentials but, at high negative membrane potentials, there was more *negative* current in the absence than in the presence of strophanthidin. This indicates that a reverse pumping mode can be induced by appropriate ion gradients and large negative transmembrane potentials, as has already been demonstrated in the squid giant axon (De Weer and Rakowski, 1984).

To determine the part of this strophanthidin-sensitive current that could be due to changes in current across the K^+ conductance rather than to the pump directly, we did the following control experiment: The pump was blocked for 4 min with ouabain, then barium was added, and under basolateral membrane voltage-clamped conditions (at -60 mV), the K^+ concentration of the bath was raised from 3 to 15 m*M*. This was a very large incease compared to the change that might have been produced in the unstirred layer by the inhibition of the pump. The change in transcellular current produced by this maneuver was only a small fraction of the pump currents measured. Thus, we may safely conclude that the current change following addition of strophanthidin is largely due to the pump current.

These results demonstrate, first, that in the Na^+-transporting tight epithelium of a kidney tubule, the Na/K pump is electrogenic and that the current generated by this pump can be measured under voltage-clamped conditions. Second, this pump current is dependent on the external K^+ concentration. Third, the pump current is voltage dependent; however, the physiological significance of the voltage dependence is not clearly established, because variations of the basolateral membrane potential in the physiological range (-50 to -80 mV (Horisberger *et al.*, 1987)) entail only minor changes of the pump activity (less than 30%), because of the small slope of the pump *I–V* curve over this range.

IV. CONCLUSION

The asymmetry in the number of Na^+ and K^+ ions carried across the membrane during a cycle of the Na^+,K^+-ATPase and the resulting electrogenicity of this transport process have been demonstrated in epithelial cells as well as in excitable cells and erythrocytes. While experimental preparations of Na^+,K^+-ATPase reconstituted in artificial membranes will certainly allow for important progress in the understanding of the mechanistic details of the pump cycle, the possibility of measuring this pump current across the basolateral membrane of intact epithelial cells

will permit investigation of the physiological relevance of the changes of membrane potential due to the pump current and of the voltage dependence of the pump activity.

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Chapter 14

Voltage Dependence of Na/K Pump Current

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I. INTRODUCTION

Electrogenic ion transporters are so called because they can generate a steady flow of current through the membrane in which they are embedded (e.g., Thomas, 1972). This means that at least one step in the transport cycle must involve net charge movement through the electric field across the membrane (Hansen *et al.*, 1981; Chapman *et al.*, 1983; De

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Weer, 1984). The rate constants governing any such charge-translocating step are necessarily sensitive to changes in the membrane potential, in a direction and to an extent determined by the sign and number of the mobile charges and the fraction of the membrane field through which they move (Reynolds *et al.*, 1985; Läuger and Apell, 1986). If a voltage-sensitive step limits the rate of the electrogenic transport cycle, then the turnover rate of the pump, and hence the steady pump current, will vary with membrane potential.

Although electroneutral transporters (or modes of transport), by definition, do not generate steady current flow, their transport reactions can nevertheless include voltage-sensitive, i.e., charge-translocating, steps as long as the net charge movement during each complete reaction cycle is zero. A simple hypothetical example of a voltage-dependent, yet electroneutral, transport cycle would be one in which two steps in the cycle involve identical charge movements over identical fractions of the membrane field, but in opposite directions. Depending on the relative sizes of the postulated voltage-sensitive and voltage-insensitive rate constants, even such electroneutral transport reactions may give rise to transient net currents in response to imposed jumps of membrane potential; the transient currents would reflect a temporary mismatch between charge influx and efflux during the redistribution of cycle intermediates that follows the change in membrane voltage (Chapman *et al.*, 1983; Hansen *et al.*, 1983; Nakao and Gadsby, 1986; Bahinski *et al.*, 1988b).

The Na/K pump is one of the best-studied electrogenic transporters; it transports three Na^+ ions in one direction across the cell membrane for every two K^+ ions pumped in the opposite direction and so generates a net membrane current (for reviews see Thomas, 1972; De Weer, 1975; Glynn, 1984). Recently, membrane potential has been shown to modulate Na/K pump rate (reviewed in De Weer *et al.*, 1988a,b) both when the pump cycle runs forward (generating outward current) and when it runs backward (generating inward current), but which reaction steps confer that voltage sensitivity, and how, is not yet fully understood. For instance, while recent measurements of transient pump currents generated during partial reactions of the Na/K pump in the absence of external K^+ (Fendler *et al.*, 1985, 1987; Nakao and Gadsby, 1986; Borlinghaus *et al.*, 1987; Apell *et al.*, 1987) indicate that Na^+ translocation includes a voltage-sensitive step (cf. Karlish *et al.*, 1985, 1988; Rephaeli *et al.*, 1986b; Goldshlegger *et al.*, 1987), just how that step contributes to the overall voltage dependence of Na/K pump rate is not clear. A difficulty arises because Na^+ translocation is not believed to limit the rate of the forward cycle (Forbush, 1984; Karlish and Kaplan, 1985; but see Goldshlegger *et*

al., 1987). Further, while it is still possible that K^+ translocation involves a voltage-sensitive step (e.g., Lafaire and Schwarz, 1986; Reynolds *et al.*, 1985; Läuger and Apell, 1986), the idea remains controversial (Rephaeli *et al.*, 1986a; Goldshlegger *et al.*, 1987; De Weer *et al.*, 1988a,b; Bahinski *et al.*, 1988a,b).

Unfortunately, the knowledge that a single net positive charge is extruded per pump during each complete, three-Na^+/two-K^+, forward transport cycle is of little help in identifying charge-translocating steps. Even if charge movement were known to be strictly limited to the translocation pathways for Na^+ and K^+ ions, the possible combinations of charge movements would still be virtually endless. For example, n positive charges (where $n \geq 1$) might move out through the membrane field during Na^+ translocation and $n - 1$ positive charges might move in during K^+ translocation; alternatively, n negative charges might move out during Na^+ translocation and $n + 1$ negative charges might move in during K^+ translocation (e.g., Chapman *et al.*, 1983; Reynolds *et al.*, 1985; Läuger and Apell, 1986). However, the steady-state current–voltage *(I–V)* relationship must be monotonic for any electrogenic transport cycle that contains only a single charge-transit step (Hansen *et al.*, 1981), in contrast to the negative slope regions expected, at extreme positive or negative membrane potentials, in the *I–V* relationship of an electrogenic cycle that contains a second voltage-sensitive step in which the charge movement occurs in the opposite sense to that of the first step (Chapman *et al.*, 1983; Reynolds *et al.*, 1985; Läuger and Apell, 1986).

The aim of the experiments reviewed here was to begin to identify and characterize voltage-dependent steps and rate-limiting steps in the Na/K pump reaction cycle. Steps of membrane potential were used to examine the voltage dependence of both transient and steady Na/K pump currents elicited under experimental conditions designed to support four separate modes of Na/K pump activity, i.e., forward and reverse Na^+/K^+ exchange, Na^+/Na^+ exchange, and K^+/K^+ exchange. Na/K pump current was measured as cardiotonic steroid-sensitive, whole-cell current in guinea pig ventricular myocytes, voltage-clamped using the gigaohm seal technique (Hamill *et al.*, 1981) with wide-tipped patch pipettes and a device for exchanging the solution inside the pipette (Soejima and Noma, 1984). This combination of techniques affords reasonable, simultaneous control of the compositions of both intracellular and extracellular solutions in addition to membrane potential. The results suggest that the Na/K pump reaction cycle includes only a single voltage-dependent step which is associated with Na^+ translocation but which, however, does not limit the rate of either the forward or the reverse transport cycle.

II. EXPERIMENTAL APPROACH

Myocytes isolated from guinea pig ventricles by collagenase digestion, and then enzyme-free incubation in a high-[K], low-[Ca] medium (Isenberg and Klöckner, 1982), were allowed to settle on the glass bottom of the recording chamber before beginning superfusion at ~36°C with normal Tyrode's solution containing 145 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.5 m*M* dextrose, and 5 m*M* HEPES/NaOH (pH 7.4). Gigaohm seals were obtained using wide-tipped (~5 μm), fire-polished pipettes filled with Tyrode's solution (pipette resistance ~1 MΩ) which was then exchanged (Soejima and Noma, 1984), just before rupture of the cell membrane, for a pipette solution containing 135 m*M* CsOH, 85 m*M* aspartic acid, 5 m*M* pyruvic acid, 2 m*M* $MgCl_2$, 10 m*M* MgATP, 5 m*M* $Tris_2$–creatine phosphate, 20 m*M* TEACl (tetraethylammonium chloride), 5.5 m*M* dextrose, 10 m*M* EGTA, and 10 m*M* HEPES (pH 7.4). The voltage-clamped cell was then superfused with nominally Ca^{2+}-free Tyrode's solution in which the 1.8 m*M* $CaCl_2$ was replaced with 1.8 m*M* $MgCl_2$ plus 2 m*M* $BaCl_2$, plus 0.5 m*M* $CdCl_2$. These intracellular and extracellular solutions were designed to minimize ion channel currents (Gadsby *et al.*, 1985; Nakao and Gadsby, 1986) and the Na^+/Ca^{2+} exchange current (Kimura *et al.*, 1987), and variations of them, as specified in the text and legends, were able to support forward or backward Na^+/K^+ exchange, or Na^+/Na^+ exchange, or K^+/K^+ exchange. The osmolarity of all solutions was ~300 mOsm.

The cardiotonic steroids, ouabain and strophanthidin, are known to be specific inhibitors of the Na/K pump and were therefore used to identify Na/K pump current. Ouabain was added from a 10 m*M* aqueous stock solution, and strophanthidin was added from a 0.5 *M* stock solution in dimethyl sulfoxide (DMSO). Control measurements showed that under the conditions of our experiments, up to 0.5% (by volume) DMSO had no effect on steady membrane currents, and neither did maximal concentrations of ouabain or strophanthidin if the pump was already inhibited, e.g., by removal of internal and external Na^+ (Fig. 8C) or external K^+ (Fig. 7).

To minimize voltage errors due to liquid junction potentials, 3 *M* KCl-filled half-cells were used to connect the voltage clamp amplifier to the pipette interior and to the experimental chamber. Up to 55% of the series resistance between pipette interior and cell membrane could be compensated by summing a fraction of the clamp amplifier output to the command potential. Current and voltage signals were low-pass filtered at 1–2 kHz (six-pole Bessel), digitized (12-bit resolution) online at 2.5–8 kHz, and stored in a computer for later analysis.

III. STEADY-STATE Na/K PUMP CURRENT IS STRONGLY VOLTAGE DEPENDENT

A. Forward Na^+/K^+ Exchange

The chart record in Fig. 1A illustrates changes in holding current during activation and inhibition of the forward Na/K pump cycle caused by raising the pipette [Na] from 1 m*M* to 34 m*M* (Na^+ substituted for Cs^+) and then exposing the cell to 10 μ*M* ouabain. The holding potential was −40 mV to inactivate Na channels, and with our experimental solutions, membrane current changes in response to the voltage steps were small (Figs. 1B and 2A) and virtually time independent (Fig. 1B). The superimposed current traces in Fig. 1B were recorded after pump activation *(a)* and

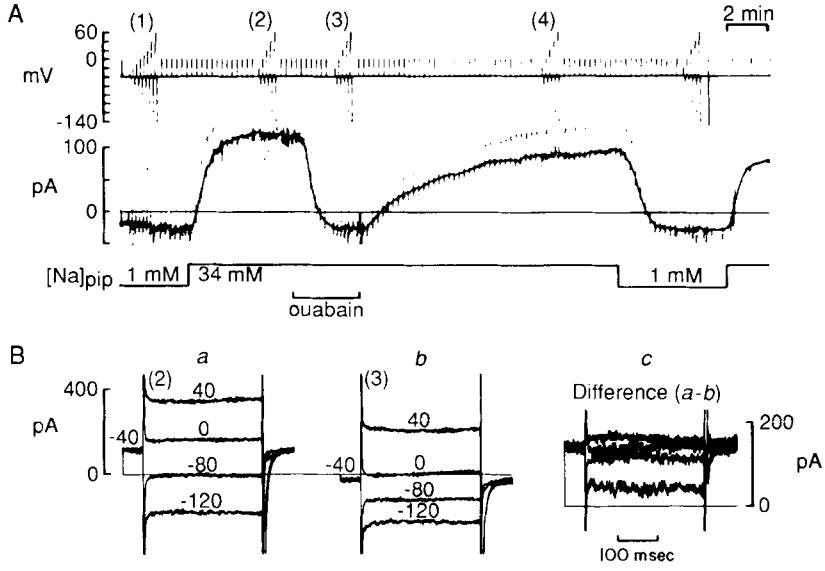


FIG. 1. Current changes on activation or inhibition of the Na/K pump in an isolated ventricular cell. (A) Chart recording of membrane potential (upper trace) and whole-cell current (lower trace); the changes in pipette Na^+ concentration, $[Na]_{pip}$, and the exposure to 10 μ*M* ouabain are indicated, respectively, by the line and the bar beneath the chart records; the numbers above the voltage trace mark the acquisition of four sets of current–voltage data. (B) Superimposed current traces recorded, just before *(a)* and during *(b)* exposure to ouabain. The pulse potential is indicated for each record; the holding potential was -40 mV. *(c)* Superimposed difference currents obtained by computer subtraction of each trace in *(b)* from its counterpart in *(a)*; pulse potentials were, from top to bottom, 0, +40, −80, and −120 mV. (From Gadsby *et al.*, 1985. Reprinted by permission from *Nature* **315**, 63–65. Copyright © 1985 by Macmillan Magazines Ltd.)

during inhibition by ouabain *(b)*; the ouabain-sensitive current traces *(c)* were obtained by appropriate computer substraction. Ouabain-sensitive, i.e., Na/K pump, current varies in size with membrane potential, and is much smaller at −120 mV [(Fig. 1B,c) lowest trace, ~50 pA] than at the holding potential, −40 mV [(Fig. 1B,c) initial current, ~150 pA].

This voltage dependence of outward Na/K pump current is more clearly illustrated by the corresponding steady-state *I–V* relationships in Fig. 2. Steady levels of whole-cell current were measured near the end of 100- to 300-msec voltage clamp steps to potentials between +60 mV and −140 mV. The lower curve in Fig. 2A is drawn through steady current levels measured with 1 m*M* pipette [Na], or with 34 m*M* pipette [Na] in the presence of ouabain; the upper curve represents current levels obtained with the pump activated by 34 m*M* pipette [Na] in the absence of

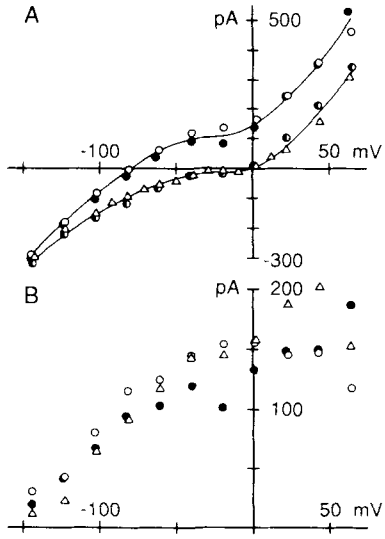


FIG. 2. Voltage dependence of current associated with Na/K pump activity. Data from the experiment in Fig. 1. (A) Whole-cell currents in the presence and the absence of Na/K pump activity. (Ordinate) Steady current levels, obtained by averaging digitalized values over the final ~100 msec of each 300-msec pulse. (Abscissa) Membrane potential during the pulse. The data were recorded during the four periods numbered in Fig. 1A: △, 1 m*M* $[Na]_{pip}$; ○, 34 m*M* $[Na]_{pip}$; ◐, 34 m*M* $[Na]_{pip}$ during exposure to ouabain; ●, 34 m*M* $[Na]_{pip}$ after recovery from ouabain. The curves drawn through the points are arbitrary. (B) Difference currents, obtained by subtracting steady current levels in the absence from those in the presence of Na/K pump activity, plotted against pulse potential. The three sets of points are: △, current activated by raising $[Na]_{pip}$; ouabain-sensitive current on application (○) and withdrawal (●) of ouabain, respectively. (From Gadsby *et al.*, 1985. Reprinted by permission from *Nature* **315**, 63–65. Copyright © 1985 by Macmillan Magazines Ltd.)

ouabain. The forward-running Na/K pump *I–V* relationships in Fig. 2B were obtained by appropriate subtraction of the whole-cell currents in Fig. 2A. All three sets of points indicate that outward pump current approaches a plateau level at positive potentials and declines steeply with voltage at negative potentials. This general shape of the outward Na/K pump *I–V* relationship is amply confirmed by the data in Figs. 4 and 7B, which also reveal a sigmoid foot to the *I–V* curve at extreme negative potentials.

Control experiments ruled out the possibility of contamination of the cardiotonic steroid-sensitive currents by changes in K^+ channel currents due to extracellular [K] changes, which may be expected to result from abolition of pumped K^+ uptake (discussed briefly in Gadsby, 1984; De Weer *et al.*, 1988a,b). Moreover, the close similarity of the substracted (Na/K pump) *I–V* relationships obtained by inhibiting the pump with ouabain (Fig. 2B), by removing intracellular Na^+ (Fig. 2B), or removing external K^+ argues that the voltage dependence of cardiotonic steroid-sensitive current reflects voltage dependence of Na/K pump current and not voltage dependence of steroid action.

B. Reverse Na^+/K^+ Exchange

To drive the Na/K pump reaction cycle backward (Garrahan and Glynn, 1967b; De Weer and Rakowski, 1984), we had to steepen the transmembrane Na^+ and K^+ gradients, and add adenosine diphosphate (ADP) and inorganic phosphate (P_i) to the pipette solution. In practice, the external solution was K^+ free, but contained 150 m*M* Na^+, while the internal solution was Na^+ free, but contained 145 m*M* K^+, 5 m*M* ATP, 5 m*M* ADP, and 5 m*M* P_i. Under these conditions, strophanthidin caused an outward shift of current at the −40 mV holding potential (see Fig. 8A and Section V, C), reflecting inhibition of inward pump current (De Weer and Rakowski, 1984; Bahinski *et al.*, 1988a,b). Steady-state levels of whole-cell current were determined during voltage-clamp pulses to various potentials in the presence and in the absence of strophanthidin, and the steady inward current generated by the backward-running Na/K pump (Fig. 3) was then computed by appropriate subtraction. Like the outward pump current just described, it had a monotonic voltage dependence, but in this case it increased in amplitude as membrane potential was made more negative, from a very small size at +40 mV toward an apparent plateau level near −100 mV. Just as with the outward pump *I–V* relationship (Figs. 2, 4, and 7), no region of negative slope conductance was evident (cf. De Weer and Rakowski, 1984). A similar voltage dependence of inward Na/K pump current has recently been demonstrated in internally dialyzed, voltage-clamped squid axons (Rakowski *et al.*, 1988).

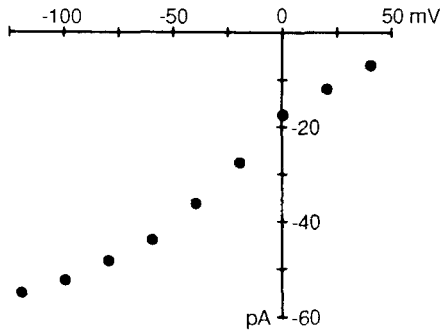


FIG. 3. Voltage dependence of steady inward pump current during reverse Na/K exchange in a cell exposed to zero K^+, 150 m*M* Na^+ fluid, and dialyzed with pipette solution including no Na^+, 145 m*M* K^+, 5 m*M* ATP, 5 m*M* ADP, and 5 m*M* P_i. Steady current was measured near the end of 100-msec voltage pulses from the −40 mV holding potential. Pump current was determined by subtracting current levels in 0.5 m*M* strophanthidin from the average of current levels obtained immediately before and shortly after exposure to strophanthidin. (From Bahinski *et al.*, 1988a. Reprinted with permission.)

IV. VOLTAGE DEPENDENCE OF OUTWARD Na/K PUMP CURRENT IS ATTENUATED AT LOW $[Na]_o$

As the forward Na/K pump *I–V* relationships in Fig. 4 show, the strong voltage dependence of steady outward current seen in Fig. 2 (see also Fig. 7) is severely attenuated after removal of most of the extracellular Na^+ (Gadsby and Nakao, 1987). This striking influence of external [Na] was first seen in *Xenopus* blastomeres (Béhé and Turin, 1984), and has recently been confirmed in the squid giant axon (De Weer *et al.*, 1987). Because simply changing the external [K] does not seem to profoundly alter the voltage dependence of pump current (see Fig. 7 and Section V,B), this marked effect of reducing $[Na]_o$ implies that the voltage dependence of pump current in the presence of 150 m*M* $[Na]_o$ is somehow related to the Na^+ translocation pathway of the Na/K pump cycle.

V. TRANSIENT PUMP CURRENTS ARE ASSOCIATED WITH SODIUM TRANSLOCATION

A. Transient Pump Currents during Sodium Translocation in K^+-Free Solution

The results illustrated in Fig. 5 provide strong support for the idea that Na^+ translocation by the Na/K pump includes at least one step which is voltage dependent and which, therefore, can be presumed to involve

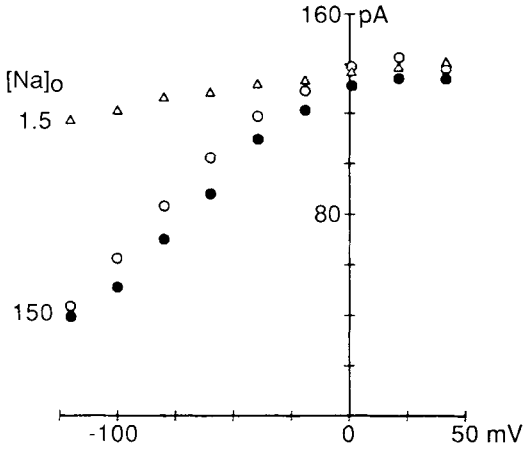


FIG. 4. Influence of external [Na] on Na/K pump current–voltage relationships determined in a single cell equilibrated with 50 m*M* Na, 10 m*M* ATP pipette solution, and exposed to 5.4 m*M* $[K]_o$. $[Na]_o$ was initially 150 m*M* (○), then 1.5 m*M* (△), and then 150 m*M* again (●). Steady current levels were measured near the end of 100-msec voltage pulses from the holding potential, −40 mV. Pump current was obtained by subtracting current recorded in the presence of 2 m*M* strophanthidin from the average of currents recorded before exposure to strophanthidin, and just after washing it out. (From Gadsby and Nakao, 1987. Reprinted with permission.)

charge movement within the membrane field. Figure 5 shows superimposed traces of whole-cell currents recorded in a cell equilibrated with 50 m*M* Na^+, 10 m*M* ATP pipette solution, and exposed to K^+-free, 150 m*M* Na^+ external solution, first in the absence *(a)*, and then in the presence *(b)*, of strophanthidin. The strophanthidin-sensitive currents, shown in *c* and *d*, were obtained by subtracting records in *b* from those in *a*. The absence of steady strophanthidin-sensitive current, at all potentials, confirms that the normal electrogenic cycle of Na^+/K^+ exchange had been interrupted by removal of external K^+, and so pump activity was presumably restricted to the steps involved in Na^+ translocation. The pump nevertheless generated exponentially decaying transient currents in response to voltage jumps; the transient pump currents were directed outward following a step to more positive voltages, and inward following a negative voltage step. The exponential decay occurred more slowly at positive potentials than at negative potentials.

The close association of these transient pump currents with Na^+ translocation steps is suggested by the findings (Nakao and Gadsby, 1986) that they required intracellular ATP, as well as both extracellular and intracellular Na^+, and that they were diminished by exposure to oligomycin B (Garrahan and Glynn, 1967a).

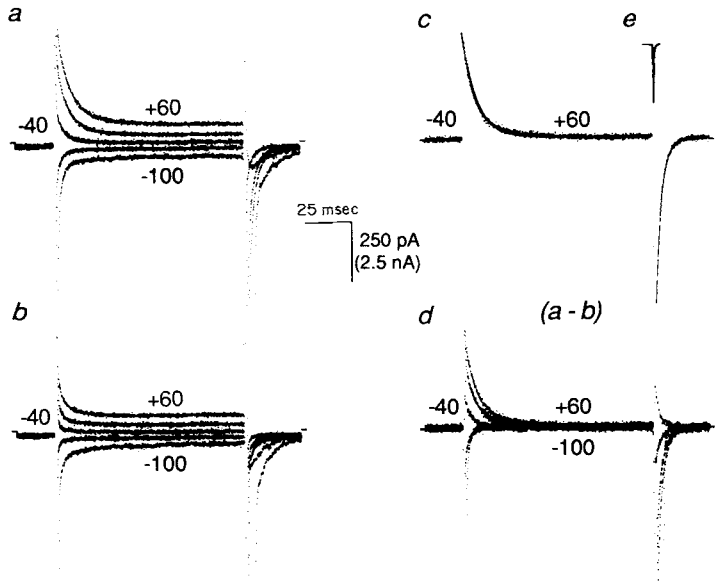


FIG. 5. Transient pump currents from a cardiac cell superfused with K^+-free, 150 m*M* Na^+ solution. *(a, b)* Superimposed traces of currents elicited by 100-msec voltage pulses from the holding potential, −40 mV, to +60, +20, −20, −60, and −100 mV in the absence *(a)* and in the presence *(b)* of 0.5 m*M* strophanthidin. *(c, d)* Strophanthidin-sensitive (i.e. pump) currents, obtained by computer subtraction of records in *(b)* from counterparts in *(a)*: *(c)* transient pump currents in response to pulse to +60 mV with superimposed, least-squares, exponential fits (τ_{on} = 8 msec, τ_{off} + 4 msec); *(d)* superimposed, transient pump currents for all pulses in *(a)* and *(b)*. [Inset *(e)*] Capacity current (decay time constant = 0.5 msec) during 10 mV hyperpolarization, without series resistance compensation; after compensation (as used for all other records), capacity currents decayed fully within 1 msec. Total cell capacitance was 156 pF. Current scale is 250 pA for all records except *(e)*, for which it is 2.5 nA. (From Nakao and Gadsby, 1986. Reprinted by permission from *Nature* **323**, 628–630. Copyright © 1986 by Macmillan Magazines Ltd.)

Voltage-dependent characteristics of these transient pump currents are presented in Fig. 6. The upper graph shows the voltage dependence of the quantity of charge moved during the transients; the time integral of the current is plotted against pulse potential. The amount of charge moved is a saturable function of voltage which can be fitted reasonably well by a Boltzmann relationship describing a two-state, voltage-dependent equilibrium governed by movement of a single charge through the entire membrane field. The rate constants for exponential decay of the transient currents are plotted against membrane potential in the lower graph (Fig. 6B). These equilibration rate constants increase as the membrane potential is made more negative, but seem to reach a minimum at positive potentials. Since depolarization gives rise to outward currents,

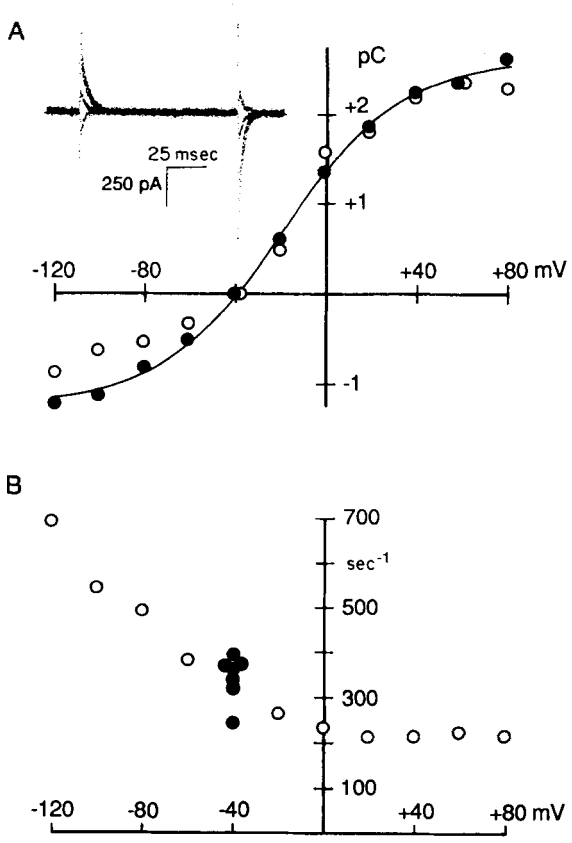


FIG. 6. Voltage dependence of charge movement (A) and of exponential decay rate constant (B) during transient, strophanthidin-sensitive currents obtained in K^+-free, 150 m*M* Na^+ external solution; holding potential, −40 mV; cell capacitance, 208 pF; temperature, 35°C. (A) (inset) Superimposed sample records of strophanthidin-sensitive currents in response to pulses to +60, +20, −20 and −60 mV; (graph) time integral [$Q(V)$] of current transients elicited by each pulse, plotted against pulse potential (V). Open circles show charge measured directly from the records, and filled circles show charge estimated by extrapolation of the exponential current decay toward the beginning of the voltage step. Only "on" charge movement is plotted. The smooth curve, derived from the Boltzmann relationship, shows $\Delta Q(V)/\Delta Q_{max} = 1/[1 + \exp(V' - V)/k]$, where $\Delta Q(V) = Q(V) - Q_{min}$, $\Delta Q_{max} = Q_{max} - Q_{min}$, V' is the potential at the midpoint, and k provides a measure of the "equivalent charge." The values used were $Q_{min} = -1.23$ pC, $Q_{max} = +2.63$ pC, $V' = -20$ mV, and $k = 26.5$ mV. (B) Rate constants of exponential fits to transient currents plotted against membrane potential: open circles show rate constants of "on" transients, and filled circles show rate constants of "off" transients, at −40 mV, following repolarization from -60 mV $< V < +80$ mV. (From Nakao and Gadsby, 1986. Reprinted by permission from *Nature* **323**, 628–630. Copyright © 1986 by Macmillan Magazines Ltd.)

the forward process should reflect outward movement of positive charge (or inward movement of negative charge) and should be accelerated at positive membrane potentials, whereas the backward process should be accelerated at negative potentials. The equilibration rate constant following a voltage jump is given by the sum of the forward and backward transition rate constants at the new voltage, and so we can conclude from the results in Fig. 6B that the backward rate constant is much more strongly voltage dependent than the forward rate constant.

Thus, Na^+ translocation by the Na/K pump seems to be voltage dependent because it is governed by movement of a single charge across the cell membrane. If that voltage dependence were to remain unaltered in the presence of external K^+, then no other step in the cycle is required to be voltage dependent (i.e., involve charge movement) because only a single net charge is transported during each complete three-Na^+/two-K^+ pump cycle. In other words, Na^+ translocation is likely to involve the only voltage-dependent step in the pump reaction cycle.

B. Transient Pump Currents during Activation of Forward Na^+/K^+ Transport Cycle by External Potassium

Figure 7 shows results from an experiment in which a cell was initially bathed in 150 m*M* Na^+, K^+-free solution and then exposed to 1 m*M* $[K]_o$ and, finally, to 5.4 m*M* $[K]_o$. In K^+-free solution, the pump's transport cycle was interrupted, as confirmed by the complete absence of steady pump current. But addition of external K^+ activated the Na^+/K^+ transport cycle in a concentration-dependent manner, as evident from the increasing levels of steady outward pump current (Fig. 7) whose monotonic voltage dependence is illustrated in the steady-state pump *I–V* relationships plotted in Fig. 7B. As steady current became larger, the area under the transient currents became smaller (Fig. 7A) but, even at 5.4 m*M* $[K]_o$, transient pump currents clearly preceded the new steady levels of pump current after voltage jumps. Just as they did at zero $[K]_o$, these transient currents reflect voltage-dependent redistribution of enzyme intermediates. In the presence of external K^+, however, these intermediates are involved in the forward Na/K pump transport cycle, whereas, at zero $[K]_o$, the intermediates are involved in a voltage-dependent (but electroneutral) equilibrium.

C. Transient Pump Currents Are Also Observed during Reverse Na^+/K^+ Transport, But Not during K^+/K^+ Exchange

Figure 8A shows a chart recording of holding current obtained under experimental conditions designed, at first, to reverse the Na/K pump reaction cycle. The external solution contained 150 m*M* Na^+ but no K^+,

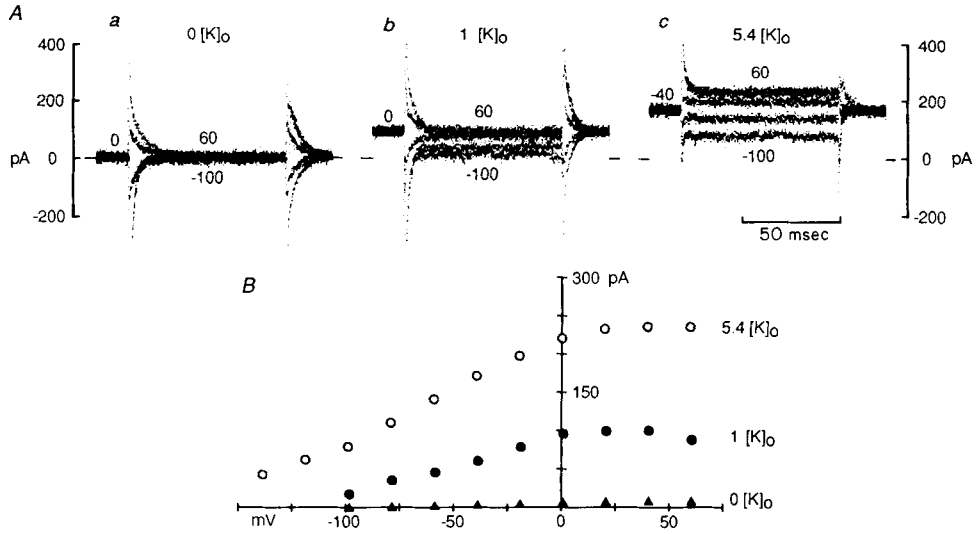


FIG. 7. $[K]_o$ dependence of transient and steady pump currents from a cardiac cell dialyzed with 50 m*M* [Na], 10 m*M* [ATP] pipette solution and exposed to 145–150 m*M* $[Na]_o$. (A) Superimposed records of strophanthidin-sensitive currents during 80-msec voltage pulses to +60, +20, −20, −60, and −100 mV, obtained by computer subtraction of whole-cell current traces recorded in the presence of 0.5 m*M* strophanthidin from the corresponding current traces recorded in its absence ~1 min earlier. Holding potential: *(a)* and *(b)* 0 mV; *(c)* −40 mV. $[K]_o$, varied by substituting K^+ for Na^+, was 0 *(a)*, 1 *(b)*, or 5.4 m*M* *(c)*. (B) Na/K pump current–voltage relationships from the results in (A). (Ordinate) Steady levels of strophanthidin-sensitive currents obtained by averaging digitalized values over the same 24-msec period near the end of each pulse. (Abscissa) Membrane potential during the pulse. ▲, 0 m*M* $[K]_o$; ●, 1m*M* $[K]_o$; ○, 5.4 m*M* $[K]_o$. Total cell capacitance, 218 pF. Initial pipette resistance, 1.0 MΩ; 0.8 MΩ of the 1.7 MΩ series resistance between pipette interior and cell membrane was compensated. (From Bahinski *et al.*, 1988b. Reprinted with permission.)

while the internal solution contained no Na^+, but 145 m*M* K^+, 1 m*M* ATP, 5 m*M* ADP, and 5 m*M* P_i, so that exposure to strophanthidin caused an outward shift of holding current due to inhibition of inward Na/K pump current. Fig. 8B shows current traces recorded before *(a)*, and during *(b)*, the exposure to strophanthidin, as well as the resulting strophanthidin-sensitive currents *(a − b)*. The latter traces reveal that, during reverse Na^+/K^+ exchange, small transient pump currents preceded establishment of the new steady levels of inward pump current following step changes in membrane potential, as already described for forward Na^+/K^+ exchange.

The steady inward pump current demonstrates that the Na/K pump was continuously cycling backward and so confirms that the 5 m*M* P_i and 145 m*M* K^+ in the pipette solution were adequate to sustain reverse K^+ translocation via the Na/K pump. Since 5.4 m*M* $[K]_o$ and millimolar levels of

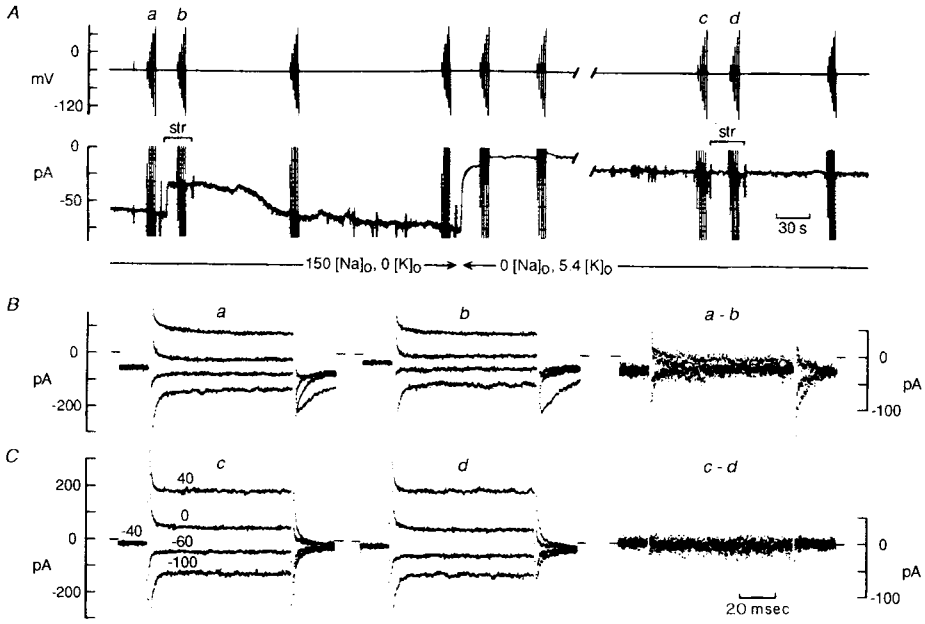


FIG. 8. Transient and steady inward pump current during reverse Na^+/K^+ exchange, and absence of strophanthidin-sensitive current during K^+/K^+ exchange. (A) Chart recording of membrane potential (upper trace) and membrane current (lower trace); holding potential, −40 mV. The gap marks omission of 9 min of record; (a)–(d) denote acquisition of current–voltage data, samples of which are presented in (B) and (C). The bars over the current record mark exposures to 2 m*M* strophanthidin (str), and the arrows below signify the switch from K^+-free, 150 m*M* Na^+ external solution, to 5.4 m*M* K^+, zero Na^+ (145 m*M* *N*-methyl-D-glucamine) solution. The internal solution was Na^+-free and included 145 m*M* K^+, 5 m*M* ADP, 5 m*M* P_i, and 1 m*M* ATP. (B) Superimposed traces of whole-cell currents elicited by 80-msec voltage pulses to +40, 0, −60, and −100 mV [from (A)] recorded during backward pumping (a) or in the presence of strophanthidin (b). The high-gain, strophanthidin-sensitive currents (a − b) were obtained by subtracting records in (b) from counterparts in (a). (C) Superimposed current records as in (B), but recorded during K^+/K^+ exchange without (c) or with (d) strophanthidin. The high-gain strophanthidin-sensitive currents (c − d)were obtained by appropriate subtraction. Total cell capacitance was 177 pF. Initial pipette resistance was 1.1 MΩ; 1.3 MΩ of the 2.5 mΩ series resistance was compensated. (From Bahinski *et al.*, 1988b. Reprinted with permission.)

pipette [ATP] were already known to be adequate to drive forward K^+ translocation (Figs. 1, 2, 4, and 7), in order to force the pump to carry out K^+/K^+ exchange, we suddenly removed all external Na^+ and added 5.4 m*M* external K^+, while retaining the high-K^+, Na^+-free pipette solution including 1 m*M* ATP and 5 m*M* P_i (Fig. 8A). Following that change of external solution, neither transient nor steady-state strophanthidin-sen-

sitive currents could be recorded (Fig. 8C,*c* – *d*), demonstrating that K^+ translocation by the Na/K pump does not involve net charge movement within the membrane field.

VI. VOLTAGE-SENSITIVE STEPS

The complete absence of strophanthidin-sensitive current under conditions appropriate for K/K exchange (Fig. 8C) suggests that K^+ transport by the Na/K pump does not include a charge-translocating, and hence voltage-sensitive, step. The same conclusion can be drawn from recent experiments carried out on partially purified Na/K pumps reconstituted into artificial phospholipid vesicles, in which a membrane potential was set with ionophores and a suitable ion gradient (reviewed in Karlish *et al.*, 1985, 1988). The observations were that large positive membrane potentials did not alter the rates of (ATP + P_i)-activated, vanadate-inhibited Rb^+/Rb^+ exchange, or of ATP-activated Na^+/K^+ transport at low [ATP] (when K^+ translocation was expected to be rate limiting) (Goldshlegger *et al.*, 1987), or of conformational changes of K^+-loaded, fluorescein-labeled enzyme (Rephaeli *et al.*, 1986a).

In contrast, the observations of transient, but not steady-state, strophanthidin-sensitive currents in cells exposed to K^+-free solution, when pump activity is presumably restricted to Na^+ translocation (Nakao and Gadsby, 1986), and of both transient and steady-state strophanthidin-sensitive currents under conditions appropriate for forward or reverse Na^+/K^+ exchange (Bahinski *et al.*, 1988b), argue that Na^+ translocation does include a charge-moving, and hence voltage-sensitive, step. Evidence in support of this conclusion comes both from further experiments on fluorescein-labeled pumps reconstituted into lipid vesicles in which the rate of the forward conformational transition ($E_1P \rightarrow E_2P$) of Na^+-loaded enzyme, phosphorylated via acetylphosphate, was increased by a large positive membrane potential (Rephaeli *et al.*, 1986b), and from experiments on Na^+,K^+-ATPase-containing membrane fragments adsorbed onto plane lipid bilayers (Fendler *et al.*, 1985, 1987; Borlinghaus *et al.*, 1987; Apell *et al.*, 1987). In the latter experiments, flash photolysis of caged ATP gave rise to a transient short-circuit current which required Na^+ but not K^+, was inhibited by ouabain, and decayed with an exponential time course. The time constant was approximately the same as that of the transient pump currents recorded in cardiac cells in K^+-free solution (described above; see also Figs. 5–7) as well as that of the transient ^{22}Na efflux initiated by photorelease of caged ATP inside tight membrane vesicles (Forbush, 1984). The overall conclusion, then, is that Na^+ translocation is voltage dependent but K^+ translocation is not.

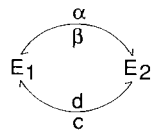
The simplest interpretation of the voltage dependence of the charge moved during Na^+ translocation (Fig. 6A) is that it involves movement of a single charge per pump site. If so, then all other charge movements during a complete forward Na^+/K^+ transport cycle must sum to zero, since only a single positive charge is extruded per cycle. In other words, any additional voltage-sensitive step(s) would require further (balancing) charge movement in the opposite sense, so that the steady-state *I–V* relationship should display regions of negative slope at large positive or negative potentials, because while those voltages would speed one or another of the steps they would simultaneously slow the step with opposite voltage sensitivity (Chapman *et al.*, 1983; Reynolds *et al.*, 1885; Läuger and Apell, 1986).

Cardiac cells provide no consistent evidence for a negative slope region in the Na/K pump *I–V* relationship between −140 and +60 mV, over a range of pump rates at various $[K]_o$, and $[Na]_i$ and $[Na]_o$ (Gadsby and Nakao, 1987): Steady pump current seems to approach a saturating outward level when the pump cycle runs forward (e.g., Figs. 4 and 7B) and a saturating inward level when it runs backward (Fig. 3). Likewise, recent experiments on squid giant axons, using improved techniques to minimize contaminating K^+ currents and electrical end effects, yielded monotonic *I–V* relationships for both the forward-running (De Weer *et al.*, 1987) and the backward-running (Rakowski *et al.*, 1988) Na/K pump, which were closely similar to those described here. Negative slope regions inferred from earlier measurements (e.g., De Weer and Rakowski, 1984; Lafaire and Schwarz, 1986) are likely to have been artifactual (Rakowski, 1987; De Weer *et al.*, 1988a,b).

An economical explanation for all of these findings is that the single charge movement associated with Na^+ translocation constitutes the only voltage-dependent step in the Na/K pump cycle (cf. Nakao and Gadsby, 1986; Goldshlegger *et al.*, 1987).

VII. RATE-LIMITING STEPS

Any multistep cycle with a single voltage-sensitive step may be represented by a reduced, pseudo-two-state kinetic scheme (Hansen *et al.*, 1981, 1983; cf. De Weer, 1984),



where empirical voltage-dependent rate constants α and β are instantaneous functions of membrane potential, α increasing and β decreasing for positive voltage jumps, and c and d are empirical, lumped rate constants representing all voltage-independent steps in the original multistep cycle. If voltage-independent steps were rate limiting, i.e., $(c + d) < (\alpha + \beta)$, steady-state concentrations of enzyme intermediates E_1 and E_2 ($[E_1]$, $[E_2]$) would be determined largely by α and β, and would therefore be voltage dependent. A positive voltage step would thus elicit an initial outward current jump, due to the instantaneous increase in α and decrease in β, followed by an exponential relaxation, with a time constant $(\alpha + \beta + c + d)^{-1}$, due to the subsequent decrease in $[E_1]$ and increase in $[E_2]$ toward their new steady levels; the ratio of the amplitudes of transient to steady current changes is given by $(\alpha + \beta)/(c + d)$ (Hansen *et al.*, 1983).

Such transient currents are analogous to the transient bursts of product that characterize the "pre-steady-state" kinetics of enzyme reactions in which product release precedes the rate-limiting step (e.g., Gutfreund, 1975; Forbush, 1984; Karlish and Kaplan, 1985). If, on the other hand, the voltage-dependent step were severely rate limiting, i.e., $(\alpha + \beta) << (c + d)$, there would be almost no transient current, because $[E_1]$ and $[E_2]$ would be controlled by voltage-independent rate constants and so would not change after a voltage jump; in that case, the new steady level of pump current would be established virtually instantaneously.

The relatively large transient pump currents shown in Figs. 5–8 therefore argue that, in the present experiments, voltage-independent steps limit the rate of both forward and reverse Na/K pump cycles. We can further conclude, then, that the overall rate of the pump cycle is voltage dependent not because the voltage-sensitive step is rate limiting, but because it controls the concentration of the enzyme intermediate that enters the rate-limiting step (De Weer *et al.*, 1988a,b). The $[K]_o$ dependence of both the saturating, voltage-independent amplitude of forward pump current (Fig. 7B) and the decay rate of the transient component (Fig. 7A) suggests that the rate-limiting, voltage-insensitive step in the forward cycle reflects K^+ translocation (Bahinski *et al.*, 1988b).

In K^+-free fluid (Figs. 5–7), steady pump current is negligible, so that the ratios of the transient to steady pump current amplitudes, given by $(\alpha + \beta)/(c + d)$, indicate that both voltage-independent rate constants c and d are also negligible. In that case, the rate constant $(\alpha + \beta + c + d)$ for the decay of transient pump currents at 0 m*M* $[K]_o$ in Figs. 5–7 approximates the sum $\alpha + \beta$, which is ~180–200 sec^{-1} at positive potentials. Since β is expected to decline at positive potentials, α should approach 180–200 sec^{-1} at sufficiently large positive voltages. Moreover, at the midpoint of the Boltzmann curve in Fig. 6, the forward and backward

rate constants should be equal. Thus, at the midpoint, -20 mV, $\alpha + \beta = 2\alpha = 270$ sec^{-1}, and so $\alpha = 135$ sec^{-1}. For comparison, the voltage-independent forward rate constant, c, can be estimated from the saturating level of steady-state outward pump current at positive potentials if the number of pump sites per cell is known (Hansen *et al.*, 1981). This can be determined from the maximum quantity of mobile charge (from a graph like that in Fig. 6A), on the assumption that a single charge moves per pump. For the cell in Fig. 7, the maximum quantity of mobile charge at 0 m*M* $[K]_o$, 4.2 pC, gives a total of 26×10^6 pumps per cell, or a density, related to linear membrane capacitance (218 pF for this cell), of 12×10^4 pF^{-1}, which yields estimates of the voltage-independent forward rate constant, c, of 1, 24, and 55 sec^{-1} at 0, 1, and 5.4 m*M* $[K]_o$, respectively (Bahinski *et al.*, 1988b).

These calculations support the above qualitative conclusion, based on the large transient currents, that the rate-limiting step of the Na/K pump reaction cycle is a voltage-independent step which is sensitive to the external [K]. Na^+ translocation, on the other hand, is faster and voltage-sensitive, and is likely to involve the only charge-translocating step in the entire Na^+/K^+ transport cycle.

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Chapter 15

Sodium–Calcium Exchange in Cardiac, Smooth, and Skeletal Muscles: Key to Control of Contractility

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I. INTRODUCTION

Calcium ions play a key regulatory role in numerous physiological processes in most types of cells (see, e.g., Campbell, 1983). Consequently, it is important to understand how cell Ca^{2+} and the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, are controlled. One of the mechanisms involved in $[Ca^{2+}]_i$ control in many types of cells (Blaustein, 1974) is the Na^+–Ca^{2+} exchanger, which was first identified in invertebrate nerve and vertebrate cardiac muscle in the late 1960s (Baker and Blaustein, 1968; Reuter and Seitz, 1968; Baker *et al.*, 1969; Blaustein and Hodgkin, 1969). Na^+–Ca^{2+}

exchange is a form of "secondary active" transport; it is powered by the Na^+ electrochemical gradient, $\Delta\bar{\mu}_{Na}$, across the plasma membrane and, thus, indirectly, by the Na^+ pump. A discussion of the properties and physiological significance of this countertransport system therefore seems most appropriate for inclusion in the present symposium. The properties of the Na^+–Ca^{2+} exchanger appear to be quite similar in most types of tissues in which it has been identified. The present discussion will, however, be limited to a review of Na^+–Ca^+ exchange in various types of muscle.

An increase in $[Ca^{2+}]_i$ is the normal trigger for contraction in virtually all types of muscle. This "trigger Ca^{2+}" may come from intracellular stores and/or from the extracellular fluid (e.g., Costantin, 1977; Chapman, 1983; Johansson and Somlyo, 1980; Ashida *et al.*, 1988). Conversely, removal of Ca^{2+} from the contractile machinery, by Ca^{2+} buffering (Pechere *et al.*, 1977; Somlyo *et al.*, 1981) and resequestration in the sarcoplasmic reticulum (SR), and/or by extrusion across the sarcolemma, is normally required to induce relaxation. In addition, in some types of muscle, other agents such as calmodulin and cyclic nucleotides may influence the sensitivity of the contractile apparatus to Ca^{2+}, and thus alter the relationship between $[Ca^{2+}]_i$ and tension (Ruegg and Paul, 1982; Schoeffter *et al.*, 1987).

The principle pathways involved in the movement of Ca^{2+} into and out of the sarcoplasmic space are diagrammed in Fig. 1. Note that the Na^+–Ca^{2+} exchanger operates in series with the calmodulin-insensitive ATP-driven Ca^{2+} pump and the Ca^{2+} release channels in the SR, and, in parallel, with the voltage-gated and agonist-operated Ca^{2+} entry channels and the calmodulin-regulated ATP-driven Ca^{2+} extrusion pump in the sarcolemma. A puzzling feature of this arrangement concerns the relative roles of the parallel Ca^{2+} transport systems in the sarcolemma; a possible explanation will be discussed below.

Ca^{2+} can be transported both inwardly (Ca^{2+} entry mode) and outwardly (Ca^{2+} exit mode) via the Na^+–Ca^{2+} exchanger (Blaustein, 1977a). Net Ca^{2+} movements mediated by the exchanger [$J_{Ca(Na/Ca)}$] are governed by the difference between the membrane potential, V_M, and the reversal potential of the exchanger, $E_{Na/Ca}$, as well as by the kinetic parameters that control the rate of exchange:

$$J_{Ca(Na/Ca)} = k(V_M - E_{Na/Ca}) \quad (1)$$

The variable k is a complex kinetic parameter that depends on: (1) the number of carriers, (2) the fractional saturation of the carrier binding sites by the activating ions and transported ions, (3) V_M, and (4) adenosine

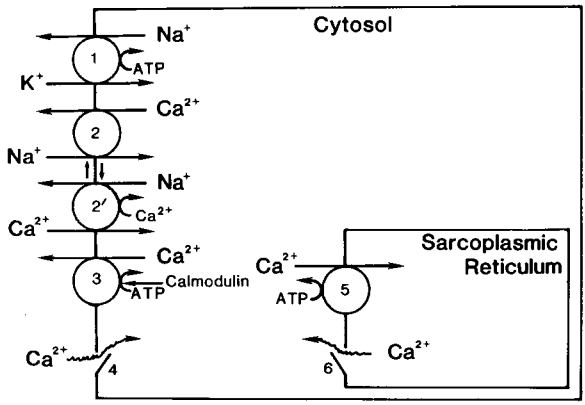

FIG. 1. Diagram of a smooth muscle cell showing the plasma membrane and sarcoplasmic reticulum (SR) mechanisms that play a role in the regulation of $[Ca^{2+}]_i$ in the cytosol. (1) Ouabain-sensitive, ATP-driven Na^+ pump; (2) Plasma membrane Na^+–Ca^{2+} exchange system operating in the Ca^{2+} efflux mode (2) and Ca^{2+} influx mode (2'); (3) ATP-driven, calmodulin-regulated plasma membrane Ca^{2+} pump; (4) Ca^{2+}-sensitive channels (both voltage gated and receptor operated); (5) ATP-driven calmodulin-insensitive SR Ca^{2+} pump; and (6) Ca^{2+} channels that mediated Ca^{2+} release from the SR. As illustrated, the Ca^{2+} entry mode of Na^+–Ca^{2+} exchange is activated by intracellular free Ca^{2+} in the dynamic physiological concentration range ($\sim 10^{-7}$ to 10^{-6} *M*). Although not shown here, the exchanger is also modulated by (internal) ATP, which affects the cation affinities (Blaustein, 1977c; DiPolo and Beaugé, 1984). (From Blaustein, 1985. Reprinted with permission.)

triphosphate (ATP), which is not used as a fuel, but does modulate the carrier kinetics (DiPolo, 1976; Blaustein, 1977c). In most preparations in which it has been studied, the Na^+–Ca^{2+} exchange coupling ratio is about 3 Na^+ : 1 Ca^{2+} (Sheu and Blaustein, 1986; Rasgado-Flores and Blaustein, 1987). Thus, the reversal potential, $E_{Na/Ca}$, is:

$$E_{Na/Ca} = 3E_{Na} - 2E_{Ca} \tag{2}$$

where

$$E_{Na} = (RT/F)\ln([Na^+]_o/[Na^+]_i) \tag{2a}$$

and

$$E_{Ca} = (RT/2F)\ln([Ca^{2+}]_o/[Ca^{2+}]_i) \tag{2b}$$

The terms in brackets refer to the respective free ion concentrations in the extracellular (o) and intracellular (i) fluids, and R, T, and F are the gas constant, absolute temperature, and Faraday's number, respectively.

II. Na^+–Ca^{2+} EXCHANGE IN BARNACLE MUSCLE

A. Direct Evidence of 3 Na^+ : 1 Ca^{2+} Coupling Ratio

Very large elongated cells, such as the squid axon and giant barnacle muscle fiber, have proven to be exceptionally useful for the study of numerous fundamental general physiological phenomena, including Na^+–Ca^{2+} exchange (e.g., Baker *et al.*, 1969; Blaustein and Hodgkin, 1969; Ashley *et al.*, 1972, 1974; Russell and Blaustein, 1974; DiPolo, 1976; DiPolo and Beaugé, 1984). These large cells are particularly well suited for transport experiments because the cytoplasmic solute composition can be conveniently controlled by internal dialysis or perfusion so that the thermodynamic and kinetic factors that regulate the various transport processes can be identified.

Internally perfused giant barnacle muscle cells were recently used to study the coupling ratio and the kinetics of Na^+–Ca^{2+} exchange during the Ca^{2+} entry mode operation of the exchanger (Rasgado-Flores and Blaustein, 1987; Rasgado-Flores *et al.*, 1989). In the Ca^{2+} entry mode, the coupled countermovements of Ca^{2+} and Na^+ could be unambiguously identified, respectively, as the Na_i-dependent $^{45}Ca^{2+}$ influx and the Ca_o-dependent $^{22}Na^+$ efflux. All of the external Na^+ was replaced by Tris to eliminate both Na^+–Na^+ exchange and Ca^{2+}–Ca^{2+} exchange, since the latter is observed only in the presence of a high external concentration of alkali metal ion (Russell and Blaustein, 1974; Blaustein, 1977c). Measurement of the stoichiometry of the Ca^{2+} exit mode exchange in this preparation is more ambiguous because physiological concentrations of internal Ca^{2+} activate an alkali metal ion conductance that is likely to lead to error in the measurement of the Ca_i-dependent Na^+ influx (Sheu and Blaustein, 1983).

The Na_i-dependent influx of $^{45}Ca^{2+}$ and the Ca_o-dependent efflux of $^{22}Na^+$ are graphed as a function of $[Na^+]_i$ at two different levels of $[Ca^{2+}]_i$: 10^{-8} and 10^{-6} M (Fig. 2). At $[Ca^{2+}]_i = 10^{-6}$ M, which is within the dynamic physiological range for activation of contraction in these cells (Hagiwara and Nakajima, 1966; Ashley and Caldwell, 1974), both the Ca^{2+} influx and the Na^+ efflux are sigmoid functions of $[Na^+]_i$. These data fit the Hill equation, with a Hill coefficient of 3. Moreover, as shown in the figure, the Ca^{2+} and Na^+ flux data at each $[Na^+]_i$ are nearly superimposable when the ordinates are scaled so that the Ca^{2+} influx equals one third of the Na^+ efflux. This indicates directly that the coupling ratio of the exchange is 3 Na^+ : 1 Ca^{2+}. A similar coupling ratio was inferred from Hill coefficient 3 for the activation, by external Na^+, of Ca^{2+} efflux from intact ^{45}Ca-injected barnacle muscle cells (Russell and Blaustein, 1974).

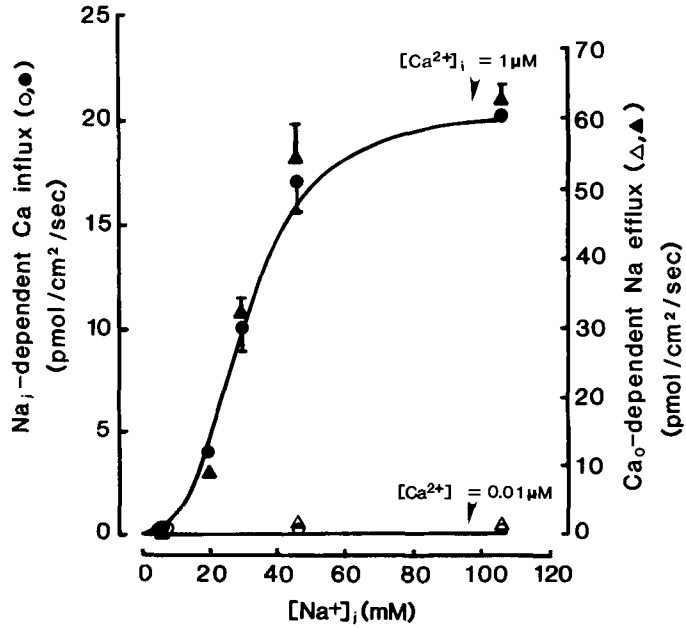

FIG. 2. Effects of $[Na^+]_i$ on the Ca_o-dependent Na^+ efflux (△, ▲; right-hand ordinate scale) and the Na_i-dependent Ca^{2+} influx (○, ●; left-hand ordinate scale) in internally perfused barnacle muscle cells. Data from 27 cells are summarized; data for at least two different $[Ca^{2+}]_i$ and/or $[Na^+]_i$ were obtained in each cell. Each symbol represents the mean of at least three flux measurements; the bars indicate ± SEM for each of the data points where the errors extend beyond the symbols. The external solution in all experiments was (Na^+-free) Tris–sea water containing 0.1 m*M* ouabain to inhibit the Na^+ pump; $[Ca^{2+}]_i$ was either 0.01 μ*M* (open symbols) or 1.0 μ*M* (solid symbols). Note that the ordinate scale for the Ca^{2+} influx is expanded threefold, relative to the scale for the Na^+ efflux. The solid line fits the Hill equation, with a Hill coefficient of 3, a K_{Na_i} of 30 m*M*, and maximal fluxes of 20.5 pmol/cm²/sec for Na_i-dependent Ca^{2+} influx and 61.5 pmol/cm²/sec for Ca_o-dependent Na^+ efflux. All data were obtained with V_M between -33 and -43 mV; more than 90% of the data were obtained with V_M = -37 ± 3 mV. These data indicate that the coupling ratio of the Ca^{2+} entry mode Na^+–Ca^{2+} exchange is 3 Na^+:1 Ca^{2+}. (Modified from Rasgado-Flores *et al.*, 1989.)

B. Activation of Ca^{2+} Entry Mode Na^+–Ca^{2+} Exchange by Internal Ca^{2+}

When $[Ca^{2+}]_i$ is reduced to 10^{-8} *M*, there is negligible Na^+–Ca^{2+} exchange, even at high $[Na^+]_i$ (Fig. 2), as is also the case in squid axons (DiPolo and Beaugé, 1986, 1987) and mammalian cardiac muscle (Kimura *et al.*, 1986). The paradoxical requirement for *internal* Ca^{2+} to activate the Ca^{2+} entry mode of exchange is illustrated in Fig. 3. Half-maximal

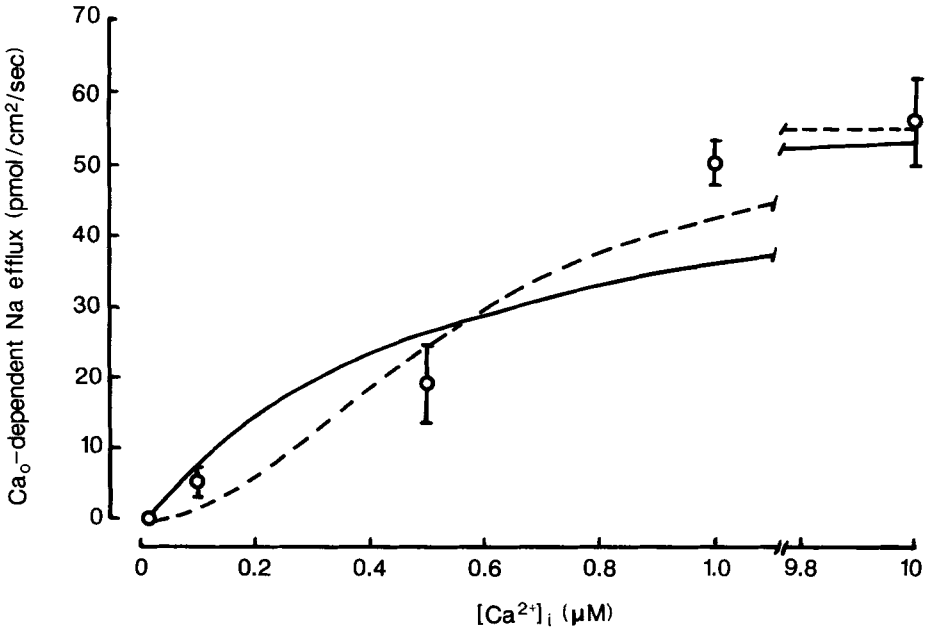

FIG. 3. Ca_o-dependent Na^+ efflux from internally perfused barnacle muscle cells graphed as a function of $[Ca^{2+}]_i$. The external solution was (Na^+-free) Tris-SW containing 0.1 m*M* ouabain; the internal perfusion fluid contained 46 m*M* Na^+ and various $[Ca^{2+}]_i$ obtained with a Ca-ethyleneglycoltetraacetic acid (EGTA) buffer containing 8 m*M* $EGTA_{total}$. The data are the means ± SEM of the efflux measurements from 4 cells. The continuous and discontinuous lines fit the Hill equation, with Hill coefficients of 1 and 2, respectively, and with $K_{Ca_i} = 0.6$ μ*M* and $J_{Na(max)} = 58$ pmol/cm²/sec. (Modified from Rasgado-Flores *et al.*, 1989.)

activation is observed at $[Ca^{2+}]_i = 0.7$ μ*M*, which is in the middle of the dynamic physiological range of $[Ca^{2+}]_i$. This internal Ca^{2+} is not transported by the exchanger during Ca^{2+} influx mode operation. Since Na^+–Ca^{2+} exchange-mediated Ca^{2+} efflux is half-maximally activated with a similar $[Ca^{2+}]_i$, at least in squid axons (Blaustein, 1977c), the implication is that the exchanger is relatively dormant in resting (relaxed) barnacle muscle cells, in which $[Ca^{2+}]_i$ is $<10^{-7}$ μ*M*. This is consistent with observations on Ca^{2+} influx and efflux in normal relaxed cells, in which both fluxes are about 1 pmol/cm²/sec, and less than half of these fluxes can be attributed to Na^+–Ca^{2+} exchange (Ashley *et al.*, 1972; Russell and Blaustein, 1974; M. P. Blaustein, unpublished observations). When the cells are activated and $[Ca^{2+}]_i$ rises into the contractile range, the exchanger should be activated; the direction of net Ca^{2+} movement will

then be determined by whether V_M is more positive (Ca^{2+} influx) or more negative (Ca^{2+} efflux) than $E_{Na/Ca}$ [see Eq. (1)]. The data in Figs. 2 and 3 imply that, during cell activation, the exchanger-mediated fluxes of Ca^{2+} may increase as much as 50- to 100-fold above the corresponding fluxes in the resting cell. This is very different from the Na^+ pump, which normally operates at about its half-maximal velocity in resting barnacle muscle cells (Nelson and Blaustein, 1980).

The precise physiological role of the Na^+–Ca^{2+} exchanger in barnacle muscle is uncertain. In resting cells, it may help to determine the size of the SR Ca^{2+} store. While much of the trigger Ca^{2+} for depolarization-activated contraction may enter the cells via voltage-gated Ca^{2+}-selective channels (Hagiwara and Takahashi, 1967), some may also enter via the exchanger when the cells are depolarized and $[Ca^{2+}]_i$ rises. Indeed, the exchanger may help to keep $[Ca^{2+}]_i$ elevated during slow tonic contractions, and may help to extrude Ca^{2+} rapidly when activation is terminated and the cells repolarize. If the exchanger turns over about tenfold more rapidly (>1000 sec^{-1}; Cheon and Reeves, 1988) than the ATP-driven Ca^{2+} extrusion pump (~50–150 sec^{-1}; Schatzmann, 1982), far fewer exchanger molecules would be required to lower $[Ca^{2+}]_i$ rapidly.

III. Na^+–Ca^{2+} EXCHANGE IN VERTEBRATE SKELETAL MUSCLE

Na^+–Ca^{2+} exchange has been studied only to a very limited extent in skeletal muscles. In twitch-type skeletal muscles, virtually all of the Ca^{2+} required to activate contraction comes from the SR (Costantin, 1977). Nevertheless, skeletal muscle is slightly permeable to Ca^{2+}, and Ca^{2+} entry is increased during excitation (e.g., Bianchi and Shanes, 1959: Curtis, 1966; Stanfield, 1977; Chiarandini and Stephani, 1983; Rios and Brum, 1987). Thus, although Ca^{2+} must be actively extruded against a very large electrochemical gradient, much of this efflux may be mediated by the ATP-driven Ca^{2+} pump in the sarcolemma (Brandt *et al.*, 1980). In addition, however, there is evidence that skeletal muscle sarcolemma contains a Na^+–Ca^{2+} exchanger.

In 1961, Cosmos and Harris demonstrated an antagonism between Na^+ and Ca^{2+} in frog skeletal muscle: Reduction of $[Na^+]_o$ increased Ca^{2+} influx (using ^{89}Sr as a tracer for Ca^{2+}) and net Ca^{2+} gain (Cosmos and Harris, 1961). These effects were reversed when Na^+ was added back. The muscle fibers also gained (net) Ca^{2+} when they were Na^+-loaded in K^+-free or ouabain-containing media, and the Ca^{2+} was reextruded when the fibers were subsequently enabled to pump the Na^+ out—e.g., by reintroduction of external K^+. Caputo and Bolaños (1978) found that ~40%

of the Ca^{2+} efflux from ^{45}Ca-loaded muscle fibers into Ca^{2+}-free media was external Na dependent.

Sarcolemmal vesicles prepared from amphibian (Donoso and Hidalgo, 1988) and mammalian (Gilbert and Meissner, 1982; Donoso and Hidalgo, 1988) skeletal muscle exhibit a Na^+–Ca^{2+} exchanger manifested by voltage-sensitive external Na^+-dependent Ca^{2+} efflux and internal Na^+-dependent Ca^{2+} influx. The exchanger is sensitive to amiloride (Donoso and Hidalgo, 1988) and the amiloride analog, dichlorobenzamil (Curtis, 1986), two agents that block the Na^+–Ca^{2+} exchanger in other tissues, albeit somewhat nonselectively (L. Smith *et al.*, 1987; Bielefeld *et al.*, 1986). The exchanger has been found in the transverse (t-) tubule and surface membranes of frog skeletal muscle, but only in the surface sarcolemma of rabbit skeletal muscle (Donoso and Hidalgo, 1988).

The physiological significance of the Na^+–Ca^{2+} exchanger in vertebrate twitch-type skeletal muscle is unclear. It may, as in other types of muscle, modulate the amount of Ca^{2+} in the SR store and thereby influence twitch amplitude. Curtis (1986, 1988) has raised the interesting possibility that the exchanger may play a role in t-tubule–SR coupling because dichlorobenzamil inhibits the repriming of the contractile apparatus and blocks twitches and contractures. The apparent absence of the exchanger in rabbit skeletal muscle t-tubules is, however, inconsistent with this hypothesis.

Tonic skeletal muscle (frog rectus abdominis), in contrast to twitch-type muscle, develops sustained contractures when $[Na^+]_o$ is reduced (Schaechtelin, 1961). This may mean that the tonic muscle has a less extensive SR than does twitch-type muscle and therefore relies more on Ca^{2+} influx across the sarcolemma for excitation–contraction coupling. Clearly, the physiological role of the Na^+–Ca^{2+} exchanger in these skeletal muscles requires further investigation.

IV. Na^+–Ca^{2+} EXCHANGE IN CARDIAC MUSCLE

The pioneering studies by Wilbrandt and Koller (1948), Lüttgau and Niedergerke (1958), and Reuter and Seitz (1968) form the background for the widely accepted view that Na^+–Ca^{2+} exchange plays a critical role in the control of cardiac contractility. This subject has been extensively reviewed in recent years (e.g., Chapman, 1983; Eisner and Lederer, 1985; Philipson, 1985; Reeves, 1985; Lederer *et al.*, 1986; Sheu and Blaustein, 1986). Therefore, only a brief description of a few key observations that demonstrate the importance of the Na^+ concentration gradient in the control of cardiac contractility seems warranted.

A. Force–Frequency Relationship

One example of this critical relationship is seen in the marked dependence of cardiac contraction strength on stimulation frequency: the "force–frequency relationship" or tension staircase (Koch-Weser and Blinks, 1963; Chapman, 1979). As illustrated by the sheep Purkinje fiber data in Fig. 4, there is an immediate increase in twitch tension when the stimulation frequency is slowed. This is presumably due to enhanced reuptake of Ca^{2+} by the SR because of the increased time available for Ca^{2+} transport between beats; this provides an increase in the releasable store of Ca^{2+}. However, the Na^+ pump now has more time to extrude Na^{2+} between beats, and $[Na^+]_i$ gradually falls. This is paralleled by a progressive decline in twitch tension with time because, as $[Na^+]_i$ falls, so does diastolic $[Ca^{2+}]_i$ (via Na^+–Ca^{2+} exchange) and, therefore, the amount of Ca^{2-} stored in the SR (Tanford, 1981). Conversely, when beat frequency is increased, the opposite effects are observed: There is an immediate fall in twitch tension, followed by a slow rise that parallels the rise in $[Na^+]_i$.

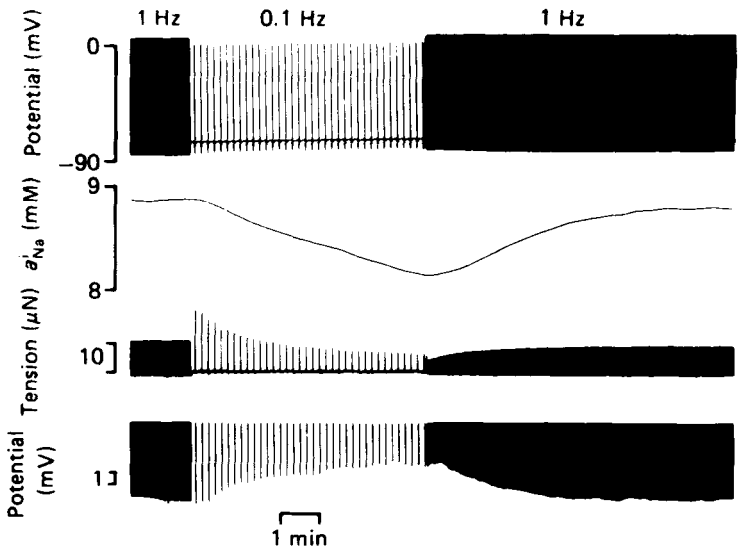

FIG. 4. Effects of stimulation rate on the intracellular Na^+ activity (a^i_{Na}), membrane potential, and twitch tension in sheep cardiac Purkinje fibers. As indicated, the stimulation frequency was reduced from 1/sec to 0.1/sec, and then increased to 1/sec. The membrane potential trace at the bottom is an amplification of the lower part of the record shown at the top. The intracellular Na^+ concentration is equal to a^i_{Na} divided by the Na^+ activity coefficient (~0.75 in free solution with an ionic strength equivalent to that of mammalian blood plasma). (From Lederer and Sheu, 1983. Reprinted with permission.)

The sequence of events responsible for these changes in tension, exemplified by the rising tension staircase in response to an increase in beat frequency, is diagrammed in Fig. 5. In these tissues, in which the SR is a major source of Ca^{2+} for contractile activation, twitch tension is regulated by the refilling of the SR Ca^{2+} store between beats. This is controlled primarily by the duration of diastole and the level of diastolic $[Ca^{2+}]_i$; the latter is, in turn, markedly influenced by $[Na^+]_i$ via its effect on Na^+–Ca^{2+} exchange (e.g., Lederer and Sheu, 1983; Bers, 1985; Sutko *et al.*, 1986).

B. Action of Cardiotonic Steroids on Heart

The critical influence of $[Na^+]_i$ on cardiac twitch tension is exemplified by the action of cardiotonic steroids on the heart (Blaustein, 1985). As

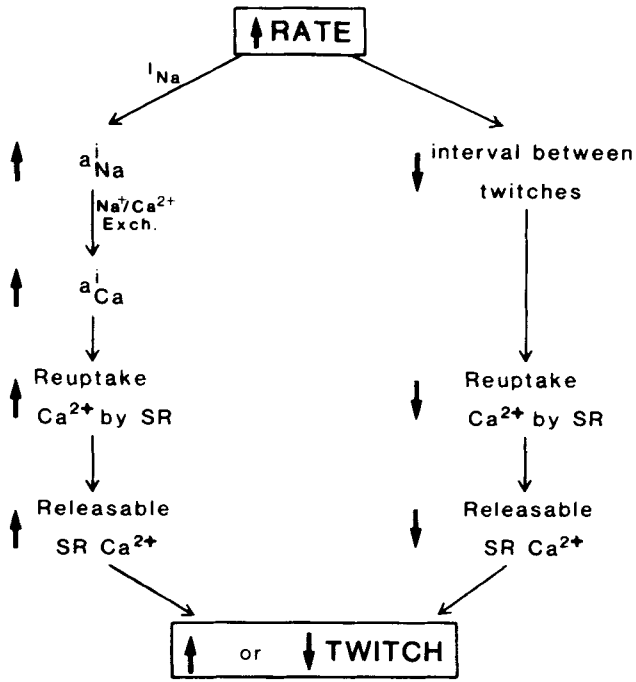

FIG. 5. Diagram illustrating the sequence of events involved in the force–frequency relationship in cardiac muscle. Following an increase in beat frequency, there may be a reduction in twitch tension initially (sequence on the right) followed by an increase in twitch tension (sequence on the left). (Data from S. -S. Sheu and W. J. Lederer, in Sheu and Blaustein, 1986. Reprinted with permission.)

shown in the data from dog cardiac Purkinje fibers in Fig. 6, a low concentration of strophanthidin may cause an initial transient *decline* in $[Na^+]_i$—presumably due to stimulation of the Na^+ pump (Lee and Dagostino, 1982; Sheu *et al.*, 1983; Hamlyn *et al.*, 1985). This is followed by a sustained increase in $[Na^+]_i$ that results from Na^+ pump inhibition (Glynn, 1964; Schwartz *et al.*, 1975). These changes in $[Na^+]_i$ are closely paralleled by changes in twitch tension: There is an initial decline, followed by a prolonged increase in which the tension amplitude follows the variation in $[Na^+]_i$. The changes in $[Na^+]_i$ are reflected by parallel changes in (diastolic) $[Ca^{2+}]_i$ (as a result of Na^+–Ca^{2+} exchange) and, thus, by the amount of Ca^{2+} stored in the SR (because this is proportional to $[Ca^{2+}]_i$; Tanford, 1981; Bers, 1985). The amount of Ca^{2+} released by the SR and, consequently, the amplitudes of the Ca^{2+} transient and the twitch (Wier and Hess, 1984) during each beat are directly related to the size of the Ca^{2+} store. This sequence of events, diagrammed in Fig. 7, therefore shows how the Na^+–Ca^{2+} exchanger serves as the functional link between the inhibition of the Na^+ pump and the positive inotropic effect of the cardiotonic steroids (Baker *et al.*, 1969; Blaustein, 1985; Smith, 1988).

In addition to the force–frequency relationship and the cardiotonic action of cardiac glycosides, a number of other aspects of cardiac function appear to depend critically on the activity of the Na^+–Ca^{2+} exchanger. These include (see Sheu and Blaustein, 1986): (1) the beat-to-beat regulation of cardiac contraction, (2) β-adrenergic relaxation, (3) calcium-in-

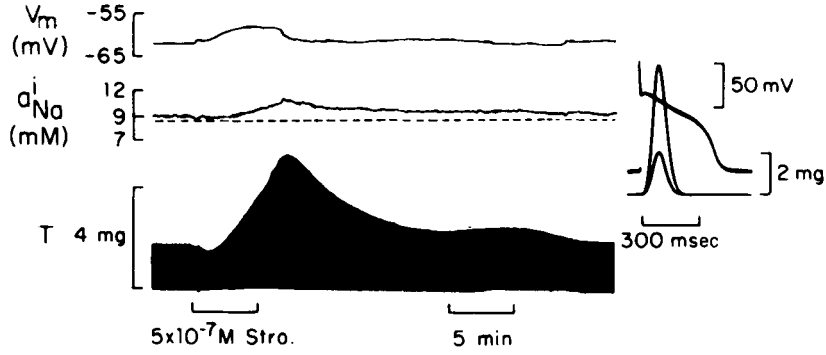

FIG. 6. Effects of strophanthidin [(Stro.) 5×10^{-7} *M*] on intracellular Na^+ activity (a^i_{Na}), twitch tension *(T)*, and filtered (0.24 *Hz* cut-off) transmembrane potential (V_M) of canine cardiac Purkinje fibers driven at a frequency of 1/sec. (Inset) Superimposed action potentials (400-msec duration) and twitch tension records (150- to 200-msec duration) before drug application and at the peak of the tension increase after drug application. (From Lee and Dagostino, 1982. Reprinted with permission.)

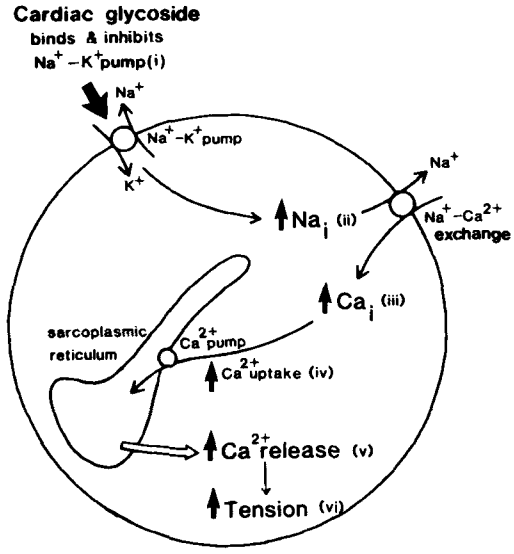

FIG. 7. Diagram of a cardiac muscle cell showing the conventional view of the mechanisms underlying the positive inotropic actions of cardiotonic steroids (the hypothesis of Na^+ pump inhibition and Na^+–Ca^{2+} exchange described in the text). These drugs bind to and inhibit the Na^+ pump (i), thereby elevating the intracellular Na^+ concentration (ii); this increases the cytosolic free Ca^{2+} concentration, Ca_i (iii). In turn, this leads to an increase in the Ca^{2+} content of the SR (iv), so that Ca^{2+} release from the SR (v) and tension (vi) are increased when the muscle is subsequently activated. (From Allen *et al.*, 1985. Reprinted with permission.)

duced arrhythmias (such as those caused by digitalis toxicity), (4) the antiarrhythmic action of local anesthetics, and (5) the calcium paradox and reperfusion injury.

C. Variation in Activity of Na^+–Ca^{2+} Exchanger during Cardiac Cycle

1. DIASTOLE

In view of this well-documented role of the Na^+–Ca^{2+} exchanger in the control of cardiac contractility, the significance of parallel Na^+–Ca^{2+} exchange and ATP-driven Ca^{2+} pump mechanisms in cardiac sarcolemma (e.g., Carafoli, 1987) has been an enigma. However, discovery that exchanger-mediated Ca^{2+} entry as well as Ca^{2+} exit is modulated by $[Ca^{2+}]_i$ in cardiac muscle (Fig. 8) (Kimura *et al.*, 1986), as it is in squid axons

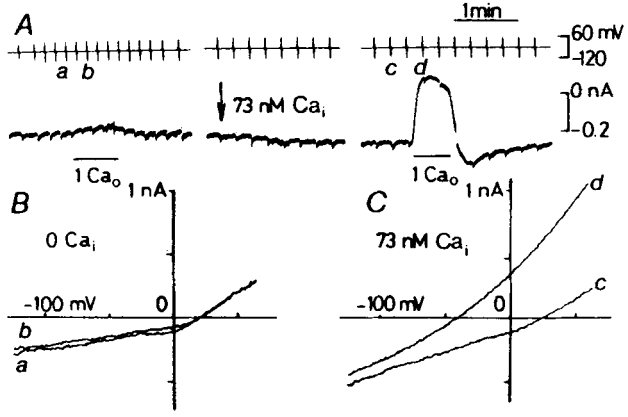

FIG. 8. Activation of an external Ca^{2+}-dependent outward (Na^+–Ca^{2+} exchange) current by internal Ca^{2+} in a single patch-clamped guinea pig cardiac myocyte. (A) Voltage-clamp records showing voltage (upper traces) and current (lower traces). Ramp pulses to +60 mV and then to -120 mV were given from a holding potential of -30 mV every 10 or 15 sec at a ramp speed of about 0.2 V/sec. In the first panel, the patch pipette (internal) solution contained 30 m*M* Na^+ and 42 m*M* EGTA (no free Ca^{2+}); the effect of external application of 1 m*M* Ca^{2+} is shown. In the second and third panels, the pipette solution contained 73 n*M* free Ca^{2+}; the third panel shows the effect of adding 1 m*M* Ca^{2+} to the external solution. The external solutions all contained 1 m*M* $BaCl_2$ and 2 m*M* CsCl to block K^+ channels, 2 μ*M* methoxyverapamil to block Ca^{2+} channels, and 20 μ*M* ouabain to block the Na^+ pump; the pipette solutions all contained 126 m*M* CsCl to reduce current through the K^+ channels. (B) and (C) The I–V (current–voltage) curves were obtained from the descending portions (+60 to -120 mV) of these ramp pulses. (B) The curves were obtained before (*a*) and during (*b*) the superfusion with 1 m*M* Ca^{2+} in the absence of free internal Ca^{2+}. (C) I–V relationship in the presence of 73 m*M* internal Ca^{2+} before (*c*) and during (*d*) superfusion with solution containing 1 m*M* Ca^{2+}. (From Kimura *et al.*, 1986. Reprinted with permission.)

(DiPolo and Beaugé, 1986, 1987) and barnacle muscle (Rasgado-Flores and Blaustein, 1987; see also Section II), may provide a resolution to this dilemma. During diastole, when $[Ca^{2+}]_i$ is below the contraction threshold, the Na^+–Ca^{2+} exchanger turns over very slowly because most of the internal Ca^{2+} activating sites are unoccupied; at this time, the sarcolemmal ATP-driven Ca^{2+} pump probably plays a major role in keeping $[Ca^{2+}]_i$ low. Nevertheless, small changes in $[Na^+]_i$ (1 m*M* or less) or in V_M (a few millivolts) will substantially alter the driving force for the exchanger, ΔV [$= V_M - E_{Na/Ca}$; Eq. (1)]. This will, in turn, bias $[Ca^{2+}]_i$ slightly—although probably not as much as predicted for equilibrium conditions [$V_M = E_{Na/Ca}$; see Eq. (1) and Blaustein, 1974, equation 3]. The change in $[Ca^{2+}]_i$ will be reflected primarily by a change in the amount of releasable

Ca^{2+} stored in the SR (see Section IV, B)—probably with a negligible (undetectable) effect on diastolic tension.

2. Systole

When the cardiac cells are activated, however, $[Ca^{2+}]_i$ rises because of Ca^{2+} influx via voltage-gated channels and/or release of Ca^{2+} from the SR (Chapman, 1983). The amount of Ca^{2+} released from the SR is directly related to the amount stored. The consequent rise in $[Ca^{2+}]_i$ activates the Na^+–Ca^{2+} exchanger. Then, because $E_{Na/Ca}$ is more negative than V_M during the early part of the action potential (Fig. 9), some Ca^{2+} may also enter the cells via the Na^+–Ca^{2+} exchanger (Lipp and Pott, 1988). During the later part of the action potential plateau, when V_M becomes more negative than $E_{Na/Ca}$ (Fig. 9), the exchanger mediates net Ca^{2+} efflux (Hume, 1987; Kenyon and Sutko, 1987; Barcenas-Ruiz *et al.*, 1987; Lipp and Pott, 1988; see also Mullins, 1979; DiFrancesco and Noble, 1985). Then, as $[Ca^{2+}]_i$ declines toward the diastolic level, the exchanger slows itself down (negative feedback), and Ca^{2+} extrusion via the ATP-driven Ca^{2+} pump assumes a relatively larger role. The relationship between the exchanger reversal potential, $E_{Na/Ca}$, and V_M during the cell activity cycle is illustrated in Fig. 9B; the primary factors that determine the rate of Ca^{2+} transport mediated by the exchanger are $[Ca^{2+}]_i$ (Fig. 9A) and the driving force, ΔV [$= V_M - E_{Na/Ca}$; see Fig. 9C and Eq. (1)].

In cardiac muscle, it is important to extrude rapidly, during diastole, all of the Ca^{2+} that enters the cells during systole. The Na^+–Ca^{2+} exchanger is ideally suited for this task because the turnover number, >1000 sec^{-1} (Cheon and Reeves, 1988), is about an order of magnitude larger than the turnover number of the ATP-driven Ca^{2+} pump (Schatzmann, 1982), so that relatively few Na^+–Ca^{2+} exchange transporter molecules need to be present in the sarcolemma to carry out this job.

In sum, the Na^+–Ca^{2+} exchanger appears to have several very important functions in cardiac muscle: (1) It controls the amount of releasable Ca^{2+} in the SR store. (2) It mediates some Ca^{2+} entry during the early part of the action potential. (3) It maintains a high $[Ca^{2+}[_i$ during the action potential plateau (i.e., it appears to "clamp" $E_{Na/Ca}$ close to V_M when $[Ca^{2+}]_i$ is high). (4) It mediates Ca^{2+} efflux during repolarization when a large amount of Ca^{2+} must be extruded during a short period of time.

The general principles of Na^+–Ca^{2+} exchanger function that are responsible for these physiological consequences are also applicable to the other types of muscle (see, for example, Section II). We shall now see how these same principles apply to smooth muscles.

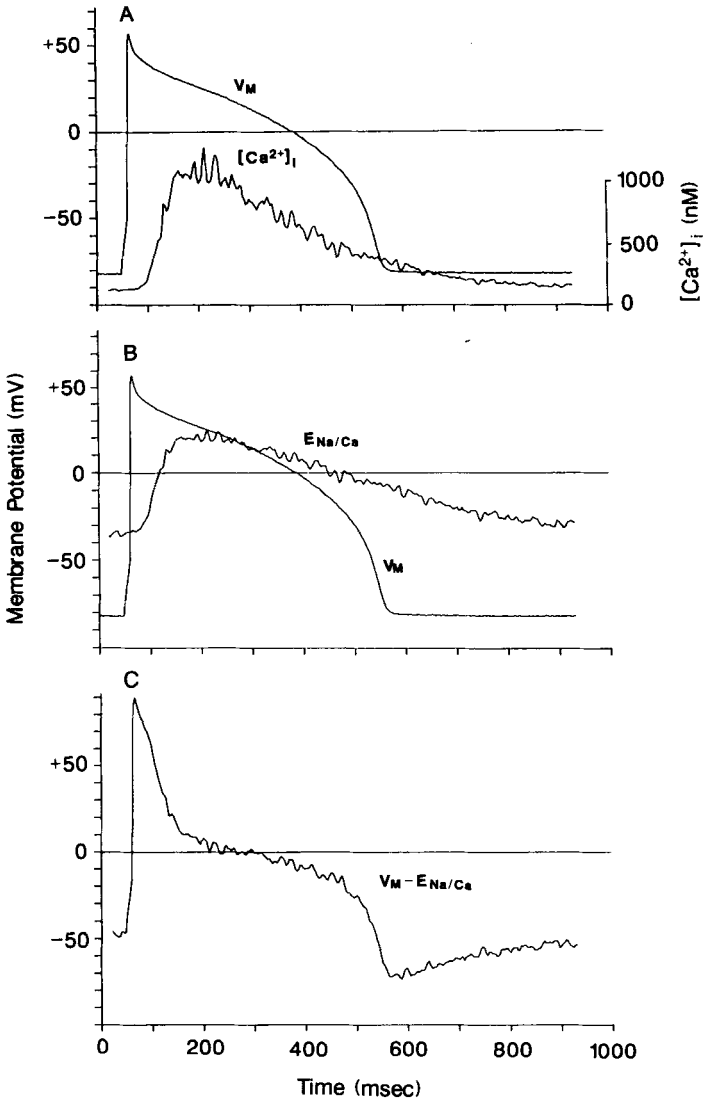

FIG. 9. The time course of changes in $[Ca^{2+}]_i$ (A); $E_{Na/Ca}$ (B); and the driving force for the Na^+–Ca^{2+} exchanger, ΔV [$= V_M - E_{Na/Ca}$; Eq. (1)] (C) in relation to the action potential [V_M in (A) and (B)] in a single rat cardiac ventricular myocyte. $[Ca^{2+}]_i$ was measured by the fura-2 method (Barcenas-Ruiz *et al.*, 1987) and V_M was determined with a patch pipette. The external solution contained 137.5 m*M* Na^+ and 2.5 m*M* Ca^{2+}; $[Na^+]_i$ was assumed to be 8.0 m*M*, slightly higher than the concentration in the pipette solution (7.5 m*M*). $E_{Na/Ca}$ and ΔV were calculated from these ion concentrations and the measured $[Ca^{2+}]_i$ and V_M. If $[Na^+]_i$ is not well controlled and is higher than the concentration in the pipette solution, as seems likely, especially during the action potential, the true $E_{Na/Ca}$ will be more negative than the calculated value. See text for further details. (Data are from an unpublished experiment by D. Beuckelmann and W. G. Wier and are reproduced with their permission.)

V. Na^+–Ca^{2+} EXCHANGE IN SMOOTH MUSCLES

In contrast to the widely accepted view that Na^+–Ca^{2+} exchange plays a central role in the control of contractility in cardiac muscle, there is substantial controversy about its physiological role in many smooth muscles (e.g., Reuter *et al.*, 1973; Blaustein, 1977a,b; van Breemen *et al.*, 1979; Brading and Lategan, 1985; Casteels *et al.*, 1985; Mulvany, 1985; Somlyo *et al.*, 1986). Therefore, it seems worthwhile to begin this section with an analysis of some of the observations that have fostered this controversy. As we shall see, when the properties of the Na^+–Ca^{2+} exchanger, elucidated above, are considered, and the pitfalls of the various experimental methods and manipulations are accounted for, it appears that Na^+–Ca^{2+} exchange does, indeed, contribute significantly to the regulation of cell Ca^{2+} and contractility in most, if not all, mammalian smooth muscles in which it has been studied.

A. Pitfalls in Identifying Na^+–Ca^{2+} Exchange in Smooth Muscles

1. Use of Tension Increase to Estimate Increases in $[Ca^{2+}]_i$

A rise in $[Ca^{2+}]_i$ is an immediate trigger for contraction in vertebrate smooth muscles (Adelstein and Klee, 1980; Johansson and Somlyo, 1980; Kamm and Stull, 1985) as in cardiac and skeletal muscles. Thus, alterations in steady (resting) tension and in the contractile response to activation may serve as indirect measures of changes in $[Ca^{2+}]_i$ and in the size of the SR Ca^{2+} store in smooth muscles (Ashida *et al.*, 1988), as in cardiac muscle (e.g., Chapman, 1983). Indeed, the most common method of studying Na^+–Ca^{2+} exchange in smooth muscle has been the measurement of resting and (in some cases) agonist-evoked tension in response to alterations in $\Delta\bar{\mu}_{Na}$. Even before Na^+–Ca^{2+} exchange was first recognized in invertebrate nerve and in mammalian cardiac muscle in the late 1960s, changes in the Na^+ concentration gradient across the plasma membrane had been observed to affect resting and/or evoked tension in several types of smooth muscles. For example, lowering $[Na^+]_o$, or inhibiting the Na^+ pump by external K^+ removal or treatment with cardiotonic steroids (to raise $[Na^+]_i$), induced contractions and/or enhanced the responsiveness to agonists in vascular smooth muscle (e.g., Leonard, 1957; Mason and Braunwald, 1964) and tracheal smooth muscle (Dixon and Brodie, 1903). Alternatively, reduction of $[Na^+]_o$ induced reversible contractures in vascular smooth muscle (e.g., Hinke and Wilson, 1962) and intestinal smooth muscle (Judah and Willoughby, 1964).

These findings fit the idea that a reduction in the transsarcolemmal Na^+

gradient tends to promote a rise in $[Ca^{2+}]_i$ and/or increased storage of Ca^{2+} in the SR. Unfortunately, this interpretation is limited by two factors: (1) Inhibition of the Na^+ pump and reduction in $[K^+]_i$ may depolarize the smooth muscle cells and promote Ca^{2+} entry via Ca^{2+} channels (e.g., Bean *et al.*, 1986; Benham *et al.*, 1987; Yatani *et al.*, 1987), and (ii) nerve terminals also have a Na^+–Ca^{2+} exchanger (e.g., Sanchez-Armass and Blaustein, 1987; Blaustein, 1987); thus, reduction of the Na^+ gradient should stimulate endogenous transmitter release (e.g., Bonaccorsi *et al.*, 1977; Katsuragi *et al.*, 1978; Lorenz *et al.*, 1980; Magyar *et al.*, 1987), which could also activate contraction. However, at least in some cases, these alternative explanations could be ruled out because the smooth muscle contractions induced by raising $[Na^+]_i$ and/or lowering $[Na^+]_o$ were observed in the presence of Ca^{2+} channel blockers (e.g., Ozaki and Urakawa, 1979; Sekine *et al.*, 1984; Ashida and Blaustein, 1987a; Johansson and Hellstrand, 1987) and/or in the presence of α-receptor antagonists (e.g., Ozaki *et al.*, 1978b; Ashida and Blaustein, 1987a).

The aforementioned observations indicate that an increase in tension can serve as a convenient measure of a rise in $[Ca^{2+}]_i$, but some potential pitfalls may cause changes in $[Ca^{2+}[_i$ to be underestimated or even missed. Tension is a nonlinear function of $[Ca^{2+}]_i$ and exhibits a threshold. Therefore, a small rise in $[Ca^{2+}]_i$, which may be induced by a small reduction in $\Delta\bar{\mu}_{Na}$, may not be detected if $[Ca^{2+}]_i$ remains below the contraction threshold; some of the entering Ca^{2+} may be sequestered in the SR (e.g., Eggermont *et al.*, 1988) so that $[Ca^{2+}]_i$ is prevented from reaching the threshold or the rise in tension is attenuated (Ashida and Blaustein, 1987a). This could account for the inability of some smooth muscles to develop contractions when they are exposed to Na^+-depleted media (e.g., Toda, 1978; Itoh *et al.*, 1982). This problem may be circumvented by inhibiting Ca^{2+} sequestration in the SR with ryanodine (Ashida *et al.*, 1988) or caffeine (Ashida and Blaustein, 1987a)—although caffeine may also elevate cyclic adenosine monophosphate (AMP), which will then reduce the sensitivity of the contractile machinery to Ca^{2+} (Ruegg and Paul, 1982; Schoeffter *et al.*, 1987). For example, Figs. 10 and 11 show that caffeine-induced contractions in rat aorta were enhanced when a low dose of strophanthidin was applied (Fig. 10B and C) or when $[Na^+]_o$ was reduced to 30 m*M* (Fig. 11), even though these treatments did not raise the baseline tension. Also, the noradrenaline-induced contractions, which depend, in part, upon Ca^{2+} release from the SR (Ashida *et al.*, 1988), were enhanced after cardiac glycoside treatment (Fig. 10A and B; see also Sekine *et al.*, 1984)—presumably because of the increased store of Ca^{2+} in the SR.

Most efforts to induce contractions by reduction of $[Na^+]_o$ are carried

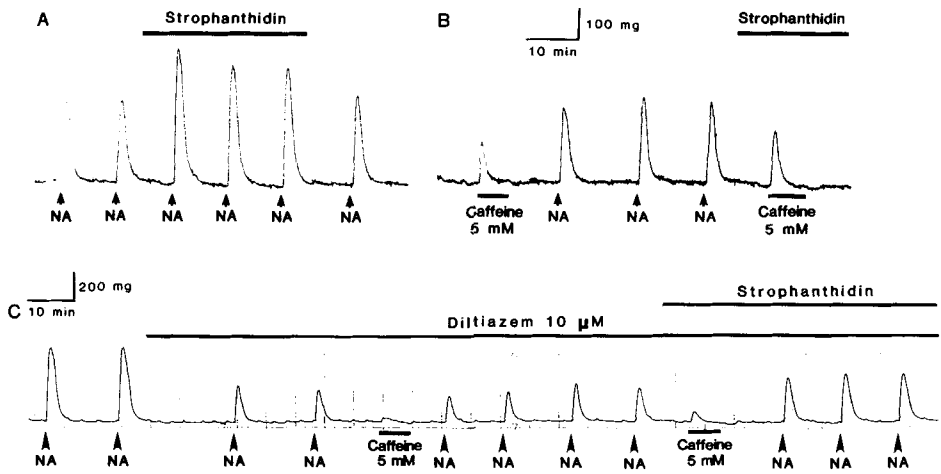

FIG. 10. Effect of strophanthidin (5×10^{-5} *M*, present during the periods indicated by the bars) on the contractile response of rings of rat aorta to brief pulses of noradrenaline [(NA) 6×10^{-8} *M*] [(A)–(C)] and to 5 m*M* caffeine [(B) and (C)]. The calcium channel blocker, diltiazem (10 μ*M*) was present during the period indicated by the bar in (C). (Data are from Ashida and Blaustein, 1987a, and are reproduced with their permission.)

out on "resting" smooth muscles. Under the conditions of low $[Ca^{2+}]_i$ and $[Na^+]_i$, the exchanger is expected to be relatively dormant (see Section II). This, too, may contribute to the need to reduce $[Na^+]_o$ markedly in order to raise $[Ca^{2+}]_i$ (via Na^+–Ca^{2+} exchange) to the contraction threshold—and this would cause the role of the Na^+–Ca^{2+} exchanger to be underestimated (see, e.g., Mulvany *et al.*, 1984; Aalkjaer and Mulvany, 1985). Such resting conditions are nonphysiological for many tonic smooth muscles, including vascular smooth muscles, which are continuously activated by sympathetic neurons (Calaresu and Yardley, 1988;

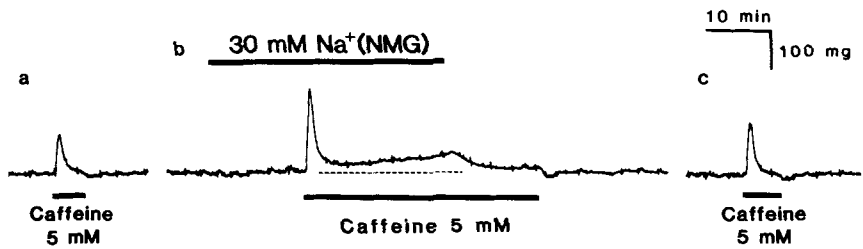

FIG. 11. Contractile responses of a ring of rat aorta to 5 m*M* caffeine during incubation in media containing the normal concentration (140 m*M*) of Na^+ [(a) and (c)], or only 30 m*M* Na^+ (b). (From Ashida and Blaustein, 1987a. Reprinted with permission.)

Jänig, 1988). *In vivo*, these smooth muscles must be maintained in a state of partial contractile activation, in which $[Ca^{2+}]_i$ is continuously held above the contraction threshold. The Na^+–Ca^{2+} exchanger should be partially activated under these circumstances (see Section IV,C,2). These conditions can be mimicked experimentally by chronically exposing the tissues to low concentrations of agonists. When this is done, the sensitivity of the tissues to small changes in $\Delta\bar{\mu}_{Na}$ appears to be markedly increased. This is illustrated by data from the perfused rat hind limb (Lang and Blaustein, 1980): A low concentration of noradrenaline in the perfusion fluid enhances the vascular tension (measured as perfusion pressure) developed in response to a reduction of $[K^+]_o$, which should inhibit the Na^+ pump raise $[Na^+]_i$ (see Fig. 12).

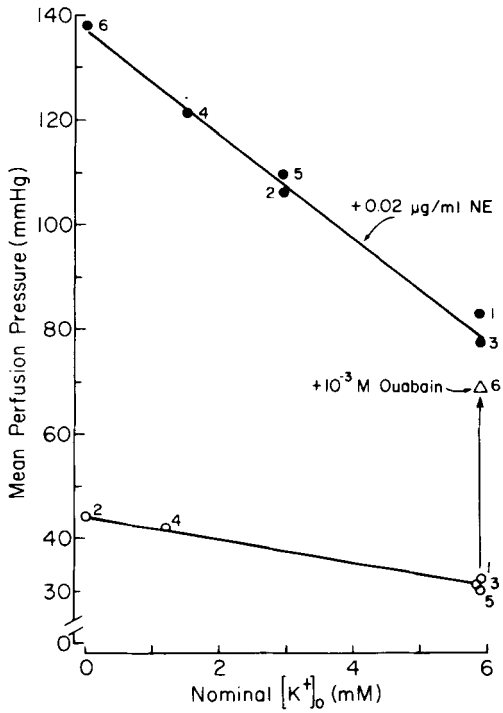

FIG. 12. Relationship between the nominal $[K^+]$ (abscissa) and the mean arterial perfusion pressure (ordinate) for two arterially perfused hind-quarter preparations from reserpine-treated rats. The open circles are from a catecholamine-free preparation; the filled circles are from a different preparation perfused with fluid containing 0.02 µg/ml noradrenaline (NE). The numbers adjacent to the circles indicate the sequence of perfusion fluid changes. (From Lang and Blaustein, 1980. Reprinted with permission.)

2. Na^+ Gradient-Dependent Changes in pH_i; the Na^+–H^+ Exchanger

Many types of smooth muscle possess a Na^+–H^+ exchanger (see, e.g., Kahn *et al.*, 1986, 1988a,b; Wray, 1988 Aalkjaer and Cragoe, 1988; Kormbacher *et al.*, 1988). A reduction in $\Delta\bar{\mu}_{Na}$ may therefore be expected to lower pH_i, especially when the incubation medium does not contain a bicarbonate buffer, so that Cl^-–HCO_3^- exchange is unable to participate in proton extrusion. A fall in pH_i should attenuate contraction and promote relaxation (Wray, 1988), and thus counteract the effect of a rise in $[Ca^{2+}]_i$. A fall in pH_i may also inhibit both Ca^{2+} entry (Baker and McNaughton, 1977) and Ca^{2+} exit (DiPolo and Beaugé, 1982) mediated by the Na^+–Ca^{2+} exchanger. Nevertheless, as discussed below, the dominant factor in mediating Na^+ gradient-dependent increases in tension appears to be the Na^+–Ca^{2+} exchange system.

Another complication of efforts to reduce $\Delta\bar{\mu}_{Na}$ by lowering $[Na^+]_o$ is that this manipulation may also lead to a rapid fall in $[Na^+]_i$ (Aickin, 1987), and thus attenuate the change in $\Delta\bar{\mu}_{Na}$. In particular, substitution of Li^+ for Na^+ may produce an especially rapid fall in $[Na^+]_i$ because of Na^+–Li^+ exchange, which may be mediated by the Na^+–H^+ exchanger (Kahn *et al.*, 1988a). This might account for differences in the effectiveness of various Na^+ replacements to promote Na^+–Ca^{2+} exchange-mediated Ca^{2+} entry and external Ca^{2+}-dependent contractions in some smooth muscles; Li^+ appears to be a particularly poor Na^+ substitute in this respect (e.g., Ozaki *et al.*, 1978b; Brading *et al.*, 1980; Ozaki and Urakawa, 1981; Ashida and Blaustein, 1987a).

3. Relaxation Rate as a Measure of a Reduction in $[Ca^{2+}]_i$

Another way to detect a change in $[Ca^{2+}]_i$ is to induce a contraction and then use the rate of relaxation to estimate the rate of removal of Ca^{2+} from the sarcoplasmic space. The Na^+ dependence of the relaxation rate may then serve as a measure of the relative contribution of Na^+–Ca^{2+} exchange to the reduction in $[Ca^{2+}]_i$. However, Na^+ entry–Ca^{2+} exit mode exchange is a sigmoidal function of $[Na^+]_o$ and is half-maximally activated by $[Na^+]_o$ = 20–30 m*M* (e.g., Ashida and Blaustein, 1987a); nearly all of the external Na^+ must be removed in order to inhibit completely Ca^{2+} extrusion via the exchanger. Furthermore, other mechanisms, including Ca^{2+} sequestration in the SR, may also help to remove Ca^{2+} from the cytosol, and these mechanisms must be taken into account when trying to estimate the relative role of the Na^+–Ca^{2+} exchanger in

Ca^{2+} extrusion (Ashida and Blaustein, 1987a). This is exemplified by the rat aorta data in Fig. 13, which indicate that relaxation from a low-Na^+ (15 m*M*) contraction, induced in the presence of caffeine, can be promoted either by Ca^{2+} sequestration in the SR when caffeine is removed or by Ca^{2+} extrusion via Na^+–Ca^{2+} exchange, when the normal $[Na^+]_o$ is restored in the presence of caffeine (which inhibits SR Ca^{2+} sequestration).

Several investigators have shown that relaxation of contracted smooth muscle is slowed in Na^+-depleted media and/or when $[Na^+]_i$ is increased (e.g., rabbit aorta: Bohr *et al.*, 1969; guinea pig taenia coli: Katase and Tomita, 1972; rat mesenteric resistance vessels: Petersen and Mulvany, 1984; rat aorta: Ashida and Blaustein, 1987a,b). However, these relaxation experiments are complicated by the fact that reduction of $[Na^+]_o$ not only inhibits Na^+ entry–Ca^{2+} exit exchange, but also promotes Na^+ exit/Ca^{2+} entry exchange. Therefore, it is important to use Ca^{2+}-free media in order to link the slowing of relaxation in low-Na^+ media directly to inhibition of Na_o-dependent Ca^{2+} extrusion (Ma and Bose, 1977; Ashida and Blaustein, 1987a). When Ca^{2+} entry is prevented and SR Ca^{2+} sequestration is minimized, the Na_o-dependent component of relaxation may serve as a measure of the relative role of the Na^+–Ca^{2+} exchanger in extruding Ca^{2+} following cell activation (Fig. 14).

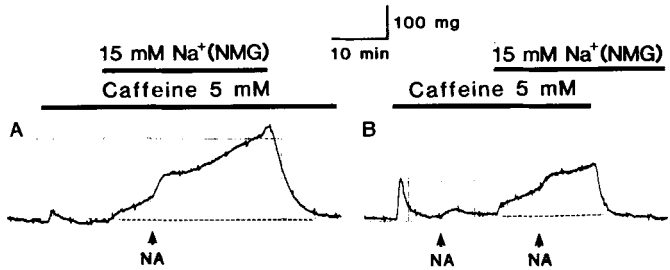

FIG. 13. The effect of Na^+ replacement and removal of caffeine on low-Na^+ contractions of a ring of rat aorta. In (A) and (B), the ring was superfused with a standard (139.2 m*M* Na^+) medium containing 5 m*M* caffeine, and low-Na^+ contractions were then induced by lowering $[Na^+]_o$ to 15 m*M* (Na^+ replaced, mole-for-mole by *N*-methylglucamine). In (A), the ring relaxed when the normal $[Na^+]_o$ was restored; in (B), the ring relaxed when caffeine was removed (to enable the SR to sequester Ca^{2+}), despite continued superfusion with media containing only 15 m*M* Na^+. In (A), the relaxation was apparently produced by the extrusion of Ca^{2+} by external Na^+-dependent Na^+–Ca^{2+} exchange because Ca^{2+} sequestration in the SR was still inhibited by the continued presence of caffeine. In (B), relaxation was apparently produced by sequestering the Ca^{2+} in the SR, since Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger was still partially inhibited because of the low $[Na^+]_o$. (Data are from an unpublished experiment by T. Ashida and M. P. Blaustein.)

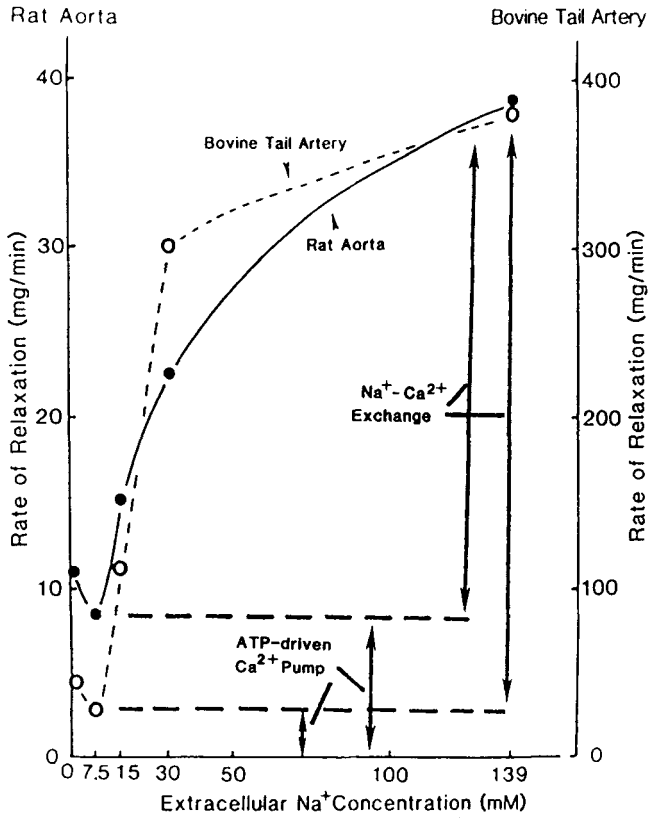

FIG. 14. Relationship between $[Na^+]_o$ and the rate of relaxation of rings of rat aorta (●; left-hand ordinate scale) and bovine tail artery (○; right-hand ordinate scale). The normal $[Na^+]_o$ was 139.2 m*M*; in solutions with reduced $[Na^+]$ (indicated on the abscissa), the Na^+ was replaced mole-for-mole by *N*-methylglucamine. All solutions contained 10 μ*M* phentolamine (an α-receptor antagonist) and 10 μ*M* verapamil (a Ca^{2+} channel blocker); in the rat aortic ring experiments, the superfusion fluid also contained 5 m*M* caffeine. The Na^+-dependent (Na^+–Ca^{2+} exchange-mediated) and Na^+-independent (ATP-driven Ca^{2+} pump-mediated) fractions of the relaxation are indicated. (Data are from Ashida and Blaustein, 1987a, and are reproduced with their permission.)

4. Direct Measurement of Ca^{2+} Movements

Tension serves only as an indirect measure of $[Ca^{2+}]_i$, and its utility is hindered by the many drawbacks mentioned above. It is therefore essential to verify conclusions about Na^+–Ca^{2+} exchange drawn from tension measurements, with direct determinations of Ca^{2+} (and Na^+) move-

ments. In 1973, Reuter *et al.* examined the influence of Na^+ gradient changes on Ca^{2+} fluxes, net Ca^{2+} movements, tension, and membrane potential in isolated rabbit blood vessels. They showed that a reduction in $[Na^+]_o$ and/or inhibition of the Na^+ pump promoted Ca^{2+} gain and caused the tissue to contract. Removal of external Na^+ (in the absence of external Ca^{2+}, to minimize tracer Ca^{2+}–Ca^{2+} exchange and net Ca^{2+} uptake) reduced Ca^{2+} efflux reversibly, but did not depolarize the vascular smooth muscle cells. Some of the experiments were carried out in chemically sympathectomized preparations to rule out the influence of endogenous catecholamine release. Although Ca^{2+}-dependent Na^+ fluxes and $[Na^+]_i$ were not measured in this study (but see Kaplan *et al.*, 1987; Aaronson and Jones, 1988), the sum of the observations appeared to provide strong evidence that the Ca^{2+} distribution in rabbit vascular smooth muscle is influenced by the Na^+ gradient across the sarcolemma.

Subsequently, Burton and Godfraind (1974) suggested that Na^+–Ca^{2+} exchange might account for the increased ^{45}Ca entry and net gain of Ca^{2+} induced by lowering $[K^+]_o$ and thereby raising $[Na^+]_i$ in guinea pig ileum longitudinal smooth muscle. However, Raeymaekers *et al.* (1974) obtained evidence that Na^+–Ca^{2+} exchange occurs at extracellular sites in guinea pig taenia coli, and Aaronson and van Breemen (1981, 1982) suggested that such an exchange might occur at intracellular binding sites in this tissue.

Unfortunately, the intact smooth muscle preparations are complex multicellular tissues with a substantial extracellular matrix that can bind ions and serve as a diffusion barrier. This greatly complicates the interpretation of tracer flux and net ion concentration measurements in these tissues. Nevertheless, it is difficult to understand how an exchange in the extracellular matrix could account for the contractile effects of changes in the Na^+ gradient observed in many of the studies mentioned above.

B. Evidence for Na^+–Ca^{2+} Exchange in Various Mammalian Smooth Muscles

The preceding discussion covers some of the difficulties encountered in evaluating the presence and the physiological role of Na^+–Ca^{2+} exchange in smooth muscles. With these considerations in mind, we will reveiw a number of reports, including many recent ones, concerning Na^+–Ca^{2+} exchange in a variety of smooth muscles. Some of the more recent studies involve new tissue preparations and methodologies in which many of the aforementioned difficulties are circumvented.

1. Vascular Smooth Muscle

a. Studies in Isolated Vessels and Cells and in Sarcolemmal Vesicles. As in indicated in Section V, A, 1 (see also Brading and Lategan, 1985), tension serves only as an indirect measure of $[Ca^{2+}]_i$ in vascular smooth muscle (VSM). However, in a number of recent studies, $[Ca^{2+}]_i$ and tracer fluxes of both Ca^{2+} and Na^+ have been measured directly in freshly dissociated VSM cells and in cultured VSM cells. Isolated VSM sarcolemmal vesicles have also been employed for tracer flux studies. For example, in cultured rat aortic VSM cells, there is a Ca_o-dependent ^{22}Na efflux and a Na_i-dependent ^{45}Ca influx, both of which are inhibited by the amiloride analog, 2′,4′-dimethylbenzamil (J. B. Smith *et al.*, 1987); the latter agent has been reported to inhibit Na^+–Ca^{2+} exchange about tenfold more effectively than it inhibits Na^+–H^+ exchange (L. Smith *et al.*, 1987). The enhanced efflux of Ca^{2+} evoked by angiotensin II in these cells is also largely external Na^+ dependent (Smith and Smith, 1987; Nabel *et al.*, 1988). In contrast to the aforementioned observations, Hirata *et al.* (1981) did not detect any Na^+ gradient-dependent Ca^{2+} influx or efflux in dissociated porcine coronary artery myocytes, although they did detect such fluxes in taenia coli (see Section V, A, 4).

Amiloride and its analogs are not selective inhibitors of Na^+–Ca^{2+} exchange (e.g., Bielefeld *et al.*, 1986). Nevertheless, it seems noteworthy that amiloride inhibits both the contraction induced by reducing $\Delta\bar{\mu}_{Na}$ and the relaxation induced by increasing this gradient in strips of guinea pig aorta, even in the presence of a Ca^{2+} channel blocker (Bova *et al.*, 1989). Reduction of the Na^+ gradient should also lower pH_i, which should antagonize the rise in tension, so that the inhibitory effect of amiloride on tension development could also be explained by its inhibition of Na^+–H^+ exchange. However, the ability of amiloride to inhibit relaxation, when the normal Na^+ gradient is restored, cannot be explained by its effect on pH_i, because a fall in pH_i should promote relaxation (Mrwa *et al.*, 1974).

While most studies of the Na^+–Ca^{2+} exchanger in smooth muscle have focused on Na^+ gradient-dependent Ca^{2+} movements, it is essential to show that these movements are coupled to countermovements of Na^+. Recent studies indicate that a component of ouabain-insensitive ^{22}Na efflux in rabbit portal vein is Ca_o dependent (Kaplan *et al.*, 1987). Also, a component of the ^{22}Na influx in rabbit aorta is Ca_i dependent (Aaronson and Jones, 1988). Although these experiments were not designed to study Na^+–Ca^{2+} exchange, this counterion dependence is circumstantial evidence that these ^{22}Na fluxes may be manifestations of Na^+–Ca^{2+} exchange. Moreover, these findings complement earlier observations on the

Na_i dependence of ^{45}Ca influx and the Na_o dependence of ^{45}Ca efflux in rabbit VSM (Reuter *et al.*, 1973).

Several investigators have employed tracer flux studies to examine the properties of the Na^+–Ca^{2+} exchanger in sarcolemmal vesicles from VSM (e.g., Daniel, 1985; Morel and Godfraind, 1984; Matlib *et al.*, 1985; Matlib and Reeves, 1987; Matlib, 1988; Kahn *et al.*, 1988b; Slaughter *et al.*, 1989). Most of these studies have been limited to the investigation of Na^+ gradient-dependent ^{45}Ca fluxes; however, Kahn and colleagues (1988b) also demonstrated Ca^{2+} gradient-dependent ^{22}Na movements both into and out of the vesicles. They and Matlib (1988) also showed that the uptake of ^{45}Ca into the vesicles was not only dependent on an outwardly directed Na^+ gradient, but was promoted by depolarization of the vesicles with an inward K^+ gradient and valinomycin. This parallels observations on the effects of depolarization in intact arterial preparations (Ashida and Blaustein, 1987a), and fits the idea that the exchange stoichimoetry is 3 Na^+ : 1 Ca^{2+} (Sheu and Blaustein, 1986; Rasgado-Flores and Blaustein, 1987).

One problem with the VSM sarcolemmal vesicle studies is that the maximum velocity of the Na^+ gradient-dependent Ca^{2+} flux in most (but not all) studies is low, and the apparent half-maximal concentration for activation by Ca^{2+} is high (Morel and Godfraind, 1984). However, the validity of this type of experiment depends on precise knowledge of the yield of nonleaky vesicles (cf. Daniel, 1985). Recent attempts have yielded vesicles with high exchanger activity (e.g., Matlib, 1988; Slaughter *et al.*, 1989) that are more campatible with the physiological observations on intact arteries preparations (Ashida and Blaustein, 1987a). The latter report clearly indicates that the exchanger operates when $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ are in the normal physiological range. Indeed, this is suggested by recent measurements of $[Ca^{2+}]_i$ with the Ca^{2+}-sensitive fluorochrome, fura-2, in dissociated single VSM cells (Goldman *et al.*, 1986a,b; Blaustein *et al.*, 1986) and in cultured aortic cells (Smith and Smith, 1987; Bova *et al.*, 1989). Digital fluorescent images of fura-2-loaded bovine tail artery cells directly demonstrate that $[Ca^{2+}]_i$ increases occur in association with contraction when $[Na^+]_i$ is increased and $[Na^+]_o$ is reduced (Fig. 15).

b. Na^+–Ca^{2+} Exchanger and Peripheral Vascular Resistance in Intact Vascular Beds. The preceding section is concerned primarily with studies carried out on isolated vascular segments, cells, and membrane vesicles. A critical question (e.g., Mulvany, 1985) is whether these findings can be extrapolated to intact systems: That is, does Na^+–Ca^{2+} exchange

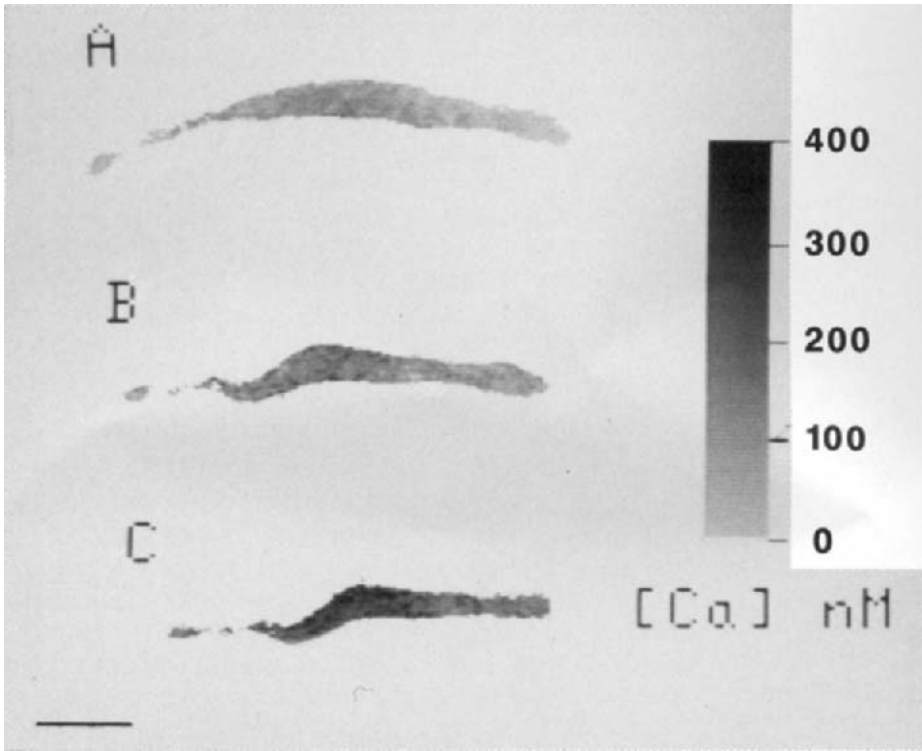

FIG. 15. "Free calcium images" of a single isolated fura-2-loaded bovine tail artery cell. The normal resting free Ca^{2+} (A), and the effects of K^+-free media (B) and K^+-free low-Na^+ media (C) on $[Ca^{2+}]_i$ are illustrated. All solutions contained 10 μM verapamil; Na^+ (139.2 mM in the control solution) was replaced mole-for-mole by *N*-methylglucamine in the low-Na^+ solution ($[Na^+]_o$ = 1.2 mM). The calculated $[Ca^{2+}]_i$ in the resting cell (A) was 98 nM. When the cell was superfused with K^+-free solution, the $[Ca^{2+}]_i$ increased to ~160 nM in the central segment of the cell. Subsequent reduction of $[Na^+]_o$ to 1.2 mM caused $[Ca^{2+}]_i$ to increase to ~310 nM in the central segment. The images were constructed by digital analysis of videotaped fura-2 fluorescent images. [Data are from W. F. Goldman, W. G. Wier, and M. P. Blaustein (see Goldman *et al.*, 1986a,b; Blaustein *et al.*, 1986).]

contribute to the regulation of $[Ca^{2+}]_i$ and contractility in blood vessels *in vivo?* It seems hard to imagine how such a prevalent transport system could not be involved, although the answers are difficult to obtain because we must rely on indirect tests, such as the responses to cardiotonic steroids and to changes in $[K^+]_o$. As an example, the operation of an Na^+–Ca^{2+} exchanger could help to explain the paradoxical ouabain-sensitive vascular relaxation and vasodilation observed with modest (5–10 mM) elevations of external K^+ (e.g., Chen *et al.*, 1972; Biamino and Wessel, 1973; Haddy, 1975; Murray and Sparks, 1978; Harker and Webb,

1987). This manipulation of the ionic environment should have a dual effect: (1) It should stimulate the Na^+ pump to lower $[Na^+]_i$. This will cause an initial *transient hyperpolarization* (Blaustein, 1981) due to the electrogenic operation of the Na^+ pump. (2) However, the less negative E_K ($= RT/F\ln[K^+]_o/[K^+]_i$) should then cause a *steady depolarization* (e.g., Casteels *et al.*, 1977; Harder, 1982; Horn, 1978). This depolarization may be expected to open Ca^{2+} channels and promote vasoconstriction (e.g., Bean *et al.*, 1986; Benham *et al.*, 1987; Yatani *et al.*, 1987; Nelson and Worley, 1989). Thus, the occurrence of ouabain-sensitive vasodilation suggests that the Na^+ pump-induced lowering of $[Na^+]_i$ may be the predominant effect of the modestly elevated $[K^+]_o$.

In a related type of experiment, peripheral vascular resistance increased when $[K^+]_o$ was reduced below 5.9 m*M* or when ouabain was introduced into the sympathectomized isolated perfused rat hind-limb preparation (Lang and Blaustein, 1980), even though varying $[K^+]_o$ in the range of ~2.0–5.9 m*M* should have little effect on the steady V_M (e.g., Casteels *et al.*, 1977). The steepness of this inverse relationship between low $[K^+]_o$ and perfusion pressure was greatly increased when the baseline resistance was increased by including a low concentration of norephinephrine in the perfusion fluids to more closely mimic the *in vivo* vascular tone. These phenomena should be especially important in tonic smooth muscles that contain relatively little SR, such as peripheral arteries (Devine *et al.*, 1972; Somlyo, 1980), in which the level of tone is controlled by continuous sympathetic discharge (Calaresu and Yardley, 1988; Jänig, 1988) and by the mechanisms that mediate the entry and exit of Ca^{2+} across the sarcolemma (Blaustein, 1977a,b: Ashida *et al.*, 1988). In these smooth muscles, Na^+–Ca^{2+} exchange is likely to bias the tone, as suggested by the data in Fig. 11.

Digitalization does not usually raise blood pressure in normal humans, but it does produce increased pressor responses to angiotensin II and norepinephrine (Guthrie, 1984). The latter effect is consistent with the aforementioned evidence that Na^+ pump inhibition increases the availability of trigger Ca^{2+} in VSM. The maintenance of normal blood pressure under these circumstances may simply indicate that the cardiovascular reflexes are able to compensate for the tendency to increase vascular tone in these individuals. In this context, it is noteworthy that long-term digoxin administration significantly elevated blood pressure in heminephrectomized rats (Fujimura *et al.*, 1984), perhaps because the cardiovascular reflexes were compromised in these animals.

c. Pathophysiological Consequences of Na^+–Ca^{2+} Exchange in Vascular Smooth Muscle. The extensive evidence that an Na^+–Ca^{2+} ex-

changer is present and can transport Ca^{2+} at a substantial rate in many VSMs suggests that this transport system plays an important physiological role. Indeed, it seems reasonable to conclude that the exchanger, operating in parallel with the sarcolemmal Ca^{2+} channels and ATP-driven Ca^{2+} pump, helps to modulate $[Ca^{2+}]_i$. In VSMs that are tonically activated, the exchanger must, therefore, participate in the modulation of vascular tone and peripheral vascular resistance (Reuter *et al.*, 1973; Lang and Blaustein, 1980). There is much evidence (e.g., Hardy and Overbeck, 1976; Blaustein, 1977a,b; deWardener and MacGregor, 1980; Hamlyn *et al.*, 1982; Blaustein and Hamlyn, 1983, 1984, 1985; Songu-Mize *et al.*, 1982) that elevated levels of a Na^+ pump inhibitor (endogenous digitalis; Hamlyn *et al.*, 1989) are present in the plasma of many hypertensive human subjects and animals. Accordingly, this Na^+ pump inhibitor may play a role in the pathogenesis of those forms of hypertension (including essential hypertension) that are associated with salt and water retention and volume expansion (Haddy and Overbeck, 1976; Blaustein, 1977a,b, Hamlyn and Blaustein, 1986) by contributing to the increased vascular reactivity and peripheral vascular resistance that is the hallmark of this disease (or group of diseases.

Another interesting consequence of these interrelationships concerns the questionable use of cardiotonic steroids in the therapy of cor pulmonale (right heart failure) secondary to chronic obstructive pulmonary disease. Recently, Salveterra *et al.* (1989) obtained evidence that $[Ca^{2+}]_i$ in small pulmonary arteries was influenced by $\Delta\bar{\mu}_{Na}$. This may explain why digitalization, by direct action on the blood vessels, often exacerbates already elevated pulmonary artery pressure in patients with cor pulmonale (e.g., Green and Smith, 1977).

2. Tracheal Smooth Muscle

It has long been known that respiratory tract smooth muscle contracts in response to inhibition of the Na^+ pump (Marco *et al.*, 1968; Souhrada and Souhrada, 1982; Chideckel *et al.*, 1987). Reduction of external Na^+ also promotes contraction in tracheal smooth muscle from a variety of mammalian species, including humans, and these findings have been ascribed to an Na^+–Ca^{2+} exchange mechanism (Bullock *et al.*, 1981; Kawanishi *et al.*, 1984; Chideckel *et al.*, 1987). This view is supported by recent evidence that sarcolemmal vesicles from tracheal smooth muscle exhibit large-capacity Na^+ gradient-dependent Ca^{2+} influx and efflux (Slaughter *et al.*, 1987). The presence of an Na^+Ca^{2+} exchange mechanism in airway smooth muscle leads to the suggestion that it may play a role in the control of bronchomoter tone (Chideckel *et al.*, 1987). Whether

or not this contributes to the production of bronchospasm in diseases such as asthma remains to be investigated. However, cardiotonic steroids have been shown to increase pulmonary airway resistance *in vivo* by a direct effect on the bronchial smooth muscle (Marco *et al.*, 1968).

3. Uterine Smooth Muscle

Reduction of Na^+ in the bathing medium has been shown to induce contractions in both nonpregnant and pregnant rat myometrium (Masahashi and Tomita, 1983; Matsuzawa *et al.*, 1987). In circular uterine smooth muscle from nonpregnant animals, reduction of $[Na^+]_o$ produces a small stimulation of contraction, but this effect is greatly enhanced during pregnancy (Matsuzawa *et al.*, 1987). Also relaxation from K^+-induced contractures of rat uterine smooth muscle is markedly slowed in Na^+-deficient media (Ma and Bose, 1977). These effects have all been attributed, at least in part, to Na^+–Ca^{2+} exchange. This view is supported by Daniel and colleagues (Grover *et al.*, 1981, 1983; Daniel, 1985), who have shown that sarcolemmal vesicles from rat myometrium exhibit Na^+ gradient-dependent Ca^{2+} influx and efflux.

The role played by Na^+–Ca^{2+} exchanged in the control of myometrial tone and in the development of uterine contractions during delivery of the fetus is unexplored.

4. Intestinal Smooth Muscle

Judah and Willoughby (1964) first showed that guinea pig ileum develops contractions in Na^+-depleted solutions; subsequent relaxation is Na^+ dependent and is inhibited by strophanthin G. However, Taniyama (1974) found that low $[Na^+]_o$ inhibits agonist-activated contractions. Low-Na^+ or Na^+-free media also induce contractions in rat ileal smooth muscle strips (Huddart and Saad, 1978; Huddart, 1981). Furthermore, Ca^{2+} efflux is inhibited by removal of external Na^+ in the absence of Ca^{2+} (to inhibit tracer Ca^{2+} exchange) (Huddart, 1981; Huddart and Latham, 1981). Morel and Godfraind (1984) subsequently identified Na^+–Ca^{2+} exchange activity (Na^+ gradient-dependent Ca^{2+} fluxes) in sarcolemmal vesicles prepared from guinea pig ileum, but the specific activity was quite low.

Replacement of external Na^+ by Tris induced contractions and antagonized agonist-induced contractions in guinea pig taenia coli (Brading *et al.*, 1980). Relaxation following K^+ contractions was inhibited by removal of external Na^+ (Katase and Tomita, 1972; Ma and Bose, 1977), and the Na^+-dependent relaxation was slowed when the tissue was treated with

ouabain or K^+-free media to inhibit the Na^+ pump (Katase and Tomita, 1972). Indeed, the latter treatments may also induce contractions in taenia coli (Ozaki *et al.*, 1978a). These findings may be correlated with the observation that Ca^{2+} influx was enhanced in low $[Na^+]_o$ solutions, and readmission of Na^+ stimulated Ca^{2+} efflux in this tissue, in dissociated cell preparations (Hirata *et al.*, 1981) as well as in multicellular preparations (Aaronson and van Breemen, 1981, 1982). In sum, these data indicate that the availability of Ca^{2+} for contraction is influenced by the Na^+ gradient across the taenia sarcolemma. More direct evidence comes from the fura-2 studies by Pritchard and Ashley (1986, 1987) on suspensions of dissociated taenia coli cells. In these studies, $[Ca^{2+}]_i$ increased when the cells were exposed to ouabain or Na^+-free media, and the rate of $[Ca^{2+}]_i$ increase was speeded up when ouabain-treated cells were subsequently exposed to Na^+-free media.

These data suggest that Na^+–Ca^{2+} exchange may contribute to the control of tone and the Ca^{2+} content of the SR store in intestinal smooth muscle. The precise physiological and pathophysiological significance, in terms of its influence on intestinal motility, is unknown. It seems logical, however, to consider the possibility that Na^+–Ca^{2+} exchange may contribute to the maintenance of tension in sphincters and to the pathogenesis of intestinal spasm or other such gastrointestinal motility disturbances.

5. Urinary Tract Smooth Muscle

a. Ureter. Smooth muscle from guinea pig ureter is a phasic muscle that normally develops contractions following action potential generation (Aickin *et al.*, 1984). Measurements of tension, membrane potential, $[Na^+]_i$, and ^{22}Na efflux in this tissue (Aickin *et al.*, 1984, 1987; Aickin, 1987) provide strong evidence that this tissue contains a Na^+–Ca^{2+} exchange system. The exchanger appears to be relatively dormant in the resting tissue (although its effect on the SR store of Ca^{2+} has not been investigated): Exposure to low-Na^+ solutions, or to K^+-free solutions or ouabain, produces little tension in relaxed tissue (see Sections II and III). After Na^+ loading with K^+-free solutions or ouabain, however, the tissue does develop contractures when $[Na^+]_o$ is reduced (Aickin *et al.*, 1984); the tension is enhanced when the Na^+-depleted solutions contain elevated K^+ concentrations (Aickin *et al.*, 1984), as expected for contractions caused by Ca^{2+} entry via an exchanger with a stoichiometry of 3 Na^+ : 1 Ca^{2+}. As in the heart (Deitmer and Ellis, 1978), the Na^+–Ca^{2+} exchanger helps to keep $[Na^+]_i$ from rising above ~20 m*M* by promoting

Ca_o-dependent Na^+ efflux when the tissue is treated with ouabain (Aickin, 1987; Aickin *et al.*, 1987). These findings raise the possibility that the Na^+–Ca^{2+} exchanger is able to extrude Na^+ at the same rate as the rate of Na^+ entry when $[Na^+]_i$ is about 20 m*M*. Alternatively, another mechanism of Na^+ entry (such as Na^+–H^+ exchange) may be activated when the Na^+ pump is inhibited, and this mechanism may subsequently be turned off by the Na^+–Ca^{2+} exchange-mediated rise in $[Ca^{2+}]_i$ (e.g., Cala *et al.*, 1986; P. M. Cala, personal communication).

Although Aickin *et al.* (1984, 1987) conclude that Na^+–Ca^{2+} exchange plays a "major role" in regulating intracellular Ca^{2+} and Na^+ in ureter smooth muscle when the Na^+ pump is inhibited, the question of its role under normal physiological conditions remains unanswered. Some possibilities are that low rate of exchanger turnover under resting conditions is nevertheless sufficient to modulate the store of Ca^{2+} in the ureter smooth muscle SR, and that the exchanger may help to extrude Ca^{2+} following cell activation (see Fig. 3 in Aickin *et al.*, 1984), as it does in the heart (Sheu and Blaustein, 1986).

b. Vas Deferens. Data on this tissue are less clear-cut. For example, Katsuragi and colleagues (1978) observed that, although ouabain induced contractions in guinea pig vas deferens, this effect was associated with enhanced noradrenaline release from the tissue; moreover, the contractions were markedly attenuated (but not abolished) by α-blockers. However, ouabain also potentiated the Ca^{2+}-dependent contractions activated by 40 m*M* K^+ (Katsuragi and Ozawa, 1978). In rat vas deferens, Na^+-free solutions induce spontaneous phasic contractions and enhance the amplitude and duration of the initial contactions induced by noradrenaline (Wakui and Inomata, 1984). These findings raise the possibility that vas deferens may have an Na^+–Ca^{2+} exchanger, but additonal data are required to resolve the uncertainty.

C. Role of Na^+–Ca^{2+} Exchange in Smooth Muscles

The data described above clearly indicate that a Na^+–Ca^{2+} exchange system is present in most, if not all types of mammalian smooth muscle that have been examined to date. However, some questions about its role in the physiology and pathophysiology of these tissues remain unanswered.

As discussed in Sections II and IV, recent studies on the regulation of the Na^+–Ca^{2+} exchanger in various types of cells, including cardiac muscle, squid axons, and barnacle muscle, indicate that the exchanger is

much more complex than was originally expected. It is regulated by $[Ca^{2+}]_i$ and ATP. It appears to be relatively dormant at resting $[Ca^{2+}]_i$ and is activated when $[Ca^{2+}]_i$ rises as a result of cell excitation (Sheu and Blaustein, 1986). Moreover, exchanger-mediated increases in $[Ca^{2+}]_i$ may be attenuated by rapid sequestration of the entering Ca^{2+} in intracellular organelles (Ashida and Blaustein, 1987a). Confusion about the presence and the role of Na^+–Ca^{2+} exchange in smooth muscles has resulted from lack of information about these phenomena, as well as from: (1) the difficulty in separating the effects of membrane depolarization (which may open Ca^{2+} and or Na^+ channels) from the effects of changing $\Delta\bar{\mu}_{Na}$ and (2) the difficulty in avoiding changes in $[Na^+]_i$ and pH_i when $[Na^+]_o$ is reduced because of the rapid efflux of Na^+ (e.g., Aickin, 1987; Kaplan *et al.*, 1987) and the inhibition of Na^+–H^+ exchange, respectively. Each of these problems has been taken into account or circumvented in at least some of the studies reviewed above. The sum of the data provides convincing evidence that substantial Na^+–Ca^{2+} exchange activity is present in the sarcolemma of all of the types of smooth muscle mentioned above. In many instances, little exchanger-mediated Ca^{2+} movement can be identified in these tissues under resting conditions (when $[Ca^{2+}]_i$ is below the contraction threshold); but even with a slow turnover rate, the exchanger may regulate (indirectly) the amount of Ca^{2+} stored in the SR and, thus, available for phasic contractions. Moreover, the exchanger may directly modulate $[Ca^{2+}]_i$ and tone when $[Ca^{2+}]_i$ is maintained above the contraction threshold. Consequently, Na^+–Ca^{2+} exchange appears to be very important, not only because of its role in normal smooth muscle function, but also because of its likely contribution to altered smooth muscle function in a variety of pathological conditions.

VI. CONCLUSION

During the past two decades, many important advances have been made in our understanding of excitation–contraction coupling in various types of muscle. The focus has been on the central role of Ca^{2+}, even though the sources of Ca^{2+} and its sites of action vary in different types of muscle. Many interesting and important questions about the regulation of intracellular calcium in muscle remain unresolved. Nevertheless, an emerging concept is that Na^+–Ca^{2+} exchange appears to play an important role in the regulation of the cytosolic $[Ca^{2+}]$ and the SR Ca^{2+} store and, therefore, in the control of contractility, in all the types of muscle in which this subject has been explored: invertebrate "skeletal" muscle and vertebrate skeletal, cardiac, and various smooth muscles.

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Chapter 16

Activation of Sodium–Hydrogen Exchange by Mitogens

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I. INTRODUCTION

Amiloride-sensitive Na^+–H^+ exchange activity has been detected in the plasma membrane of virtually all mammalian cell types studied to date. The exchanger, or antiport, catalyzes the electroneutral countertransport of Na^+ for H^+. Though the mechanism is reversible, entry of Na^+ in exchange for intracellular H^+ is the predominant transport mode under physiological conditions (Aronson, 1985). This net extrusion of acid equivalents tends to offset the spontaneous tendency of the cells to become acidic, which results from metabolic acid production and from

the electrodiffusive accumulation of H^+ (or depletion of OH^-) driven by the internally negative plasma membrane potential. The Na^+–H^+ antiport is virtually quiescent at physiological cytosolic pH (pH_i), but is markedly activated when the cytoplasm becomes acidic (see Section II). Such behavior, together with the direction of net acid transport, suggests that the antiport plays an important role in the regulation of pH_i in resting (nonproliferating) cells.

In addition to being activated by cytosolic acidification, the antiport can also be stimulated without prior pH_i change by the addition of mitogenic or comitogenic agents such as growth factors and phorbol esters, respectively (Moolenaar, 1986; Grinstein and Rothstein, 1986). In these instances, the stimulated antiport drives pH_i upward, to a new steady state that, in nominally HCO_3^--free media, is 0.2–0.3 units more alkaline than the resting pH_i. The ubiquity and early onset of this response have led to the suggestion that the cytosolic alkalosis induced by stimulation of the antiport plays a central role in mediating cell proliferation (L'Allemain *et al.*, 1984; Mendoza, 1987). The purpose of this chapter is to review the mechanism whereby the antiport is activated by mitogens and to assess critically whether such stimulation is required for the initiation of cell growth.

II. ACTIVATION OF ANTIPORT BY MITOGENS AND COMITOGENS

The rate of forward (Na^+ inward, H^+ outward) Na^+–H^+ exchange across the plasma membrane is a sharp function of pH_i. This has been determined by acid-loading the cytoplasm to varying degrees in the absence of Na^+, followed by the addition of external Na^+ and the measurement of countertransport activity. The rate of exchange can then be estimated from the fraction of Na^+ uptake that is amiloride sensitive (Grinstein *et al.*, 1984b) or from the Na^+-induced cytoplasmic alkalinization, which is largely blocked by amiloride (Grinstein *et al.*, 1984a). Net H^+ equivalent efflux can be computed from the alkalinization by multiplying the rate of pH_i change times the cellular buffering power. Such experiments revealed that, while very active at pH_i near 6.3, the Na^+–H^+ antiport becomes gradually less active as pH_i becomes more alkaline, reaching virtual quiescence at physiological pH_i. Similar observations have been reported in a variety of nonepithelial mammalian cell types (for review see Aronson, 1985).

This peculiar behavior of the antiport is not predicted on a thermodynamic basis. The inward Na^+ gradient, which drives the net ejection of H^+, is capable of alkalinizing pH_i well beyond the resting level (for details

see Aronson, 1985; Grinstein and Rothstein, 1986), as can be readily demonstrated by adding monensin, an exogenous Na^+–H^+-exchanging ionophore. Monensin can rapidly elevate pH_i nearly 0.5 units above the physiological level, a process that depends on the Na^+ gradient. These findings, together with the unusually steep pH_i dependence of the antiport, led to the suggestion that endogenous Na^+–H^+ exchange is stringently controlled by an allosteric "modifier" site (Aronson *et al.*, 1982). The existence of the proposed modifier was confirmed by the observation that, under certain conditions, Na^+ *efflux* can be stimulated by elevating the cytoplasmic concentration of H^+ ($[H^+]_i$) (Aronson *et al.*, 1982). In the absence of allosteric control, elevating $[H^+]_i$ would be expected to compete with Na^+ binding to the inwardly facing transport site, inhibiting Na^+ efflux.

The modifier site, which seems to be located at or near the cytoplasmic face of the antiport, determines the pH_i dependence of Na^+–H^+ exchange. As such, the modifier seems to play a central role in the activation of the exchanger by growth-promoting agents. As mentioned above, treatment of cells with mitogens or comitogens induces an elevation of pH_i by 0.2–0.3 units. Several lines of evidence indicate that this alkalosis is due to activation of the otherwise nearly quiescent antiport. First, the pH_i change is accompanied by accelerated extrusion of H^+ into the medium, indicating that transmembrane H^+ transport takes place. Concomitantly, the rate of Na^+ uptake by the cells increases. Moreover, the internal alkalinization is precluded by omission of extracellular Na^+ or by the addition of amiloride or amiloride analogs that are known to block antiporter activity. These drugs also eliminate the excess Na^+ uptake observed upon addition of mitogen.

How can the antiport, which is quiescent in unstimulated cells, account for the increase in pH_i? The explanation lies in a change in the pH_i sensitivity of the exchanger. Acid-loading experiments like those described above have revealed that, following treatment with growth promoters, the pH_i at which antiport activity becomes negligible is shifted upward. This change in "set point" must be attributed to an alteration in the properties of the modifier site, inasmuch as this moiety is believed to dictate the pH_i dependence of transport.

In rat thymic lymphocytes, activation of the Na^+–H^+ exchanger has been observed following addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Grinstein *et al.*, 1985). This effect is most likely due to stimulation of protein kinase C activity, since it is mimicked by a variety of activators of this kinase, but not by other phorbol derivatives that fail to stimulate phosphotransferase activity. Moreover, the effect of TPA is blocked by H7, a putative inhibitor of protein kinase C. Perhaps the most

convincing evidence, however, was obtained in cells depleted of protein kinase C. Depletion can be accomplished by prolonged preincubation in the presence of high concentrations of biologically active phorbol esters. For reasons that are poorly understood, this treatment results in down-regulation of the enzyme, with virtual disappearance after 24 hr, in the case of lymphocytes. In protein kinase C-depleted cells, addition of TPA failed to stimulate the antiport, indicating that this enzyme mediates the activation process.

In thymocytes, Na^+–H^+ exchange is also stimulated by concanavalin A (Con A), a polyclonal mitogenic lectin, but not by phytohemagglutinin (PHA), which is ineffective as a mitogen in these cells. Even though Con A activates phospholipase C, releasing diacylglycerol and stimulating C kinase, the latter enzyme does not seem to be responsible for the activation of the antiport. This was concluded from the findings that neither H7 nor depletion of the kinase by down-regulation impaired the response to the lectin (for review see Grinstein and Dixon, 1988). Similarly, though in some cell types an elevation in cytoplasmic Ca^{2+} stimulates the antiport (Villereal, 1981), this mechanism cannot explain stimulation in lymphocytes by Con A. In cells loaded with Ca^{2+} buffers, the lectin does not change $[Ca^{2+}]_i$, while stimulation of the Na^+–H^+ antiport persists. It must be concluded that a third, Ca^{2+}- and protein kinase C-independent mechanism underlies stimulation of the exchanger by lectins in thymocytes.

The antiport is stimulated by mitogens in many other cell types as well (for reviews see Moolenaar, 1986; Grinstein and Rothstein, 1986). In all cases, stimulation is accompanied by activation of protein kinase C-, tyrosine kinase-, or Ca^{2+}–calmodulin-dependent kinase and perhaps other kinases as well. We have therefore speculated that phosphorylation of the antiport at or near the modifier site may be the mechanism underlying the shift in the pH_i dependence of transport and the resultant alkalinization of the cytosol.

III. Na^+–H^+ EXCHANGE AND INITIATION OF PROLIFERATION

In recent years, evidence has accumulated suggesting that the activation of the Na^+–H^+ antiport by mitogens and the associated cytoplasmic alkalosis are necessary steps and perhaps the signal leading to cellular proliferation. This evidence includes the observation that successful completion of the proliferative cascade requires the presence of extracellular Na^+ (Deutsch *et al.*, 1981). However, because a number of processes, including other transport systems, require external Na^+, this evidence is

very circumstantial. More direct evidence was obtained using amiloride. A variety of laboratories have found that blocking the antiport with this diuretic drug or with the more potent 5-*N*-disubstituted analogs of amiloride resulted in inhibition of mitogen-induced thymidine incorporation (L'Allemain *et al.*, 1984; Baliga *et al.*, 1987; Yamaguchi *et al.*, 1986; Shibanuma *et al.*, 1987). The effect of the blockers was more pronounced in low-Na^+ media, consistent with the competitive nature of the interaction between this cation and the amilorides. Finally, it has been shown that fibroblast mutants devoid of the Na^+–H^+ antiport fail to reproduce at physiological pH in the nominal absence of bicarbonate. This was interpreted to mean that the antiport is essential for the cells to attain the critical pH_i required to induce proliferation. These observations, together with the nearly universal activation of the antiport by growth promoters, suggest that stimulation of Na^+–H^+ exchange is a key event in mitogenesis.

Most of the experiments cited above assessed cellular proliferation by measuring the incorporation of tritiated thymidine. This required prolonged (up to 72-hr) incubations in the presence of inhibitors or in media of reduced Na^+ concentration, increasing the likelihood of nonspecific effects. Indeed, in addition to blocking Na^+–H^+ exchange, amiloride also intercalates into DNA (Besterman *et al.*, 1987) and can inhibit DNA topoisomerase (Besterman *et al.*, 1987), a number of protein kinases (Besterman *et al.*, 1985; Davis and Czech, 1985), the Na^+–K^+ pump (Soltoff and Mandel, 1983), and protein synthesis in cell-free systems (Leffert *et al.*, 1982). Moreover, there is the possibility that the effects of blockade of the antiport on proliferation are not due to prevention of the mitogen-induced cytosolic alkalosis, but rather to a subsequent drop in pH_i below the physiological (unstimulated) range, to inhibitory or even cytotoxic levels. This effect would be even more pronounced in the absence of other pH_i regulatory systems. This condition was in fact brought about in most of the above experiments by the omission of bicarbonate from the medium, an essential substrate for pH_i regulation by the cation-dependent and -independent anion exchangers.

IV. Na^+–H^+ EXCHANGE AND INCREASED PROTOONCOGENE RNA LEVELS

To overcome some of the interpretive difficulties discussed above and to more rigorously analyze the role of the antiport in proliferation, we have studied earlier manifestations of the response to mitogens. One of the earliest events associated with mitogenesis in a variety of cell types

is the increased expression of the cellular protooncogene c-*fos*. Expression of this gene increases minutes after stimulation by growth factors. The protein it encodes for has been localized to the nucleus, where it associates with chromatin and is believed to operate as a "master switch," regulating the subsequent expression of other genes relevant to the proliferative cascade (Marx, 1987). Because of the very rapid onset of c-*fos* expression after stimulation, we were able to assess the early events of the proliferative sequence under conditions in which pH_i and the cellular Na^+ concentration were continuously controlled or monitored.

Two biological systems were tested: rat thymic lymphocytes and human tonsillar T lymphocytes. The latter were stimulated with PHA, whereas the former, which are not very responsive to the lectin, were activated by a combination of TPA and ionomycin. The same genomic probe was used to quantify c-*fos* in both instances. Northern blot analysis showed that a 2.2-kilobase RNA was selectively identified in both systems, consistent with the reported size of the c-*fos* mRNA.

As discussed above and as illustrated in Fig. 1, addition of TPA induced a clear cytoplasmic alkalinization in thymocytes. The subsequent addition of ionomycin produced only a small acidification. A similar acidification was earlier attributed to increased H^+ uptake driven by the hyperpolarized membrane potential and to metabolic acid generation in response to the elevated cytoplasmic Ca^{2+} concentration. Hyperpolarization is known to occur as a result of opening of Ca^{2+}-sensitive K^+ channels. As shown in Fig. 1, the alkalosis induced by the combination of TPA and ionomycin was accompanied by an increase in the intracellular Na^+ content, consistent with activation of the antiport.

Addition of TPA plus ionomycin to thymocytes increased the level of c-*fos* mRNA, as shown by the slot blots of Fig. 1. Compared to untreated cells, the level of the protooncogene RNA increased over tenfold (Fig. 1D). When the blots were rehybridized using a probe for the constitutively expressed enzyme glyceraldehyde-3-phosphate dehydrogenase, no differences were found between control and stimulated cells, indicating that the effect of the mitogens on c-*fos* was not due to a generalized effect on cellular RNA. Moreover, this result also indicates that the amount of RNA blotted was comparable in both samples.

To assess the roles of pH_i and the antiport on c-*fos* levels, Na^+–H^+ exchange was inhibited throughout the stimulation period using 5-(*N*-ethyl-*N*-propyl)amiloride (EPA). This analog of amiloride was first shown to be a potent inhibitor of acid-induced Na^+–H^+ exchange, with an apparent K_i of 24 n*M* when the external pH and Na^+ concentrations were 7.3 and 70 m*M*, respectively. As shown in Fig. 2, EPA eliminated the

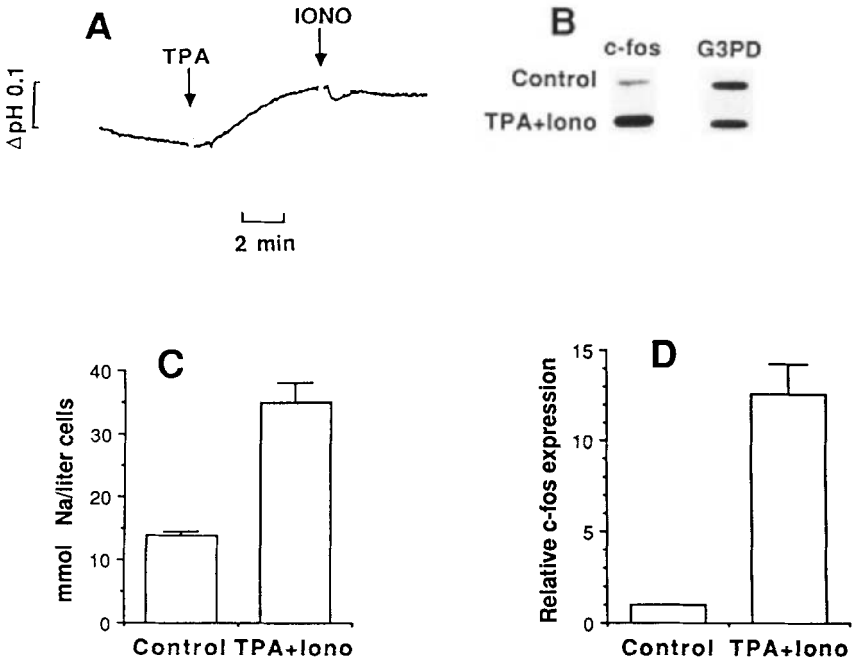

FIG. 1. TPA and ionomycin increase cytoplasmic pH (pH_i), intracellular [Na^+], and the levels of c-*fos* RNA in rat thymic lymphocytes. (A) Cells loaded with the pH-sensitive dye bis(carboxyethyl)carboxyfluorescein (BCECF) were suspended in Na^+-rich solution, and pH_i was recorded fluorimetrically. Where indicated, TPA (0.2 μM) and ionomycin [(iono) 0.5 μM] were added. (B) Cells suspended in Na^+-rich solution were incubated for 30 min in the presence or absence of TPA plus ionomycin and were used for the extraction of total RNA. The extract was then analyzed for c-*fos* and glyceraldehyde-3-phosphate dehydrogenase RNA by slot blotting. A typical radiogram is illustrated. (C) Cells were treated with TPA and ionomycin as described in (B). The cells were sedimented, washed rapidly in cold Na^+-free solution, and used for the photometric determination of intracellular Na^+ content. Intracellular Na^+ concentration was calculated using a volume of 114 μm^3 per cell. Means ± SE of three experiments are shown. (D) The relative increase in c-*fos* in experiments like that in (B) was estimated by densitometry and the means ± SE of three separate experiments are presented.

cytosolic alkalinization induced by TPA, supporting earlier data that demonstrated inhibition by amiloride (Grinstein *et al.*, 1985) and confirming that the antiport is responsible for the pH_i change. Subsequent addition of ionomycin produced a significant acidification of ~0.1 pH units. In contrast to the complete elimination of the alkalinization induced by the mitogens, the change in $[Na^+]_i$ was only partially inhibited by EPA (Fig. 2C). Because the Na^+ gain induced by TPA alone is almost completely

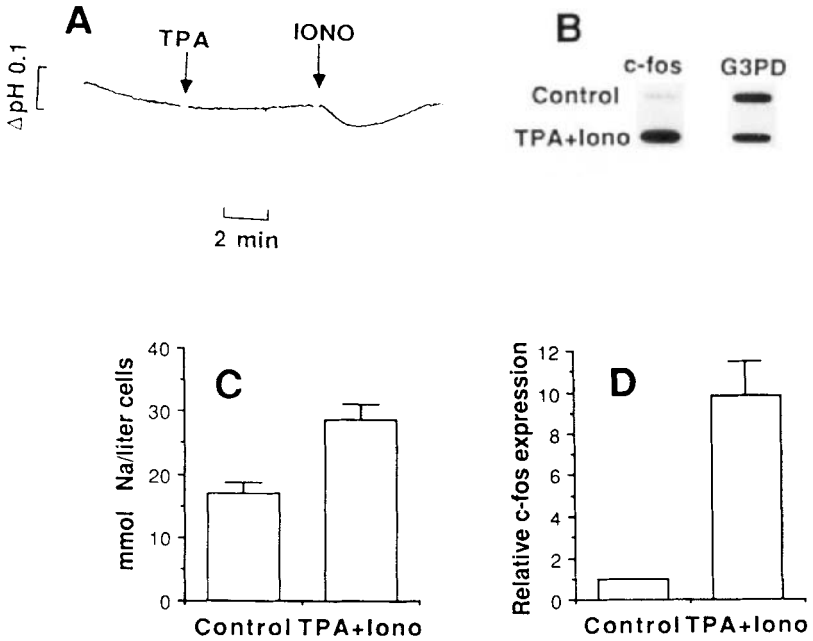

FIG. 2. Effect of 5-(*N*-ethyl-*N*-propyl)amiloride (EPA) on the stimulation of Na^+–H^+ exchange and on the increased levels of c-*fos* RNA induced by TPA and ionomycin in thymic lymphocytes. (A) BCECF-loaded cells were suspended in Na^+-rich solution containing 5 μM EPA, and pH_i was recorded fluorimetrically as in Fig. 1. Where indicated, TPA (0.2 μM) and ionomycin [(Iono) 0.5 μM] were added. (B) Cells suspended in Na^+-rich solution with 5 μM EPA were incubated for 30 min in the presence or absence of TPA plus ionomycin and were used for the extraction of total RNA. The extract was then analyzed for c-*fos* and glyceraldehyde-3-phosphate dehydrogenase RNA by slot blotting as in Fig. 1. A typical radiogram is illustrated. (C) Cells were treated with TPA and ionomycin as described in (B). The cells were then used for the photometric determination of cellular Na^+ concentration. Means ± SE of three experiments are shown. (D) The relative increase in c-*fos* in experiments like that in (B) was estimated by densitometry and the means ± SE of three separate experiments are presented.

blocked by amiloride and its analogs, it is likely that treatment with ionomycin increases Na^+ "leakage" (or reduces active Na^+ efflux). Indeed, measurements of transmembrane potential using bisoxonol demonstrate that, under the conditions used, a slowly developing depolarization follows addition of ionomycin, which is consistent with an elevated Na^+ conductance.

Despite the inhibition of the Na^+–H^+ antiport by EPA, the increased level of c-*fos* RNA induced by TPA and ionomycin was not affected significantly (cf. Figs. 1D and 2D). The combination of mitogens stimulated

a 9.95 (± 1.8)-fold increase in the level of the RNA coding for the protooncogene. These data suggest that stimulation of c-*fos* RNA levels can proceed in the absence of Na^+–H^+ countertransport activity.

Further evidence supporting this view was obtained using human tonsillar T lymphocytes activated by PHA. Unlike rat thymocytes, these T cells respond to the addition of the lectin with synthesis of interleukin 2, expression of receptors to this lymphokine, and eventual proliferation (for review see Gelfand *et al.*, 1987). As shown in Fig. 3E, one of the early responses to the addition of PHA is an increased level of c-*fos* RNA. Unlike other growth factors, however, PHA did not produce a detectable cytoplasmic alkalosis in human T cells (Fig. 3A). That the antiport was functional and capable of elevating pH_i in these cells could be demonstrated by making the medium hypertonic. This procedure, which is known to stimulate Na^+–H^+ exchange in a variety of cells, was associated with a rapid and pronounced rise in pH_i (Fig. 3A). Together, these

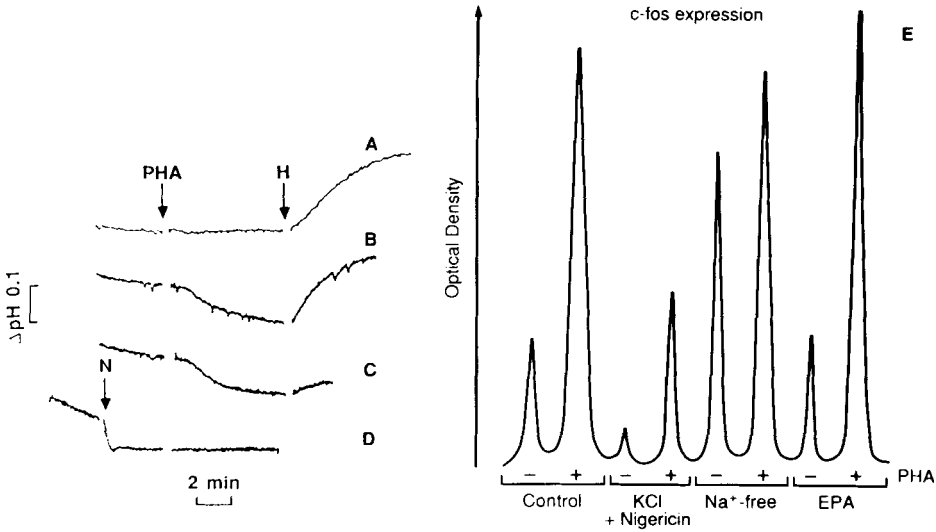

FIG. 3. Effects of phytohemagglutinin (PHA) on pH_i and on the level of c-*fos* RNA in human tonsillar T lymphocytes, isolated by E-rosetting. (A)–(D) Intracellular pH recordings using BCECF. Where indicated, the cells were stimulated with 10 μg/ml PHA. To stimulate the antiport, the medium was then made hypertonic [H in traces (A)–(C)] by addition of an extra 100 m*M* NaCl. (A) Cells suspended in normal, Na^+-rich medium. (B) Cells suspended in Na^+-free, *N*-methyl-*D*-glucamine$^+$ solution. (C) Cells suspended in Na^+-rich solution containing 5μ*M* EPA. (D) Cells suspended in K^+-rich solution were first treated with nigericin (2 μg/ml; indicated by N on the trace) and then with PHA. (E) c-*fos* RNA levels in human T cells treated for 30 min with PHA under the conditions described in (A)–(D). Total RNA was then extracted, blotted, and probed for c-*fos* as in Fig. 1. A typical densitometric scan of an autoradiogram is illustrated.

results indicate that in human T lymphocytes, as in rat thymocytes, c-*fos* increases can be induced without an elevation in cytosolic pH.

This was further confirmed in experiments using EPA or Na^+-free media. When suspended in Na^+-free solutions (*N*-methyl-D-glucamine$^+$ was used as a substitute), the cells responded to the addition of PHA with a considerable cytoplasmic acidification (Fig. 3B). This acidifying process seems to be associated with the concomitant elevation of the free cytosolic Ca^{2+} concentration, since it is markedly depressed by omission of external Ca^{2+} and by the introduction of Ca^{2+} chelators into the cytoplasm. For reasons that are presently unclear, incubation of the cells in *N*-methyl-D-glucamine$^+$ solution in the absence of the lectin increased the cytoplasmic level of c-*fos* RNA (Fig. 3E). Nevertheless, the presence of PHA during this period further elevated the amount of the RNA encoding for the protooncogene. A marked decrease in pH_i was also observed in cells stimulated with PHA in Na^+-containing medium in the presence of EPA, at concentrations that entirely block the antiport. The presence of the amiloride analog did not affect the basal c-*fos* RNA levels and, more importantly, the effect of PHA on the expression of the protooncogene RNA was similarly unaffected (Fig. 3).

We also performed experiments in which pH_i was maintained constant throughout the treatment with the mitogen, using nigericin. In K^+-rich solutions (where $[K^+]_i \approx [K^+]_o$) this ionophore, which catalyzes the exchange of K^+ for H^+ across membranes, will maintain pH_i at the same level as the external pH (pH_o), since the electroneutral exchange reaction will attain equilibrium when $[K^+]_i/[K^+]_o \approx [H^+]_i/[H^+]_o$. Under these conditions, the subsequent addition of PHA failed to alter pH_i (Fig. 3). The basal level of c-*fos* RNA (in the absence of PHA) was significantly reduced by incubation in nigericin/K^+. However, the relative increase elicited by the lectin was comparable to that recorded in control cells. It seems clear, therefore, that the pH_i changes associated with activation of the Na^+–H^+ antiport are not essential for the early stages of the mitogenic process in human T lymphocytes. Also, inasmuch as the intracellular Na^+ concentration could not have increased in either K^+ or *N*-methyl-D-glucamine$^+$ solutions, it is evident that a Na^+ gain is not required for the cells to initiate proliferation.

V. CONCLUDING REMARKS

Our results indicate that the mitogen-induced activation of Na^+–H^+ exchange and the associated cytoplasmic alkalinization are neither sufficient nor necessary to initiate proliferation in rodent and human lympho-

cytes. Despite reported findings in sea urchin eggs (Eppel, 1980) and in mammalian fibroblasts (Zetterberg and Engstrom, 1981) indicating that mitogenesis could be induced by simply elevating pH_i, we have been unable to induce c-*fos* RNA in lymphocytes by alkalinizing the cytoplasm with monensin. Moreover, we have similarly failed to induce proliferation, measured by the incorporation of tritiated thymidine, either with monensin or by incubating lymphocytes in alkaline media, under conditions in which pH_i is known to increase. In addition, the data presented above indicate that the alkalinization generally observed upon addition of mitogens does not appear to be necessary for the induction of c-*fos*. Finally, treatment of human lymphocytes with PHA under conditions that lead to increased c-*fos* RNA levels and eventual incorporation of thymidine and proliferation was not associated with cytoplasmic alkalinization, despite the activation of the antiport. It can therefore be concluded that, although the Na^+–H^+ exchanger is indeed stimulated by growth-promoting agents, the resulting alkalinization (when present) is not essential for the cells to proliferate.

This conclusion runs contrary to the accepted notion that stimulation of Na^+–H^+ exchange and cytoplasmic alkalosis are necessary for proliferation. It is conceivable that, although the early stages of the proliferative cascade, such as c-*fos* RNA expression, are independent of internal alkalinization, later steps require the pH_i change. However, it is also possible that some of the earlier results were misinterpreted. It is conceivable that, in experiments in which Na^+–H^+ exchange was precluded and HCO_3^- was absent, pH_i fell below the resting physiological value, reaching levels that were not permissive for the development of the proliferative response. These effects were, in all likelihood, more pronounced in experiments requiring long incubations, such as those involving determinations of proliferation by thymidine incorporation. Thus, inhibition of proliferation may have resulted from the imposition of unphysiologically low pH_i levels, rather than from the elimination of the alkalinization generated by stimulation of the exchanger by the mitogens.

Based on the c-*fos* data, we can conclude that stimulation of the Na^+–H^+ antiport is not essential for the development of the early stages of the mitogenic cascade in lymphocytes. It remains to be determined whether the stimulation of the antiport, if it persists at longer times, is required for the successful completion of later phases of the mitogenic process. The data obtained with human lymphocytes, where alkalosis was never observed, and earlier data obtained with amiloride analogs in HCO_3^--containing media (Mills *et al.*, 1986) suggest that this is probably not the case. Thus, our present notions regarding the involvement of Na^+–H^+ exchange in proliferation need to be reassessed.

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Chapter 17

Sodium Pump Regulation by Endogenous Inhibitors

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I. BACKGROUND

A. Natriuretic Hormone

Experimental observations which ultimately led to the concept of the existence of an endogenous regulator of mammalian Na^+,K^+-ATPase began more than 25 years ago in laboratories of renal physiology. De Wardener and co-workers (1961) reported in 1961 that intravascular expansion using saline in dogs produced a brisk natriuresis despite the facts that

renal perfusion pressure did not rise (inflation of aortic balloon above the renal arteries), glomerular filtration rate remained unchanged or diminished, and decreases in mineralocorticoid activity could not occur (after administration of supraphysiological doses of mineralocorticoid during the course of volume expansion). Since perfusion pressure and filtration rate did not change, and the natriuretic effects of extracellular fluid volume expansion in one animal also occurred in a second animal cross-circulated with the blood of the first, the presumption was that the natriuresis was due in part to a circulating substance that exerted its effects directly on the renal tubular Na^+ reabsorptive process, without affecting renal hemodynamics. The initial studies by de Wardener and colleagues were not fully accepted because the saline infusion used to expand the circulation changed the composition of the blood, altering physical factors such as peritubular oncotic pressure and renal vascular resistance. But more persuasive evidence came out of a second generation of cross-circulation experiments and experiments using transplanted kidneys or isolated perfused kidneys while at the same time controlling blood composition. Such experiments again showed an increase in Na^+ excretion with expansion of blood volume, despite the absence of nervous system activity or measurable changes in glomerular filtration rate or renal blood flow (Bahlman *et al.*, 1967; Kaloyanides and Azer, 1971; Lichardus and Nizet, 1972).

Since crude urine or plasma samples might contain many substances that could produce the observed *in vivo* effects, fractionation of extracts was carried out prior to physiological testing. These early purification attempts were, however, limited largely to deproteination, desalting, and some concentration of activity using gel filtration chromatography, and the emphasis in the field remained mainly on physiological testing.

It was not clear whether this putative humoral substance acted by altering intrarenal hemodynamics or by directly inhibiting Na^+ transport in the renal tubule. Therefore, investigators turned to *in vitro* approaches to demonstrate biological activity consistent with the natriuretic hormone hypothesis. The bioassays involved the demonstration of changes in active Na^+ transport either across anuran membranes such as the toad urinary bladder, which is considered a functional analog of the human distal nephron (Bourgoignie *et al.*, 1971; Kaplan *et al.*, 1974; Haupert and Sancho, 1979), or in isolated, perfused segments of mammalian renal tubule (Fine *et al.*, 1976). Results of these experiments confirmed that active extracts from plasma, urine, and tissue sources that were natriuretic *in vivo* exerted their effects by a direct action on transepithelial sodium transport. Hillyard *et al.* (1976) first documented directly that the observed effects on cation transport were associated with inhibition of the Na^+,K^+-ATPase.

B. Na^+,K^+-ATPase Inhibition and Essential Hypertension

At the same time, in another area of circulatory physiology, evidence was beginning to accumulate linking the kidney, the brain, and the cardiovascular system in the genesis of certain forms of experimental animal and human essential hypertension. It had been observed that the development of volume-expanded (low-renin) hypertension in animal models followed a predictable sequence of events. Initially, there is an increase in cardiac output due to an increase in cardiac contractility and an increase in venous return of blood to the heart (decreased venous capacitance). In a short time, however, cardiac output returns to normal, associated with an increase in total peripheral vascular resistance which is sustained and results in tonic elevation of arterial pressure. Na^+,K^+-ATPase activity in cardiovascular tissues in animals with volume-expanded hypertension was found to be decreased (Haddy and Overbeck, 1976). Haddy and Overbeck (1976) postulated that the physiological sequence of events and the Na^+,K^+-ATPase measurements could be explained by the appearance of a circulating Na^+,K^+-ATPase inhibitor that might be linked to pathogenesis of the hypertension. Indeed, Overbeck *et al.* (1976) showed that volume expansion was associated with the elaboration of a heat-stable substance in plasma that inhibited ouabain-sensitive cell membrane Na^+ transport in vascular muscle. Brody and colleagues (1978), seeking a locus of anatomical control, demonstrated that lesions in the region of the anteroventral third ventricle of the brain prevented the hypertension of volume expansion. Pamnani *et al.* (1981), Songu-Mize *et al.* (1982), and Bealer *et al.* (1983) subsequently showed that these lesions prevented the secretion of the Na^+ transport inhibitor. It now seemed possible to explain a number of puzzling physiological phenomena, including both the natriuresis and hypertension of volume expansion, on the basis of a circulatory substance released by the midbrain in response to volume expansion. The postulated mechanism of action of this substance(s) was the modulation of renal tubular Na^+ reabsorption and vascular smooth muscle tone by regulation of Na^+,K^+-ATPase activity.

The overall hypothesis (de Wardener, 1987) proposes that, in hereditary forms of hypertension, there is a persistent tendency toward renal retention of Na^+. This may be due to increased Na^+–K^+ cotransport in the proximal tubule, as found in the Milan hypertensive strain of rats (Bianchi *et al.*, 1986), or to augmented Na^+–H^+ exchange in the proximal tubule, occurring as a local manifestation of a generalized genetic defect in Na^+–Na^+ (Na^+–Li^+) countertransport which exists in the erythrocytes of some essential hypertensives and their first-degree normotensive relatives (Canessa *et al.*, 1980; Canessa, 1986). The renal Na^+ retention leads to a transient increase in extracellular fluid volume which serves as

stimulus for the release of a Na^+,K^+-ATPase inhibitor, probably from the hypothalamus. The sodium pump inhibitor acts on the renal tubule to promote Na^+ excretion, thus restoring extracellular fluid volume to normal (or even low) levels, but with similar inhibitory effects on the Na^+,K^+-ATPase in vascular smooth muscle cells resulting in a tonic increase in vascular tone, increased total peripheral resistance and arterial hypertension. The persistence of the Na^+,K^+-ATPase inhibitor in the circulation in the absence of increased intravascular volume (which is measured to be normal or low in established essential hypertension) appears paradoxical. London *et al.* (1985) have suggested that this could be related to a persistent increase in intrathoracic vascular pressures acting as the afferent stimulus.

How Na^+,K^+-ATPase inhibition in vascular smooth muscle results in increased cytosolic free calcium concentrations, which must occur to produce the arterial vasoconstriction, remains unclear. Blaustein (1977) has proposed and defended (Blaustein *et al.*, 1986) the hypothesis that altered Na^+–Ca^{2+} exchange resulting from partial sodium-pump inhibition can account for the increased intracellular free Ca^{2+} concentration in vascular smooth muscle. Other authorities maintain that under physiological conditions there is little evidence for intracellular Ca^{2+} regulation by Na^+–Ca^{2+} exchange in vascular smooth muscle (Somlyo *et al.*, 1986), although a role in extreme situations of Ca^{2+} overload remains a possibility (Mulvany, 1985).

The effects of sodium-pump inhibition in excitable tissues nevertheless provides for other possibilities. Increased intracellular free Ca^{2+} concentration could occur through membrane depolarization effects of Na^+,K^+-ATPase inhibition via the voltage-dependent Ca^{2+} channel (Mulvany *et al.*, 1984). Pamnani *et al.* (1982) have measured such a change in potential, using a microelectrode in rat tail arteries bathed in hypertensive rat plasma containing a Na^+ transport inhibitor. Since norepinephrine uptake into adrenergic nerve terminals is sodium dependent and is inhibited by ouabain (Vanhoutte and Lorenz, 1984), another possibility is that the circulating Na^+,K^+-ATPase inhibitor acts indirectly on smooth muscle cells through actions on sympathetic nerve endings. Normal canine plasma has been shown to block uptake of [^{3}H]norepinephrine in isolated saphenous vein, and this ability of the plasma was resistant to boiling, suggesting that the effects were due to a heat-stable, nonprotein component (Freas *et al.*, 1982).

C. Antidigitalis Antibodies

Although there was no reason to assume a structural identity between the postulated endogenous Na^+,K^+-ATPase inhibitor and the cardiac gly-

cosides, digitalis is a potent inhibitor of Na^+,K^+-ATPase, and has been shown to cause both natriuresis (Hook, 1969) and an increase in vascular resistance (Vatner, *et al.*, 1971), although these are not its major pharmacological effects. The widespread availability of the digoxin radioimmunoassay (Smith *et al.*, 1969) stimulated the measurement of immunoreactivity in plasma in situations in which the inhibitor might be elevated. Klingmuller *et al.* (1982) found digitalislike immunoreactivity in the urine of Na^+-loaded normal human subjects. Graves *et al.* (1983) made a similar observation when they examined the plasma of uremic subjects and suggested that this phenomenon was of prevalence and magnitude sufficient to distort the interpretation of the digoxin assay in clinical situations. Cross-reactivity in digoxin radioimmunoassays was also shown in the plasma of volume-expanded dogs (Gruber *et al.*, 1980), in the plasma and urine of hypertensive patients (Crabos *et al.*, 1984), and in the plasma of volume-expanded hogs and in normal human subjects purified by high-performance liquid chromatography (Tamura *et al.*, 1985; Kelly *et al.*, 1986). In the latter two cases, the responsible compounds were identified as nonesterified fatty acids and lysophospholipids, illustrating the problem of specificity of effects in the radioimmunoassays.

If antibodies specific for the digitalis glycosides are indeed capable of binding the Na^+,K^+-ATPase inhibitor, one would expect that the adminstration of high-affinity antibodies *in vivo* would inhibit the activity of the inhibitor in the same way that such antibodies inhibit the pharmacological effects of digoxin (Smith *et al.*, 1982). In a hypertensive model, in which rats were heminephrectomized and then treated with deoxycorticosterone and salt, Kojima *et al.* (1982) showed that the administration of antidigoxin antibody caused a marked decreased in blood pressure. Because whole antibody was used in this study rather than Fab fragments, the possibility that the reduction in blood pressure was due to immune complex-mediated release of vasodilators cannot be excluded. Control animals, nevertheless, did not manifest a similar hypotensive response when infused with the same antibody solution. Subsequently, the same investigators showed that digoxinlike immunoreactivity, as well as Na^+,K^+-ATPase binding activity, was present in the plasma of these hypertensive animals (Kojima, 1984).

The background of these experimental and clinical observations has stimulated a concerted effort by a number of laboratories to isolate and identify the endogenous Na^+,K^+-ATPase inhibitor. Although considerable effort has been expended over a number of years, a purified substance of known structure is not yet in hand. Controversy still exists even about the chemical nature of the substance; some maintain it is a peptide, while others affirm that it has properties manifestly inconsistent with this class of compounds. It should be remembered, however, that vertebrates

are capable of synthesizing substances closely analogous in structure and pharmacological properties to the digitalis glycosides. The bufodieneolides, present in the skin of certain toads, are, like the digitalis glycosides, steroid lactone compounds that inhibit Na^+,K^+-ATPase (Flier *et al.*, 1980) and are potent cardiac inotropic agents (Shimoni *et al.*, 1984).

D. Criteria for Physiological Significance

In evaluating the various efforts to isolate the endogenous Na^+,K^+-ATPase inhibitor, it is important to keep in mind that certain criteria must be met before a putative substance can be considered a physiological regulator. Like all enzymes, Na^+,K^+-ATPase is readily inhibited by a large number of substances. For example, the common fatty acids, linoleic and linolenic, are effective inhibitors (Bidard *et al.*, 1984; Tamura *et al.*, 1985), although they exhibit a rather high inhibitory constant (K_i) well beyond their physiological range of concentration. For these reasons, a believable, physiologically relevant inhibitor should have a very high binding affinity for the enzyme. Ouabain, one of the digitalis glycosides, exhibits a K_i of 2 n*M*. One must expect the K_i of an endogenous Na^+,K^+-ATPase inhibitor to be at least within that order of magnitude.

If the function of the putative inhibitor is to regulate the sodium–potassium pump, such inhibition must be reversible. Na^+,K^+-ATPase regeneration by protein synthesis would seem too sluggish a mechanism to allow quickly changing homeostatic needs to be met. Specificity for membrane Na^+,K^+-ATPase is also essential, since it would be cumbersome for the inhibitor to regulate several enzymes at the same time. Finally, one would like to observe appropriate changes in inhibitor plasma concentration in response to relevant stimuli. None of the substances put forward as candidates for the endogenous Na^+,K^+-ATPase inhibitor meets all these criteria, and most have not even been convincingly tested for any of them.

As inferred above, many investigators have hypothesized that the endogenous inhibitors of Na^+,K^+-ATPase may have a chemical structure analogous to the digitalis glycosides, or may at least share immunological cross-reactivity with them. Although this structural similarity would simplify measurement or purification of the inhibitor, it cannot be considered a necessary criterion for identification of the substance. Nor would it be necessary for the mechanism of enzyme inhibition to duplicate exactly that of the cardiac glycosides.

Various laboratories have used plasma, urine, or tissue as potential sources of the Na^+,K^+-ATPase inhibitor. Most of these efforts are at a preliminary stage. To date, none has yielded molecular characterization, few have addressed the essential tests just mentioned, and conclusions as to the chemical nature of the material are often contradictory. Neverthe-

less, a few groups have succeeded in purifying to a considerable degree compounds that demonstrate potent Na^+,K^+-ATPase inibitory activity, and efforts in structural analysis are geing actively pursued. The efforts of a number of the laboratories involved in this work have been detailed in a recent review (Haber and Haupert, 1987).

E. Source of Endogenous Na^+,K^+-ATPase Inhibitor

The precise source of the endogenous Na^+,K^+-ATPase inhibitor also remains uncertain, but the brain has been a favored organ, since the natriuretic effects of extracellular fluid volume expansion appear to depend on an intact central nervous system (Lichardus *et al.*, 1969; Kaloyanides *et al.*, 1978), and a number of investigators have been able to extract and partially purify Na^+,K^+-ATPase inhibitory activity from cerebral (primarily midbrain) tissue (Haber and Haupert, 1987). Although endogenous Na^+ transport inhibitory activity need not be restricted to the central nervous system, most investigators have been unable to find it in other organs. Alaghband-Zadeh *et al.* (1983), using a cytochemical assay for Na^+,K^+-ATPase activity, processed numerous organ tissues from the rat (including cerebral cortex tissue) and found inhibitory activity only in extracts from the hypothalamus. However, De Pover *et al.* (1982) have extracted and partially purified Na^+,K^+-ATPase inhibitory activity from guinea pig heart.

Two additional lines of evidence suggest that the brain is an important source of this inhibitory factor: Two groups (Halperin *et al.*, 1983, 1988; Lichtstein *et al.*, 1985) have isolated ouabainlike compounds from human cerebrospinal fluid, and Morgan *et al.* (1985) have recovered and partially characterized a Na^+,K^+-ATPase inhibitor from fetal rat hypothalamic cells in culture. Finally, Jandhyala and Ansari (1986) have recently reported that perfusion of cerebral ventricles in the dog, with artificial cerebral spinal fluid containing an elevated Na^+ concentration, leads to release into the circulation of a Na^+,K^+-ATPase inhibitor with resulting inhibition of sodium–potassium pump activity in the animal's blood vessels. To this point, the bulk of evidence suggests that the brain, and more specifically, the hypothalamus, represents an enriched source of the endogenous inhibitor of Na^+,K^+-ATPase, if not the site of production.

II. HYPOTHALAMIC Na^+,K^+-ATPase INHIBITOR

Drawing on the observations cited above, that volume expansion natriuresis appeared to depend on an intact central nervous system, and focusing on the hypothalamus as a rich source of other regulatory hormones,

we began a decade ago to prepare partially purified extracts of bovine hypothalamus and test them for Na^+ transport-inhibitory activity. Small amounts of these extracts, when applied to the serosal (circulatory) side of the toad urinary bladder, produced a reversible, nontoxic inhibition of active Na^+ transport across the membrane (Haupert and Sancho, 1979). The inhibited Na^+ transport was accompanied by an increase in membrane resistance, indicating that the inhibition occurred through the active transport pathway and not because of a toxic effect on the preparation. When applied to the mucosal (noncirculatory) side of the membrane, there was no inhibition of Na^+ transport. When the hypothalamic inhibitor (HI) was applied to frog urinary bladder, a tissue that binds ouabain tightly, HI caused inhibition of tritiated ouabain binding to its cellular receptor, the Na^+,K^+-ATPase. Na^+,K^+-ATPase prepared from the renal medulla of the rabbit was then shown to be directly inhibited by HI (Haupert and Sancho, 1979). In unpublished experiments, infusion of HI into an isolated perfused rat kidney increased fractional excretion of Na^+ by 10%. These early studies thus indicated that the inhibitor from hypothalamus had the characteristics of the putative natriuretic hormone and, more generally, some of the actions of the cardiac glycosides.

A. Purification and Partial Chemical Characterization of Hypothalamic Inhibitor

The activity of interest in extracted from bovine hypothalamus, which is dissected fresh and quick-frozen on dry ice. Following homogenization in methanol, lipids are extracted and discarded and the remaining aqueous phase, which contains the inhibitor, is desalted and then further purified by lipophilic gel and ion-exchange chromatography (Haupert *et al.*, 1984). HI is a low-molecular-weight, nonpeptidic substance, since it elutes on a Sephadex G-25 column in the included volume and is resistant to acid hydrolysis (Haupert and Sancho, 1979). Activity is lost after ashing or base hydrolysis. The molecule is highly polar and appears to act as a zwitterion. At acid pH, it behaves as a weak base and can be absorbed and eluted from strong cation-exchange resins (Haupert and Sancho, 1979). At neutral pH, it appears to be anionic by its retention on anion-exchange resins. The anionic behavior may be due to the presence of a carboxylic acid group, since the biological activity is lost after methylation by diazomethane and can be restored by gentle acid hydrolysis (as would be expected for a methyl ester). The partially purified HI is free of proteins, salts (flame photometry, atomic absorbance spectroscopy), vanadium (emission spectroscopy), ammonium ion, and fatty acids and lysolipids (mass spectroscopy), the latter having recently been extracted

from plasma and found to be relatively low-affinity, nonspecific inhibitors of Na^+,K^+-ATPase (Tamura *et al.*, 1985; Kelly *et al.*, 1986). Complete understanding of the structure of HI is in progress, using mass spectroscopy on samples further purified by high-performance liquid chromatography.

B. Criteria for Physiological Significance

In the absence of a definitive structure, it became particularly important to characterize HI with regard to biochemical criteria considered essential for *in vivo* physiological relevance. Central among these, as noted in Section I. D, are high binding affinity, reversibility of enzyme inhibition, and specificity for the target enzyme/receptor, in this case, the Na^+,K^+-ATPase. Using highly purified canine renal Na^+,K^+-ATPase, we determined that the apparent affinity for inhibition of the enzyme by HI was in a physiological range (K_i = 1.4 n*M*) (Haupert *et al.*, 1984). Thus, HI is a potent inhibitor of the Na^+,K^+-ATPase *in vitro*, being somewhat more potent than ouabain (K_i = 2 n*M*) (Schwartz *et al.*, 1982) or vanadate (K_i = 6 n*M*) (Josephson and Cantley, 1977). The inhibition of the purified enzyme *in vitro* was facilitated by Mg^{2+} and inhibited by Na^+, and by adjusting the concentration of these ligands in the binding assay it was possible to show reversibility of binding *in vitro* (Haupert *et al.*, 1984). This was consistent with our earlier findings of reversibility of inhibition of actual sodium pumping studied in toad urinary bladder (Haupert and Sancho, 1979). In collaboration with Carilli and Cantley (1985), we further determined that HI could inhibit sodium pumping in a human cell (erythrocyte), and that inhibition was specific for Na^+,K^+-ATPase since, at concentrations of HI that inhibited Na^+,K^+-ATPase by 75%, other ATPases in the human erythrocyte plasma membrane were not affected. Thus, three of the cardinal criteria for a physiologically relevant inhibitor of Na^+,K^+-ATPase—affinity, reversibility, and specificity—were satisfied by the endogenous inhibitor extracted from hypothalamus.

C. Mechanism of Inhibition of Na^+,K^+-ATPase

Both the mechanism by which HI inhibits enzyme activity (an *in vitro* observation) and active sodium pumping in intact transporting epithelia (an activity that translates enzyme inhibition into a relevant physiological function) required further study. To address this question, it is useful to briefly review the biochemical events considered to account for the hydrolysis of ATP by Na^+,K^+-ATPase, and the resulting transport of Na^+

and K^+ across the plasma membrane. Figure 1 represents a model that provides a working hypothesis and incorporates much of the current thinking for the mechanism of Na^+ and K^+ transport by the Na^+,K^+-ATPase. The model is discussed in detail and critically evaluated by Cantley (1981), but the salient features can be summarized as follows: The enzyme is felt to exist in two major conformational states, E_1 and E_2. The E_1 conformation binds and is stabilized by Na^+ and ATP; the E_2 conformation, by K^+ or a covalently bound phosphate. In the presence of Na^+, Mg^{2+}, and ATP, E_1 becomes phosphorylated [(B) to (C) in Fig. 1]. The phosphorylated protein relaxes into the E_2 state, the change in conformation resulting in the outward transport of three Na^+ ions. The outwardly directed cation-binding sites now possess a higher affinity for K^+ than for Na^+, and the resulting K^+ binding catalyzes dephosphorylation of the enzyme [(D) to (F)]. The model proposes that during this process that K^+ ions become trapped at their binding site and are released to the interior of the cell only after a shift in conformation from E_2 back to E_1. This shift is accelerated by the presence of ATP inside the cell [(F)

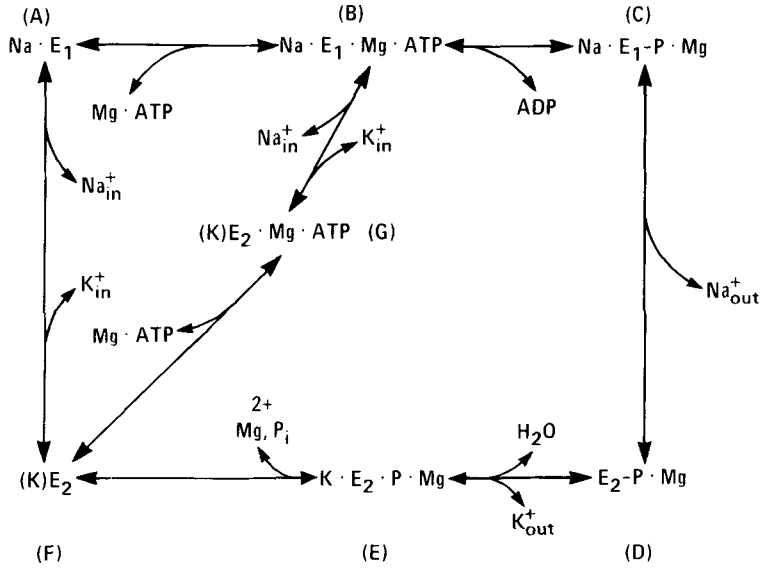

FIG. 1. A kinetic model for the mechanism of ATP hydrolysis by Na^+,K^+-ATPase and the transmembrane active transport of Na^+ and K^+. See text for explanation. The stoichiometries for Na^+ and K^+ binding sites are omitted. (From Cantley, 1981. Reprinted with permission.)

to (G)]. The driving of the enzyme from E_2 to E_1 following ATP binding [(G) to (B)] releases two K^+ ions inside the cell and reestablishes the conformation, which permits phosphorylation of the enzyme in the presence of Na^+, thus perpetuating the cycle, resulting in directed, active Na^+ and K^+ transport.

The model permits us to ask several questions regarding the biochemical interaction of HI with the cycling enzyme: (1) Does HI bind to the inside (like vanadate) or the outside (like ouabain) of the cell? (2) Does binding occur to stabilize the E_1 conformation (like Na^+ and ATP) or the E_2 conformation (like K^+ or ouabain)? (3) Could the inhibitory effects of HF be related to enzyme phosphorylation events? (4) Is the reversibility of binding of HF related in some way to the cycling of Na^+,K^+-ATPase through E_1 and E_2?

1. HI Binding to Extracellular Surface

To determine the sidedness of HI binding, human inside-out red cell vesicles were prepared and the ATPase activity of these vesicles was determined in the presence of HI (Carilli *et al.*, 1985). As shown in Table I, HI had little effect on the Na^+,K^+-ATPase activity of inside-out red cell vesicles, but almost completely inhibited the Na^+,K^+-ATPase activity in vesicles permeabilized by detergent Nonidet P-40 (NP-40), thus exposing a binding site on the extracellular surface. Na^+,K^+-ATPase activity of intact vesicles was defined as that ATPase activity which is resistant to ouabain (which does not permeate the vesicles) but is sensitive to strophanthidin (which is sufficiently hydrophobic to permeate the vesicles). Thus, in human erythrocytes HI acts only from the extracellular surface

TABLE I
ATPase Activity of Inside-Out Vesicles[a]

Preparation	ATPase activity (nmol/mg/min)		
	Total	Strophanthidin sensitive	Strophanthidin resistant
IOV	3.8	1.1	2.7
IOV + HI	4.2	0.9	3.3
IOV + NP-40	3.6	1.7	1.9
IOV + NP-40 + HI	2.4	0.1	2.3

[a]Error in data was ± 15% (triplicate determinations). IOV, Inside-out vesicles; HI, hypothalamic inhibitor; NP-40, Nonidet P-40. Adapted from Carilli *et al.* (1985).

of the cell. This finding is consistent with previous experiments which showed that HI inhibited active Na^+ transport in toad urinary bladder when applied to the serosal (circulatory) but not the mucosal side of the membrane (Haupert and Sancho, 1979).

2. HI Stabilization of Na^+,K^+-ATPase in E_2 Conformation

The two conformational states of the Na^+,K^+-ATPase, E_1, and E_2, can be distinguished fluorometrically following labeling of the purified enzyme by fluorescein 5′-isothiocyanate (FITC) (Karlish *et al.*, 1979). The conformational state can be manipulated *in vitro* by altering monovalent cations in the bathing buffer: K^+ stabilizes the E_2 conformation, while Na^+ shifts the conformation to E_1, as indicated by an increase in relative fluorescence (Carilli *et al.*, 1982). Table II shows that, in the absence of monovalent cations, the fluorescence of FITC-labeled enzyme that had been incubated with HI is similar to that of the control enzyme. The addition of 20 m*M* KCl to either control or HI-treated enzyme caused only a slight decrease in fluorescence, indicating that in 20 m*M* Tris–Cl, pH 7.4, both enzymes were predominantly in the E_2 form. NaCl (250 m*M*) reversed the effect of KCl on the control enzyme and caused an additional 30% increase in fluorescence within 30 sec (a shift to E_1). However, NaCl had only a slight effect on the HI-treated enzyme, causing a return to its original fluorescence intensity within 30 sec, but with no further change observed over the next 5 min. Choline chloride (250 m*M*) had no effect on FITC fluorescence, suggesting that the changes observed were not simply due to an ionic-strength effect. Thus, HI stabilized the enzyme in an E_2 conformation and prevented the shift to E_1 ordinarily caused by addition of Na^+ ions.

TABLE II
Fluorescence of Fluorescein-5′-Isothiocyanate-Labeled Na^+,K^+-ATPase[a]

Sample	Control	+ HI
20 m*M* Tris–Cl	1.00	1.04
+ 20 m*M* KCl	0.95	0.98
+ 250 m*M* NaCl	1.30	1.04
+ 250 m*M* choline chloride	1.00	1.01

[a]Data are expressed in relative fluorescence units. Error in data was ±2%. HI, Hypothalamic inhibitor. Adapted from Carilli *et al.* (1985).

3. HI Inhibition of Phosphorylation of Na^+,K^+-ATPase

The active site aspartate residue of Na^+,K^+-ATPase can be phosphorylated in two ways: (1) from ATP, Mg^{2+}, and Na^+ or (2) by forming a covalent bond with inorganic phosphate in the presence of Mg^{2+}, a reaction which is supported by ouabain and results in a protein conformation similar to, but not identical to, the E_2 state (Post *et al.*, 1975). Table III shows the results of this "back-door" phosphorylation from [γ-^{32}P]ATP of purified Na^+,K^+-ATPase treated with ouabain and HI. HI was found to block phosphorylation of the enzyme from inorganic phosphorus and Mg^{2+}; and HI prevented ouabain from supporting this phosphorylation, consistent with earlier findings that HI blocks ouabain binding to the Na^+,K^+-ATPase (Haupert and Sancho, 1979). The phosphorylation was decreased only when HI was allowed to equilibrate with the enzyme before the addition of ouabain. This result was expected, in view of the slow rate of HI binding to and release from the purified enzyme (Haupert *et al.*, 1984) and because of the high concentration of ouabain used (1 m*M*). Thus, although HI stabilizes an E_2-like conformation, it does not stabilize the E_2–P bond and prevents ouabain from stabilizing this bond.

D. Inhibition of Sodium Pump Activity in Renal Tubular Epithelial Cells

Although HI appeared to satisfy several essential criteria for physiological relevance as noted above, kinetic analysis of the binding of HI to purified Na^+,K^+-ATPase raised on additional conceptual problem for HI as a physiological regulator *in vivo*. Like ouabain, on and off rates for HI binding to purified membrane preparations were relatively long (off rates = 60 min) (Haupert *et al.*, 1984). Since HI has been proposed as a natriuretic hormone, and since such a slow rate of dissociation after binding

TABLE III
Phosphorylation of Na^+,K^+-ATPase by [^{32}P]H_3PO_4[a]

Conditions	^{32}P bound (nmol/mg)
Ouabain (10 min)	1.2 ± 0.1
Ouabain + HI (10 min)	1.3 ± 0.1
HI (30 min), then ouabain + HI (10 min)	0.04 ± 0.1
HI (40 min)	0.0 ± 0.1
Ouabain (40 min)	1.2 ± 0.11

[a]HI, Hypothalamic inhibitor. Adapted from Carilli *et al.* (1985).

might seem inconsistent with short-term regulation of renal sodium handling, it was of interest to determine whether binding reactions in intact cells would show a time frame more consistent with physiological regulation *in vivo*. Using ouabain-sensitive $^{86}Rb^+$ uptake (J_{Rb}) as a measure of sodium pump activity, we studied the binding and release of HI to cultured porcine renal tubular epithelial cells (LLC-PK_1) (Haupert *et al.*, 1986). These cells are particularly suitable for study since they are of renal origin, they have been well characterized (Handler, 1983), they contain a large number of sodium–potassium pump sites (10^6 sites per cell) (Mills *et al.*, 1981), and 80–90% of their K^+ transport is through the Na^+,K^+-ATPase (Cantiello *et al.*, 1986). Results of the binding studies are shown in Fig. 2. As with membranes, a 60-min incubation with HI inhibited Na^+,K^+-ATPase activity in LLC-PK_1 cells. In contrast to membranes, no prior incubation with LLC-PK_1 cells was needed. Saturating HI produced an immediate 33% reduction of J_{Rb}. When the cultured renal cells were quickly washed with cold buffer following incubation with HI, the subsequent $^{86}Rb^+$ influx indicated a rapid reversal of inhibition (dissociation of HI from the receptor) and a doubling of pump activity (K^+ influx) (Fig. 2). Ouabain binding studies in HI-treated LLC-PK_1 cells before and after "washout" show that the enhanced transport is due to an increase in pump velocity and not to recruitment of additional pump units into the cell membrane (Cantiello *et al.*, 1988). Note that ouabain does not demonstrate this rapid reversal of binding in the renal tubular cells (Fig. 2).

E. Target Organ Specificity for Endogenous Na^+,K^+-ATPase Inhibitor

Is there target organ specificity for the endogenous Na^+,K^+-ATPase inhibitor, and what could be the mechanism for particular effects in various target tissues? In a preliminary set of experiments, we have addressed these issues for HI by measuring its inhibitory effects on Na^+,K^+-ATPase purified from three putative target organs in the same animal (Haupert *et al.*, 1987). Guinea pig Na^+,K^+-ATPase was purified from kidney and brain, using the method of Jørgensen (1974), and from heart by the method of Jones and Besch (1984). Enzyme activity was measured as the hydrolysis of ATP by using both a kinetic coupled-enzyme assay (Haupert *et al.*, 1984), and the release of the [γ-^{32}P]ATP. The amount of protein in the experiments was adjusted so that HI effects could be determined on enzymes with equal specific ATP hydrolytic activity. Results for 1 and 2 U of HI (a concentration close to the K_m for

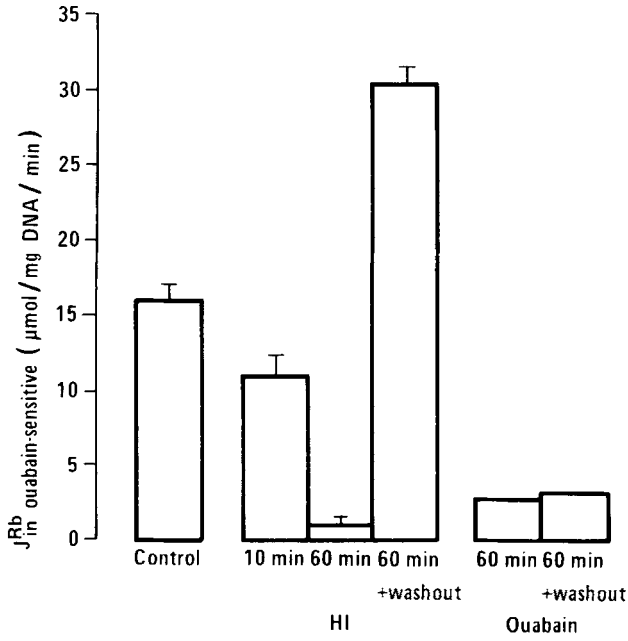

FIG. 2. Effects of HI (20 U/ml) and ouabain (1 m*M*) on sodium pump activity in cultured renal tubular cells (LLC-PK_1), measured as $^{86}Rb^+$ influx (J_{Rb}) as a function of time of incubation with the inhibitors and following washout. Data for HI are ouabain-sensitive $^{86}Rb^+$ influx; data for ouabain are total $^{86}Rb^+$ influx. In washout experiments, cells were washed rapidly twice in cold buffer following the incubations with HI (n = 9) or ouabain (n = 2), with subsequent addition of $^{86}Rb^+$ to run the flux. (From Haupert *et al.*, 1986. Reprinted with permission.)

inhibition of purified canine kidney Na^+,K^+-ATPase) are shown in Table IV. The enzymes from brain and kidney were clearly more susceptible to inhibition by HI than the enzyme from heart, even though inhibitory effects on the latter were significant. Furthermore, the apparent steepness of the dose–response relationship differed for Na^+,K^+-ATPase from kidney and brain.

One possibility for the differences observed in this experiment, and for the rationale for regulation by an endogenous inhibitor in general, could lie in organ-specific isoenzyme composition of the Na^+,K^+-ATPase (Sweadner. 1979). For this reason, we assayed the effects of HI on purified $\alpha+$ and α from rat axolemma and kidney, respectively (kindly sup-

TABLE IV
INHIBITION BY HYPOTHALAMIC INHIBITOR OF Na^+,K^+-ATPASE PURIFIED FROM GUINEA PIG

HI	Kidney	Brain	Heart
1 U[a]	23 ± 5 (n = 6)	80 ± 6[b] (n = 8)	16 ± 2 (n = 6)
2 U	95 ± 2[c] (n = 5)	100 ± 0[c] (n = 4)	23 ± 3 (n = 5)

[a]One unit of hypothalamic inhibitor (HI) is defined as the amount required for 50% inhibition of purified Na^+,K^+-ATPase from canine renal medulla under standard assay conditions at 37°C (Haupert *et al.*, 1984).
[b]$p < 0.0001$ versus kidney and heart.
[c]$p < 0.0001$ versus heart.

plied by K. Sweadner). Initial results show that concentrations of HI which inhibit $\alpha+$ by 82% inhibit purified α by only 18%, a direction parallel to that of ouabain (1.0 m*M*), which inhibited $\alpha+$ and α by 90% and 45%, respectively, using the same experimental protocol.

III. DISCUSSION

Bovine hypothalamus contains a low-molecular-weight, nonpeptidic molecule that inhibits the hydrolysis of ATP by mammalian Na^+,K^+-ATPase. Initial studies of this substance following partial purification showed it to be a nontoxic inhibitor of active Na^+ transport across toad urinary bladder (a functional analog of the human distal nephron) (Haupert and Sancho, 1979) and in intact human erythrocytes (Carilli *et al.*, 1985). This HI also prevented the binding of the cardiac glycoside, ouabain, to its receptor, the Na^+,K^+-ATPase, and directly inhibited Na^+,K^+-ATPase prepared from rabbit renal medulla (Haupert and Sancho, 1979). HI thus possesses some of the characteristics ascribed to an endogenous, ouabainlike compound which has been proposed to act as a natriuretic hormone, a pathogenetic factor in human essential hypertension, and perhaps as an even more general regulator of Na^+,K^+-ATPase in mammalian cells (for review see Haber and Haupert, 1987).

Chemical characterization to date has shown HI to be a highly polar, low (<1000)-molecular-mass molecule with zwitterionic properties due in part to a carboxylic acid group necessary for biological activity (Carilli *et al.*, 1985). Purified extracts containing HI have been shown to be free of known inhibitors of Na^+,K^+-ATPase, such as monovalent and divalent cations, fatty acids, lysolipids, and vanadium.

HI has been shown to satisfy several essential criteria for a physiologically relevant regulator. It inhibits purified Na^+,K^+-ATPase with high affinity (K_i = 1.4 n*M*). This *in vitro* inhibition of the Na^+,K^+-ATPase is reversible (nontoxic) (Haupert *et al.*, 1984), just as is the inhibition of active Na^+ transport across amphibian epithelial cell membranes (Haupert and Sancho, 1979) and cultured renal tubular epithelial cells (Haupert *et al.*, 1986). Studies of plasma membrane ATPases in human erythrocytes showed the inhibitory effects of HI to be specific for theNa^+,K^+-ATPase (Carilli *et al.*, 1985). Thus, HI meets the essential criteria—high binding affinity, reversibility, and substrate specificity—required of a physiological regulator of the sodium pump.

The studies detailed in this review further characterize HI as to mechanism of inhibition of the Na^+,K^+-ATPase and the kinetics of binding and dissociation to the sodium pump in mammalian renal tubular cells (LLC-PK_1). With reference to the model of active cation transport associated with ATP hydrolysis by the Na^+,K^+-ATPase (Fig. 1), the findings from these experiments permit the following hypothesis: HI binds with high affinity to the extracellular surface of the cell (Table I), consistent with the concept of a circulating inhibitor of the sodium pump. HI may inhibit Na^+,K^+-ATPase activity by influencing the phosphorylation site on the enzyme (Table III), and may alter active Na^+ transport by "locking" the enzyme in E_2, thus preventing the cycling of the enzyme between E_1 and E_2, with an attendant interruption of the translocation of Na^+ and K^+ across the plasma membrane (Table II and Fig. 1).

While off rates for HI inhibition of purified Na^+,K^+-ATPase *in vitro* were relatively slow (60 min), binding and dissociation reactions in intact renal cells were quite different from those in isolated membranes. HI produced immediate inhibition of the sodium pump in the intact cells, and removal of HI after 1 hr of incubation showed rapid reversal of the inhibitory effect, and an actual stimulation of pump activity (Fig. 2). [^{3}H]Ouabain binding studies in the HI-treated cells under conditions of binding and washout indicate that the enhanced transport activity is due to an increase in pump velocity and not to an increase in pump number (Cantiello *et al.*, 1988). This pump stimulation is probably due to the accumulation of intracellular sodium during the period of pump inhibition by HI. Ouabain does not show the rapid dissociation of binding from the renal cells following washout (Fig. 2). This observation reveals a further mechanistic difference between ouabain and HI, and would appear to indicate that HI behaves more physiologically than ouabain in regulation of Na^+,K^+-ATPase in these cells.

Since HI appears to bind to the Na^+,K^+-ATPasein the E_2 conformation (Table II), and since inhibition of the pump by HI would favor the intra-

cellular accumulation of ligands (Na^+ and ATP) which are known to pull the enzyme from E_2 into E_1, it is possible that the rapid dissociation time in a sided preparation (intact cells) relative to isolated membranes is due to the shift in enzyme conformation from E_2 to E_1, the latter being a conformation that does not support HI binding (Haupert *et al.*, 1984). Thus, the available data suggest that HI may generate its own feedback cycle of binding and dissociation reactions for regulation of Na^+,K^+-ATPase activity in the plasma membrane of intact renal epithelial cells. These effects could play an important role in the transepithelial movement of cations in the kidney that would account for a natriuretic effect *in vivo*.

Although data are preliminary, organ specificity for an endogenous Na^+,K^+-ATPase inhibitor may be governed by binding affinity differences in various target organs, and such differing affinities may be due in part to isoenzyme composition of the Na^+,K^+-ATPase.

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