SUBCELLULAR BASIS OF CONTRACTILE FAILURE

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SUBCELLULAR BASIS OF CONTRACTILE FAILURE

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To our wives
Marta Korecky and Ranjit Dhalla
for their understanding and support

CONTENTS

PREFACE

This monograph contains 20 selected papers presented at the Symposium on Subcellular Basis of Contractile Failure which was held in Ottawa during May 11-13, 1989 and is designed for the benefit of those who were unable to attend this event. It is now increasingly becoming clear that an excessive amount of calcium is intimately involved in the pathogenesis of a wide variety of heart diseases. Accordingly, the investigations concerning the role of calcium channels and their regulatory mechanisms in heart function as well as of the intracellular calcium overload in cardiac dysfunction are presented here. Since sodium is also considered to influence the cardiac contractile force by changing the intracellular concentration of calcium through the Na^+-Ca^{2+} exchange mechanism in the cell membrane, the role of Na^+-Ca^{2+} exchange in heart function as well as pathology of contractile failure is discussed. In view of the newly discovered implications of the oxygen free radicals in cellular injury, papers concerning the role of these radicals in heart disease are included in this book. For the purpose of clarity, different chapters have been organized under three main headings: (I) Role of cations in heart function, (II) Cardiac hypertrophy and cardiomyopathies, and (III) Ischemic heart disease and cardiac failure. These chapters by expert investigators represent a multidisciplinary approach for defining the events occurring during the development of contractile failure and include electrophysiological, functional, biochemical and pharmacological aspects. It is hoped that this book will be of some use to both students and researchers in the area of cardiovascular sciences and will generate new ideas for further investigations on problems associated with heart disease.

Borivoj Korecky and Naranjan S. Dhalla

I

ROLE OF CATIONS IN HEART FUNCTION

1

ROLE FOR SODIUM CHANNELS AND INTRACELLULAR SODIUM IN REGULATION OF THE CARDIAC FORCE-FREQUENCY RELATION AND CONTRACTILITY.

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INTRODUCTION

The force of contraction in cardiac muscle is markedly dependent upon the frequency of stimulation (1). In most animal species, an increase in the stimulation frequency leads to an increase in twitch tension amplitude that requires a few minutes to reach a steady-state. Lowering of the stimulation frequency results in an opposite effect with a gradual decrease in twitch tension amplitude. Therefore, over a wide range of stimulation frequencies the force-frequency relation has a characteristic positive shape (2-5). In cardiac Purkinje fibers, twitch tension amplitude increases with increasing stimulation frequency over the range 0.2 to 3 Hz, and up to several minutes is required to reach a new steady-state following a change in rate (5-7).

In cardiac muscle, the stimulation of repetitive action potentials causes the intracellular sodium ion activity (a^i_{Na}) to increase above the unstimulated level (6,8-10). The magnitude of the increase is a function of the stimulation frequency with small increases in a^i_{Na} observed at stimulation frequencies as low as 0.2 Hz, and at higher frequencies of stimulation a^i_{Na} is increased by up to 30% (6). The duration of stimulation also is important, with a few minutes required in Purkinje fibers for a^i_{Na} to rise to a new steady-state value during stimulation, or for a^i_{Na} to return to a resting value following cessation of stimulation (6,10). Using a voltage clamp to control membrane potential, January and Fozzard (10) investigated the mechanism of the stimulation induced rise in a^i_{Na}, and concluded that the frequency-dependent rise in a^i_{Na} resulted primarily from increased Na^+ entry through Na^+ channels during the Na^+ current.

An important role in the regulation of twitch tension development has been postulated for a^i_{Na} and its influence on Ca^{2+} through the Na-Ca exchange mechanism (7,9-13). When a^i_{Na} is increased (usually by inhibiting the Na-K pump), a steep relation between twitch tension amplitude and a^i_{Na} is found (9,14-19).The purpose of the present study was to test the hypothesis that frequency- and time-dependent changes in a^i_{Na} are important regulatory mechanisms of frequency- and time-dependent changes in cardiac twitch tension amplitude. Thus, by serving an important role in the regulation of a^i_{Na}, Na^+ channels also participate in the regulation of cardiac twitch tension amplitude. Preliminary results of these studies have appeared (20,21).

METHODS

Single unbranched free-running sheep or occasionally canine Purkinje fibers having diameters of 100-300 μm were used. Tyrode's solution (10) was oxygenated and warmed before entering (2-5 ml/min) the chamber (1.5 ml volume) and contained 5.4 mM K^+ and 1.8 mM Ca^{2+}. When indicated tetrodotoxin (TTX, Calbiochem) was added to the bath in a concentration of 1×10^{-5} M. Experiments were performed at 37°C.

The experimental methods for the control of membrane potential and the measurement of a^i_{Na} were previously described in detail (10). Voltage control was achieved using the single rubber membrane gap voltage clamp technique. Na^+ electrodes were fabricated from thick- or thin-wall borosilicate glass and contained the liquid ion exchange resin ETH 227. Electrodes were calibrated after and usually before each experiment. Driven shields and capacitance neutralization were carefully applied in order to improve the response time of the electrodes. The difference channel in most records was filtered (-3 db at 35 Hz).

In order to measure twitch tension, a fine wire ligature was first tightly tied around each Purkinje fiber. The Purkinje fiber was then passed through the constricting hole in the rubber membrane leaving a short segment less than 2 mm in length in the test endpool terminated by the fine wire tie. The wire tie was attached to a force transducer (Cambridge Technology, Inc, Model 403) capable of resolving tension to <0.01 mg. The other end of the fiber was held by the rubber membrane gap which had been stiffened by gluing it to a thin lucite wafer containing a small perforation. Fiber extending into the current injection endpool was trimmed away. Fibers were stretched 20 to 30% beyond their slack length.

Fibers were allowed to recover in the experimental chamber before initiation of voltage clamp. The holding potential (V_h) used in all experiments was -80 mV unless otherwise indicated, and during voltage clamp the current injection endpool was. Trains of voltage clamp steps (step duration 50 to 200 msec) to 0 mV were applied at frequencies of 0.5 to 4 Hz. Pulse trains were separated by a 5 to 7 minute interval of rest or low frequency stimulation (0.1 Hz) in order to allow a^i_{Na} to return to the unstimulated level before application of the next pulse train (6,10). In a few experiments, each depolarizing step to 0 mV in a pulse train was preceded by a prepulse to a constant voltage (range -67 to -100 mV) in order to shift the voltage-dependent availability of Na^+ channels. The prepulse duration used was 50 msec. Makielski et al (22) studied isolated single Purkinje cells at 23°C and reported that for subthreshold voltage steps from -140 mV to -80 and -100 mV, the time constants for the development of inactivation were 27 and 90 msec, respectively. Thus, the prepulses are of sufficient duration to permit the Na^+ conductance to shift towards a new level of inactivation before application of the depolarizing step to 0 mV. Prepulses of this duration also do not alter the amplitude of the associated twitch

(23). These prepulse voltages also should be sufficiently negative to avoid altering steady-state inactivation of conventional Ca^{2+} current (24,25). Nonlinear least-squares regression analysis for curve fitting was performed using a Masscomp model 5500 computer (Masscomp, Westford, Mass.).

RESULTS

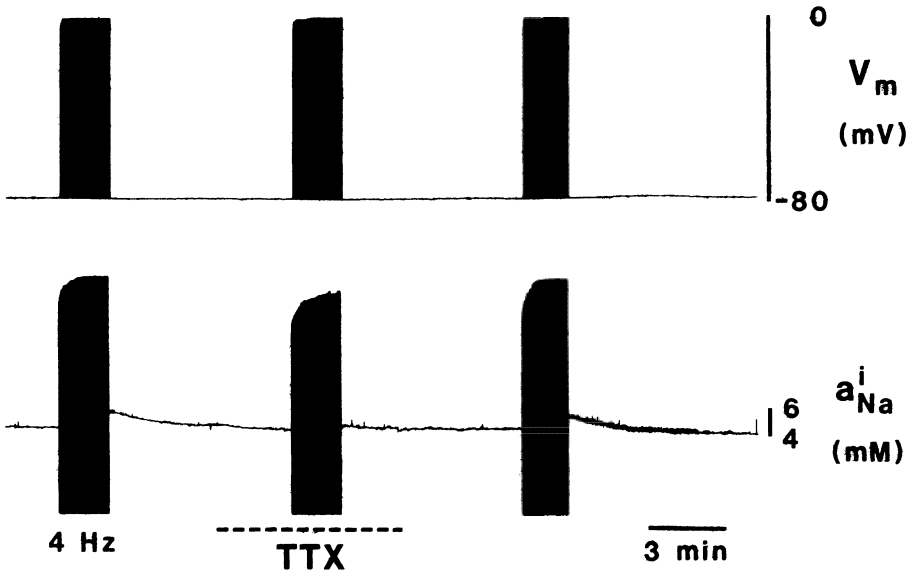

Figure 1. The frequency-dependent rise in a^i_{Na} in a sheep Purkinje fiber in response to 3-minute duration voltage clamp pulse trains, and its blockade by TTX (1×10^{-4} M). Individual voltage steps were to 0 mV for 100 msec at 4 Hz. The dashed line indicates the period of TTX exposure.

We have previously shown that Na^+ channel block with TTX at a concentration of 4×10^{-4} M diminished the rise in a^i_{Na} which occurred with repetitive depolarization (10). Figure 1 shows records from a fiber where the frequency-dependent rise in a^i_{Na} was studied and the ability of a higher concentration of TTX to block its rise was tested. From a resting value of 4.3 mM, a^i_{Na} had increased to 5.8 mM at the end of the initial pulse train. a^i_{Na} then gradually returned to the baseline value over a few minutes. TTX was then introduced into the chamber at a concentration of 1×10^{-4} M and this resulted in a small decline in the resting a^i_{Na} of about 0.4 mM. When the voltage clamp pulse train was repeated in the presence of TTX, the frequency-

dependent rise in a_{Na}^{i} was abolished. TTX was then washed from the chamber for 4 minutes and when the voltage clamp pulse train was reapplied the frequency-dependent rise in a_{Na}^{i} was again present. Similar results showing TTX block of the frequency-dependent rise in a_{Na}^{i} were obtained in three additional experiments. From the results of Cohen et al (26), it may be calculated that the concentration of TTX used in these experiments blocked nearly completely the Na^{+} current.

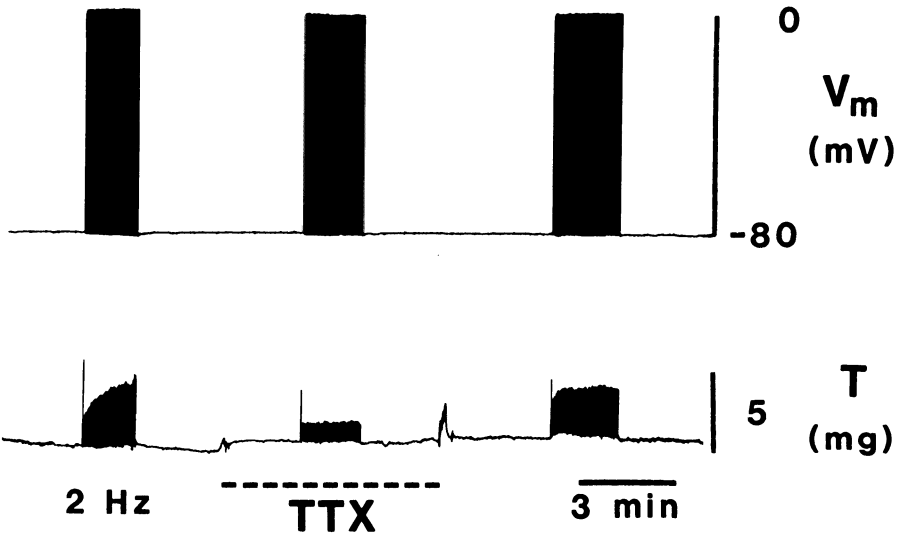

Figure 2. Twitch tension recordings during 3-minute voltage clamp pulse trains. With the initial train, a large amplitude rest contraction was followed by a positive tension staircase. Repeating the pulse train during exposure to TTX (1×10^{-5} M, dashed line) resulted in a smaller rest contraction and the positive staircase was abolished. Partial recovery occurred with a 2 1/2 minute TTX washout period.

Figure 2 shows results from an experiment similar to that in the previous figure except that tension was recorded while trains of voltage clamp pulses were applied. From rest, trains of voltage clamp pulses (to 0 mV for 200 msec) were applied at 2 Hz. After the initial "rest" contraction, the subsequent twitches were of smaller amplitude but gradually increased in size as a positive tension staircase developed. TTX then was added to the perfusate at a concentration of 1×10^{-5} M. When the voltage clamp pulse train was repeated, two changes were apparent. First, the amplitude of the rest contraction was reduced. Secondly, the development of a positive tension staircase was almost completely abolished. After washout of TTX for

2 and 1/2 minutes, partial recovery of the positive tension staircase and the amplitude of the rest contraction is shown. Nearly complete recovery of the tension staircases could be obtained with longer washout periods. Similar results with TTX abolishing almost entirely the positive tension staircase were obtained in five additional experiments.

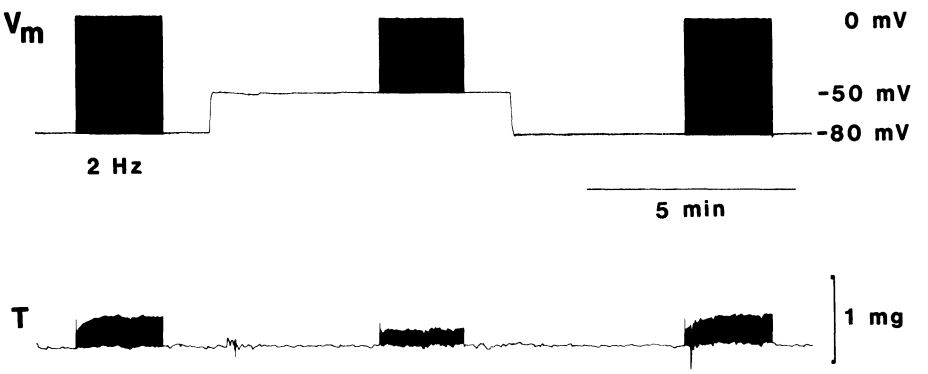

Figure 3. Effect on the positive tension staircase of applying voltage clamp pulse trains from -80 mV and -50 mV is shown. At V_h = -50 mV, where voltage inactivation of the Na^+ current should be complete, the positive staircase was nearly abolished.

Effect of Altering the Na^+ Current Voltage-Inactivation Relation on a_{Na}^i and the Positive Tension Staircase. The magnitude of Na^+ current in response to a depolarizing pulse is dependent upon the membrane potential prior to the application of a depolarizing step, as expressed in the Na^+ current steady-state inactivation relation (22,27,28). Steady-state inactivation of Na^+ current is complete at membrane potentials positive to -50 mV, conditions where the frequency-dependent rise in a_{Na}^i also are abolished. The effect on the positive tension staircase of voltage inactivation of the Na^+ current by changing V_h to -50 mV is shown in Figure 3. Initially, from a V_h of -80 mV, a 3 minute long train of voltage clamp pulses was applied at 2 Hz and resulted in a rest contraction of approximately 0.35 mg which was followed by a positive tension staircase. V_h was then changed to -50 mV in order to inactivate Na^+ current. When the voltage clamp pulse train was repeated, the amplitude of the rest contraction was reduced to 0.25 mg and a positive tension staircase failed to develop. After returning V_h to -80 mV, reapplication of the voltage clamp pulse train resulted in return of the rest contraction amplitude and the positive tension staircase to the

control level. Similar findings of nearly complete block of the positive tension staircase at a V_h of -50 mV were confirmed in two additional fibers.

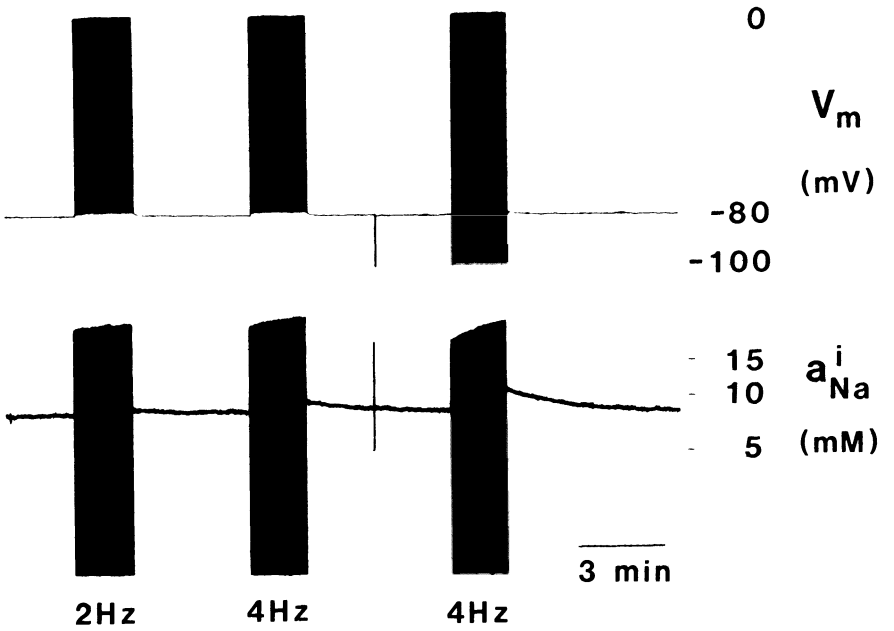

Figure 4. The initial two voltage clamp pulse trains were applied at 2 and 4 Hz and caused frequency-dependent increases in a^i_{Na}. The third pulse train was identical to the second except that a 50 msec long prepulse to -100 mV preceded each depolarizing step in order to increase voltage-dependent availability of Na^+ current. In response to the third pulse train, the frequency-dependent rise in a^i_{Na} was increased nearly 2-fold.

The effect on the frequency-dependent rise in a^i_{Na} and tension development of applying voltage clamp pulse trains from hyperpolarized membrane potentials was studied. Because maintained hyperpolarization of membrane potential will cause a large rise in a^i_{Na}, voltage-dependent Na^+ channel inactivation was manipulated using brief duration prepulses applied from a constant V_h of -80 mV. Each prepulse preceded the depolarizing voltage step to 0 mV. Figure 4 shows records from one fiber where hyperpolarizing prepulses to -100 mV were applied in order to diminish Na^+ channel inactivation, and changes in a^i_{Na} were recorded. Initially, a 3 minute long train of voltage clamp pulses was applied at 2 Hz and resulted in a rise in a^i_{Na} from 8.4 to 9.1 mM at the end of the pulse train. After a period of rest, the pulse train was reapplied at a higher frequency of 4 Hz which resulted in a greater rise in a^i_{Na} to about 9.9 mM. After another period of rest, the pulse train was repeated at 4 Hz except that each depolarizing voltage clamp step in the pulse train was preceded by

a 50 msec long hyperpolarizing prepulse to -100 mV. From a resting value of 8.5 mM, the rise in a^i_{Na} was nearly doubled to 11.5 mM at the end of the pulse train, and had returned to baseline level within 5 minutes. Similar results, showing an increase in the frequency-dependent rise in a^i_{Na} with the application of hyperpolarizing prepulses were obtained in two additional fibers. Application of trains of hyperpolarizing prepulses but without the subsequent depolarizing voltage steps (to activate the Na^+ current) resulted in a rise of a^i_{Na} of no more than 0.3 mM (n=2).

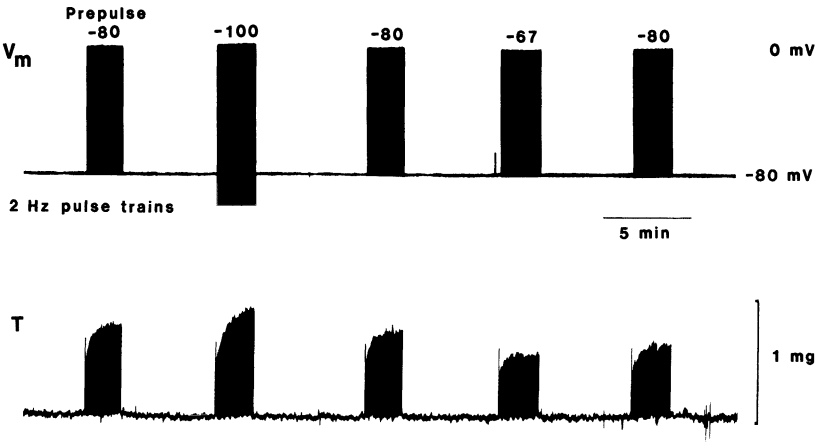

Figure 5. Effect on the positive tension staircase of including prepulses in the voltage clamp pulse trains. Five trains were applied (V_h = -80 mV). As indicated above each pulse train, for 50 msec preceding each depolarizing step to 0 mV, the prepulse voltage was stepped to -100 mV or -67 mV in order to alter voltage-dependent inactivation of Na^+ current, or the voltage was maintained at -80 mV.

Protocols similar to those used to manipulate voltage-dependent inactivation of the Na^+ current and a^i_{Na} also were studied during twitch tension recordings. Figure 5 shows records from one experiment and illustrates the effect of the prepulses. Five 2 and 1/2 minute long voltage clamp pulse trains and the resulting twitch tension responses are shown. With the first pulse train (control, prepulse voltage -80 mV), the rest contraction amplitude was 0.60 mg. This was followed by the development of a positive tension staircase with twitch tension gradually rising from a minimum of 0.45 mg to 0.72 mg. After a five minute rest period, the second pulse train was applied. This pulse train differed from the control pulse train only in that the voltage during each prepulse was -100 mV, a condition previously shown to augment the frequency-dependent rise in a^i_{Na}. The rest contraction amplitude was 0.60 mg

which was unchanged from control. This was followed by the development of a larger positive tension staircase which increased from a minimum value of 0.45 mg to 0.88 mg. The third pulse train was identical to the first, and the twitch tension response returned to the control level. The rest contraction amplitude was 0.60 mg. and was followed by a positive tension staircase with the amplitude increasing from a minimum of 0.45 mg to 0.70 mg. With the fourth pulse train, the prepulses were to -67 mV in order to increase voltage inactivation of Na^+ channels and blunt the frequency-dependent rise in a_{Na}^i. At the end of the rest interval, a brief train of prepulses to -67 mV was applied without the subsequent voltage steps to 0 mV. This brief train of subthreshold prepulses failed to elicit a mechanical response and did not trigger Na^+ current. When the fourth 2 and 1/2 minute long pulse train was applied, the rest contraction amplitude was 0.58 mg. A positive tension staircase followed but was of reduced amplitude, increasing from a minimum of 0.38 mg to 0.50 mg. The final pulse train shows the return to the control conditions (prepulse voltage -80 mV). The rest contraction amplitude was 0.58 mg and the positive tension staircase returned nearly to the control level. Similar findings were found in two additional fibers.

Effect of TTX on the Force-Frequency Relation. The role of frequency-dependent changes in a_{Na}^i in the force-frequency relation was studied. In Figure 6 are shown twitch tension records from one experiment. Initially, depolarizing voltage clamp steps were applied at 0.1 Hz and elicited twitches of 2.6 mg amplitude. The frequency was then increased every 2 to 2 1/2 minutes to 0.5, 1, 2, and 3 Hz. After increasing the depolarization frequency to 0.5 Hz, twitch tension amplitude fell to 1.9 mg and was constant. Increasing the frequency to 1 Hz resulted in a slight initial decrease in twitch tension amplitude which was followed by a small positive tension staircase with twitch tension amplitude rising to 2.0 mg. Increasing the frequency to 2 Hz resulted in a positive tension staircase and a further increase in twitch tension amplitude to 3.0 mg. At 3 Hz, a slight initial fall in twitch tension amplitude was followed by a positive tension staircase and a peak twitch tension amplitude of 3.0 mg. The frequency was then returned to 0.1 Hz which resulted in a prominent negative staircase with the twitch tension amplitude gradually returning to the control level. The twitch tension amplitude was measured at the end of each period at a constant depolarization frequency when twitch tension amplitude was nearly at steady-state conditions, and is plotted as the control force-frequency curve (O) in Figure 6. This curve shows the typical shape found in 11 sheep Purkinje fibers and illustrates the progressive increase in steady-state twitch tension amplitude found with increasing the frequency from 0.5 to 3 Hz. The record also shows an increase in twitch tension amplitude that is present at low frequencies, below about 0.2 Hz.

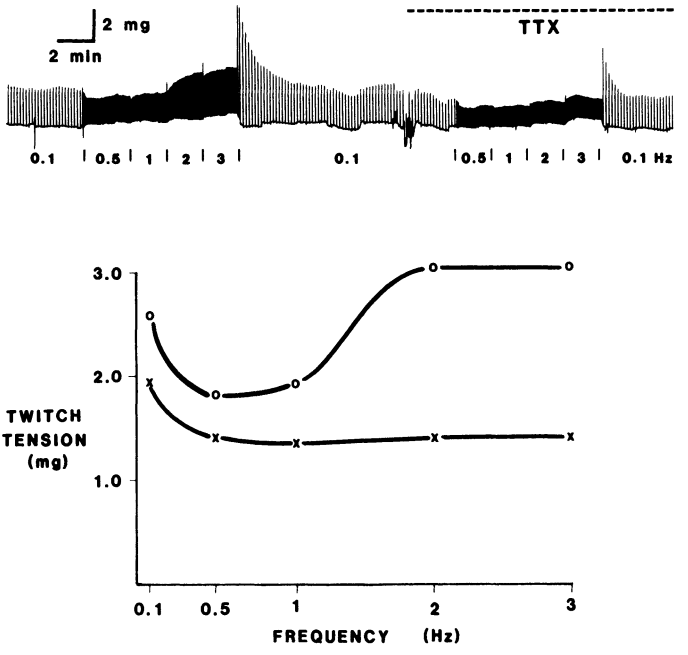

Figure 6. The force-frequency relationship and the effect of TTX (1 x 10^{-5} M, dashed line). The continuous twitch tension record is shown above the plots of the force-frequency relationships during control (O) and TTX (X) exposure.

TTX (1 x 10^{-5} M, see Fig 6) was then added to the perfusate at a concentration of and the voltage clamp protocol was repeated. Following the addition of TTX, there was a gradual decline of twitch tension amplitude from 2.6 mg to 2.0 mg. When the frequency at which voltage clamp steps were applied was increased to 0.5 Hz, twitch tension amplitude declined to 1.4 mg. Increasing the frequency to 1 Hz caused a slight fall in the twitch tension amplitude to 1.35 mg which was constant. With further increases in pulse frequency to 2 and 3 Hz, TTX block of the positive tension staircase resulted in no change in twitch tension amplitude. As with the control force-frequency curve, the twitch tension amplitude was measured at the end of each period at a constant depolarization frequency and is plotted as the TTX force-frequency curve (X) at the bottom of Figure 6.

Effect of TTX on the Negative Tension Staircase. After returning from a high to a low depolarization frequency, a negative tension staircase is elicited, as shown in Figure 6. Twitch tension amplitude was measured during the decline of the negative tension staircase to the steady-state level. Using nonlinear least-squares regression analysis, the data could be fit with a single exponential decay having time constants

of decay of about one minute. When the force-frequency protocol was repeated in the presence of TTX, a negative tension staircase was again present but it was of reduced amplitude and declined more rapidly to a steady-state. The decline in contraction after TTX could be fit with single exponentials having time constants half of those of control. This difference in the rate of decay may suggest that the negative tension staircase remaining in the presence of TTX represents a component of twitch tension not dependent on the frequency induced change in a_{Na}^{i}.

DISCUSSION

Na$^+$ Channels and Stimulation Dependence of a_{Na}^{i}. In cardiac muscle the repetitive stimulation of action potentials leads to a frequency- and time-dependent rise in a_{Na}^{i} that, after termination of stimulation, gradually declines to baseline. Several lines of evidence suggest that Na$^+$ channels are involved in regulation of the frequency-dependence of a_{Na}^{i}. The magnitude of the rise in a_{Na}^{i} can be decreased by intermediate concentrations of the Na$^+$ channel blocking agent TTX (4 x 10^{-6} M, ref 10). With higher concentrations of TTX (1 x 10^{-5} M) which nearly completely block the Na$^+$ current (26), the frequency-dependent rise in a_{Na}^{i} was virtually abolished as was shown in Figure 1. The magnitude of the frequency-dependent rise in a_{Na}^{i} also is dependent upon the membrane potential from which depolarizing pulses are initiated. When compared to trains of pulses initiated from a V_h of -80 mV, pulse trains initiated from a more negative membrane potential elicited a larger rise in a_{Na}^{i}, whereas virtually no change in a_{Na}^{i} is present with pulse trains elicited from a membrane potential of -50 mV (10). The dependence upon voltage of the magnitude of the stimulation induced rise in a_{Na}^{i} is similar to the voltage-dependence of Na$^+$ current in cardiac tissue (22,27,28) and suggests a role for the Na$^+$ current steady-state inactivation relation in the frequency-dependent regulation of a_{Na}^{i}. Falk and Cohen (29) studied the electrogenic current attributed to the Na-K pump current following periods of rapid drive from different V_h and during exposure to TTX. They also concluded that Na$^+$ current was important in Na$^+$ loading of the cell interior, but identified another component of Na$^+$ loading which they attributed to Ca^{2+} current and Na-Ca exchange. Similar conclusions were drawn by Brill et al (30).

a_{Na}^{i} and the Positive Tension Staircase. The mechanisms underlying the positive tension staircase have remained uncertain. In the present study, the application of trains of depolarizing voltage clamp pulses that resulted in a gradual rise in a_{Na}^{i} also increased twitch tension amplitude. When TTX was applied, it abolished the stimulation dependent rise in a_{Na}^{i} and the positive tension staircase. TTX also decreased the amplitude of the rest contraction. This later finding is not unexpected since TTX has been shown to slightly reduce resting a_{Na}^{i} (8,10,31) which, because of the Na-Ca exchange mechanism, also might be expected to reduce a_{Ca}^{i} and Ca^{2+}

available for contraction. When voltage clamp pulse trains were applied from a V_h of -50 mV which voltage inactivates Na^+ channels and blocks the frequency-dependent rise in $a_{Na}^{\downarrow}$ (see 10), the positive tension staircase was again abolished and the amplitude of the rest contraction was reduced. Interpretation of these results, however, is complicated by the change in V_h. As previously noted, reducing V_h from -80 to -50 mV should voltage inactivate Na^+ current and produce a small decline in $a_{Na}^{\downarrow}$ to a new steady-state level. Because of the Na-Ca exchange mechanism, a decline in $a_{Ca}^{\downarrow}$ and Ca^{2+} available for contraction also may occur. However, voltage dependence of the Na-Ca exchange mechanism also may influence $a_{Ca}^{\downarrow}$ depending on its electrogenicity and proximity to its reversal potential. Thus, following a change in V_h, the influence of both $a_{Na}^{\downarrow}$ and membrane potential may contribute to the magnitude and direction of change in twitch tension. In addition, at a V_h of -50 mV partial voltage-dependent inactivation of the conventional L-type Ca^{2+} current can not be completely excluded (24,25) and could contribute to a reduction in twitch tension amplitude by reducing the trigger for internal Ca^{2+} release (32,33). Inactivation of T-type Ca^{2+} current also could contribute to changes in twitch amplitude (34).

Brief duration prepulses were added before each step to 0 mV in the voltage clamp pulse trains in order to modify voltage-dependent inactivation of Na^+ channels yet avoid the effects of prolonged changes in V_h. When the prepulses were to -67 mV, which should increase voltage inactivation of Na^+ channels and reduce the stimulation dependent rise in $a_{Na}^{\downarrow}$, the amplitude of the positive tension staircase was reduced. In contrast, larger positive tension staircases were elicited when hyperpolarizing prepulses were applied. Hyperpolarizing prepulses increase the availability of Na^+ channels and the frequency-dependent rise in $a_{Na}^{\downarrow}$. As shown in Figure 4, the inclusion of hyperpolarizing prepulses to -100 mV caused nearly a two-fold increase in the magnitude of the stimulation dependent rise in $a_{Na}^{\downarrow}$.

The Negative Tension Staircase: Evidence for a non-$a_{Na}^{\downarrow}$ Dependent Tension Component. Upon returning from a high frequency of stimulation to a low frequency of stimulation, $a_{Na}^{\downarrow}$ decreases (Figs 1,4) and the amplitude of twitch tension gradually declines to a baseline level (Fig 6). Whereas TTX blocked almost completely the positive tension staircase, only part of the negative tension staircase was blocked. The residual component of negative tension staircase differed from the control staircase not only in that the amplitude was reduced, but also the time constant of decay was less than one-half of the control negative tension staircase. The component of tension remaining in the presence of TTX is not likely to result from an unblocked fraction of Na^+ channels producing a lesser rise in $a_{Na}^{\downarrow}$ because at the concentration of TTX used, block of Na^+ current should be nearly complete (26) and shortening of the decay time constant would not be expected (6). Thus, under these experimental conditions the negative tension staircase can be separated into two

components; one that decays rapidly and one that decays more slowly. The slower decaying component is likely to represent twitch tension dependent upon the gradual decay of a_{Na}^{i} to baseline. Its time course of decay is similar to that previously found for the decay of a_{Na}^{i} (6,10) or the electrogenic Na-K pump current (29) following periods of rapid drive. The mechanism of the faster decaying component is less certain but it may represent enhanced availability of Ca^{2+} for contraction at low frequencies of stimulation and could result from loading of the cell during the higher frequency stimulation with Ca^{2+} by mechanisms such as voltage-dependent Ca^{2+} channels or the Na-Ca exchange mechanism.

The Force-Frequency Relation. In most cardiac tissues increasing the frequency of stimulation over a wide range causes a positive tension staircase leading to heightened steady-state twitch tension and a positive force-frequency relation. Several cellular mechanisms could account for this including, 1) increased Ca^{2+} loading caused by elevated a_{Na}^{i} and Na dependence of the Na-Ca exchange mechanism, 2) increased Ca^{2+} resulting from voltage-dependent loading of the cell by Na-Ca exchange mechanism, and, 3) increased Ca^{2+} loading of the cell resulting from the repetitive activation of voltage-dependent Ca^{2+} channels. These experiments show that the increase in twitch tension amplitude with increasing voltage clamp pulse train frequency could be almost completely eliminated by TTX. Since TTX block of Na^{+} channels also blocks the frequency-dependent rise in a_{Na}^{i}, this strongly suggests that frequency-dependent accumulation of Na^{+} in cells, and its effect on Ca^{2+} via the Na-Ca exchange mechanism, is a major component of the force-frequency relation in heart muscle. Particularly at higher frequencies, Ca^{2+} loading also might occur by voltage dependence of the Na-Ca exchange mechanism or by entry through the repetitive activation of Ca^{2+} channels.

Our results suggest that Na^{+} channels are an important component in the regulation of cardiac force-frequency behavior, and there are several implications for cardiac function from this work. Because the voltage inactivation relation is steep in the range of normal resting potentials (27,28), even small changes in membrane resting potential may have significant effects upon Na^{+} entry into heart cells and the resulting dependence of a_{Na}^{i} on frequency. Other properties of Na^{+} channels (e.g., activation, development of inactivation, recovery from inactivation, etc.) as well, may be important. In addition, agents such as antiarrhythmic drugs that bind to Na^{+} channels may alter the frequency-dependent accumulation of Na^{+} and modify the shape of the force-frequency relation.

SUMMARY

The role of Na^{+} channels and frequency-dependent changes in the intracellular sodium ion activity (a_{Na}^{i}) in the regulation of the cardiac force-frequency relation was

investigated in voltage clamped cardiac Purkinje fibers. Trains of voltage clamp pulses produced frequency-dependent increases in a_{Na}^{i} and positive tension staircases. These were abolished by the addition of TTX (1 x 10^{-6} M) to block Na^+ channels. Reducing the holding potential (V_h) to -50 mV in order to voltage-inactivate Na^+ channels also abolished the positive tension staircase. When voltage clamp pulse trains contained prepulses to different voltages to modify voltage-dependent inactivation of Na^+ channels, the amplitude of the positive staircase also was altered. From control (V_h = -80 mV, no prepulse), when the voltage clamp pulse trains contained hyperpolarizing prepulses to -100 mV the frequency-dependent rise in a_{Na}^{i} was increased, as was the amplitude of the positive tension staircase. When pulse trains contained prepulses to -67 mV, the positive tension staircase was present but was reduced in size below that of the control staircase. When a range of frequencies was studied (0.1 - 3 Hz) and TTX was applied, twitch tension amplitude was decreased at all frequencies but the major effect was to nearly completely eliminate the rise in twitch tension amplitude that normally occurs at higher stimulation frequencies. These results suggest that a central mechanism in the cardiac force-frequency relation is Na^+ entry through Na^+ channels leading to frequency-dependent loading of the cell interior with Na^+, and through Na^+ dependence of the Na-Ca exchange mechanism an increase in intracellular Ca^{2+} available for contraction.

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2

REGULATION OF CARDIAC CALCIUM CURRENT DURING SUPPRESSION OF SECOND MESSENGER INTRACELLULAR ENZYMATIC PATHWAYS

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INTRODUCTION

It is well established that membrane ionic channels in heart cells are up-regulated by enhanced activation of intracellular enzymatic cascades (1-3). However, less is known about channel regulation and modulation under conditions of depressed enzymatic activity such as might occur in the failing heart. In this study, we have developed protocols to suppress pertinent enzymatic activity in guinea pig ventricular myocytes, and examined the consequences on whole-cell calcium current (I_{Ca}) and its modulation.

I_{Ca} in mammalian cardiomyocytes is affected by three major enzymatic pathways. (a) I_{Ca} is enhanced by stimulation of adenyl cyclase leading to cAMP accumulation, activation of protein kinase A, and phosphorylation of Ca channels (2,4). This intracellular cascade is coupled to the β-adrenergic receptor by the guanosine nucleotide binding (G) transducer protein, G_s (5,4,6). (b) The second system involves a different transducer protein, G_i, which couples muscarinic and adenosine receptors to the adenyl cyclase pathway (5). Activation of G_i inhibits adenyl cyclase, and this is the mechanism whereby muscarinic agents and adenosine can inhibit the stimulatory action of β-adrenergic agents on I_{Ca} (7,8). (c) The third system involves a different membrane G-protein, G_p, that is coupled to a number of membrane receptors including those that bind muscarinic agonists (9,3). Activation of G_p stimulates the generation of diacylglycerol (DAG) and inositol trisphosphate (IP_3) (10). DAG increases the activity of Ca-activated phospholipid-sensitive protein kinase C, and this kinase can phosphorylate ionic channels (11). It

has been reported that protein kinase C can increase Ca channel current in neuronal cells (12), and possibly in cardiac ventricular cells as well (13). The other product of G_p activation, IP_3, may also act directly to stimulate Ca channel activity (14).

We have used single and dual tight-seal pipette methods to voltage-clamp and dialyse guinea pig ventricular myocytes. The solutions employed to dialyse the cells were formulated for a number of single or multiple purposes that included block of various intracellular enzymatic cascades, stimulation and inhibition of adenyl cyclase, stimulation and inhibition of G-proteins, and stimulation and inhibition of protein kinases. Extracellular agents used as probes included forskolin, isoproterenol and acetylcholine. The results indicate the importance of intact phosphorylation pathways for the maintenance of cardiac Ca channel activity, and the extent to which neurohumoral regulation is dependent on functional intracellular enzymatic pathways. They also suggest that the stimulatory regulatory protein G_s can have an action on Ca channels that is independent of the stimulation of adenyl cyclase.

METHODS

Guinea pigs weighing 250-500 g were anesthetized with pentobarbital sodium (30 mg/kg, i.p.). The heart was cannulated in situ, and sequentially perfused with Ca-free Tyrode solution, 50 μM Ca-Tyrode solution containing collagenase Type I (Sigma, St. Louis, MO, USA), and a high K, low Na solution ("KB": 15). The ventricles were cut into chunks and stored in KB solution at room temperature prior to the experiments.

During the experiments, cells were superfused with either (a) modified Tyrode solution containing (mM) NaCl 120, KCl 4, $CaCl_2$ 3.6, $MgCl_2$ 2, glucose 10, and HEPES 5 (pH 7.4), (b) K-free Tyrode (K replaced by Cs) or (c) Na, K-free solution containing (mM) either Tris (OH) or tetramethylammonium Cl 120, and CsOH 4, $CaCl_2$ 3.6, $MgCl_2$ 2, glucose 10, and HEPES 5 adjusted with HCl to pH 7.4.

Pipettes were filled with a minimum intracellular solution (MICS) of the following composition (mM): CsCl 80, CsOH 40, $MgCl_2$ 2, EGTA 10, and HEPES 10, adjusted with HCl to pH 7.4. Agents added to dialysates to test for stimulatory action on I_{Ca} included 1 mM GTP (guanosine 5′-triphosphate), 100 μM GTP-γ-S (guanosine-5′-0-(3-thiotriphosphate)), 100 μM GMP-PNP (5′-guanyl-imidodiphosphate), 100 μM GDP-β-S (guanosine 5′-0-(2-thiodiphosphate)), 10 μM IP_3 (inositol trisphosphate), and 1 μM TPA (12-0-tetradecanoylphorbol-13-acetate). Agents added to inhibit intracellular phosphorylation pathways included 5 mM AMP-PNP (5′-adenyl-imidodiphosphate), 0.1 mM R_p-cAMPs (R_p-adenosine 3′,5′-monothionophosphate), 50 μM DNP (2,4-dinitrophenol), 10 mM ADP-β-S (adenosine 5′-0-(2-thiodiphosphate)), and 100 μM cGMP (guanosine 3′,5′-cyclic monophosphate). Agents added to external solutions to test for stimulatory action on I_{Ca} included 1-5 μM forskolin, 0.1 μM isoproterenol, and 10 μM acetylcholine. Forskolin was dissolved in ethanol and added to the external solution; the final ethanol concentration was <0.2% which, by itself, had no effect on I_{Ca}. Isoproterenol and acetylcholine were added from 1 mM stock solutions (isoproterenol solution also contained 1 mM ascorbic acid). All agents were purchased from Sigma (St. Louis, MO, U.S.A. or Munich, F.R.G.) except R_p-cAMPs which was a gift from Dr. B. Jastorff (Universität Bremen).

Electrophysiological studies were conducted on myocytes transferred to the experimental chamber in an aliquot of the KB medium. After the myocytes had settled on the Perspex bottom of the chamber positioned atop an inverted microscope stage, the chamber was perfused with modified Tyrode solution heated to 34±1°C. Heat-polished pipettes were prepared from thick-walled borosilicate glass capillaries (Jencons, Bedfordshire, UK) according to Hamill et al. (16). They had an inside tip diameter of 2-4 μm, and a resistance of 1-3 MΩ when filled with MICS. After establishing a giga-ohm seal by suction, the membrane patch under the pipette tip was disrupted by additional suction. In single-

pipette experiments, a pipette (P#1) was connected to a patch-clamp amplifier (EPC-7, List Medical Electronic, Darmstadt, FRG). In dual-pipette experiments, a second pipette (P#2) was connected to a voltage-follower of the type described by Dreyer and Peper (17). When both pipettes were in the whole-cell configuration, a positive pressure of 15-30 cm H_2O was applied to the back side of P#2, and a similar negative pressure to P#1, to facilitate cell dialysis (18).

Currents elicited by depolarizing clamp steps were displayed on a storage oscilloscope (Tektronix 5113) after analog compensation of leak current (19). Current and voltages were recorded on an FM tape recorder (Hewlett Packard 3964), and replayed for analysis and pen recorder output using a Nicolet digital oscilloscope (model 3091) and a Nicolet NIC MED-80 computer. The amplitude of I_{Ca} was measured as the difference between zero current and peak inward (or outward) I_{Ca}.

RESULTS

Fig. 1A shows the "rundown" of I_{Ca} in a single-pipette experiment when a guinea pig ventricular myocyte was dialysed with the ATP-free minimal intracellular solution (MICS) and pulsed from -60 to +10 mV for 160 ms at 0.5 Hz. In this experiment, and in most of the others detailed here, the time of initial patch breakthrough was referenced as 0 min, and replacement of external Tyrode by external Na, K-free solution began at the same time. Prior to complete exchange (within 1-2 min), overlapping Na current precluded accurate determination of I_{Ca} amplitude. The I_{Ca}-time plot in Fig. 1A shows that I_{Ca} declined from 1.6 nA at 2 min to 1 nA at 8 min and 0.8 nA at 16 min in this myocyte dialysed with MICS. Similar rundown occurred in dual-pipette experiments with MICS dialysates.

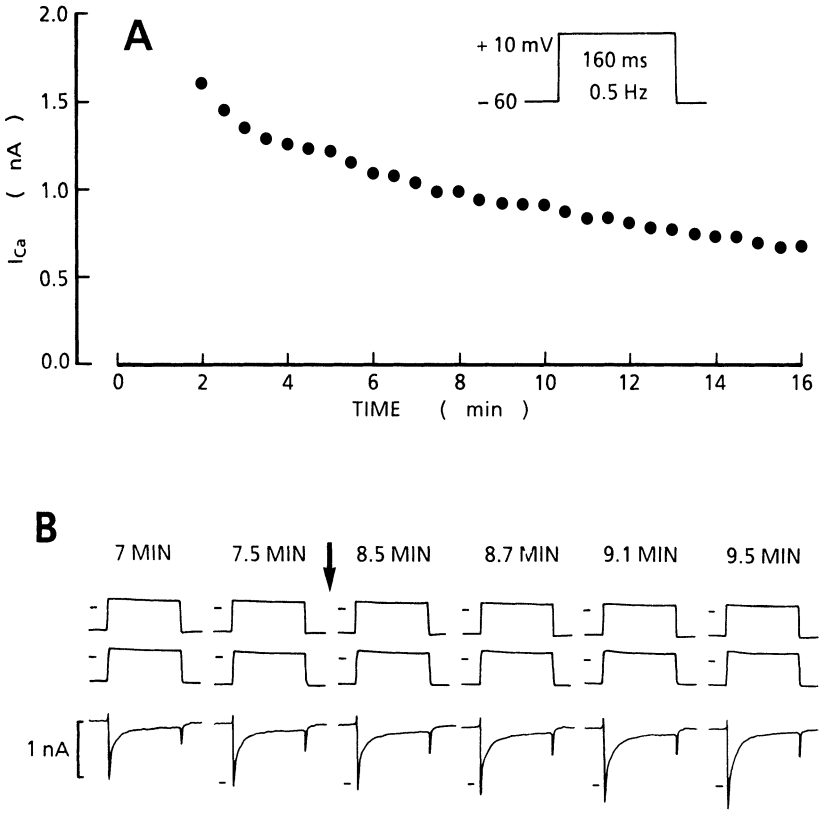

Figure 1. I_{Ca} in guinea pig ventricular myocytes dialysed with ATP-free MICS. The myocytes were pulsed from -60 to +10 mV for 160 ms at 0.5 Hz after patch breakthrough at 0 min. The external Tyrode solution was switched to Na, K-free solution at the same time, and measurements began at 2 min. (A) Rundown of I_{Ca} amplitude during a single-pipette experiment. (B) The effect of 100 μM cAMP during a dual-pipette experiment. The myocyte was dialysed for 7 min from the first pipette prior to dialysis with MICS + cAMP from the second pipette. Vertical arrow indicates onset of positive pressure on the latter solution. The tracings are (top to bottom) clamp command potential, membrane potential sensed by the second pipette, and membrane current. The horizontal bars on the potential traces indicate 0 mV. Note the increase in I_{Ca} (downward transient) during dialysis of cAMP-MICS (horizontal bar indicates pre-cAMP amplitude).

Since channel phosphorylation is a major determinant of the availability of I_{Ca} (1,2), it is likely that the rundown of I_{Ca} in Fig. 1A was at least in part related to compromised channel phosphorylation due to low intracellular ATP concentration caused by cell dialysis with ATP-free MICS. If so, one would expect that stimulation of the channel phosphorylation pathway at reaction steps preceding ATP-dependent kinase action on the channels, might not be as effective during MICS dialysis as during dialysis with ATP-containing solutions. We examined this supposition by measuring I_{Ca} in myocytes that were dialysed with 100 μM cAMP to stimulate the phosphorylation pathway. Under control conditions (ATP-containing dialysate), the inclusion of cAMP increased I_{Ca} by about 3-fold (not shown, cf. ref. 4). In the test (dual-pipette) experiments, myocytes were dialysed with MICS solution from the first pipette for 5-8 min. The records in Fig. 1B indicate that the stimulatory action of cAMP is much reduced under these conditions. In fact, the increase in I_{Ca} produced by cAMP was less than 2-fold in each of 4 experiments.

In a large number of other experiments, myocytes were dialysed with MICS or with one of three "PI-MICS" dialysates (MICS containing agents known to inhibit intracellular phosphorylation pathways). The first of these, PI(1)-MICS, contained 5 mM AMP-PNP and 0.1 mM R_p-cAMPs. AMP-PNP is a non-hydrolysable ATP analogue (20) that binds to the active site of protein kinase A and produces dead-end inhibition by formation of an unproductive enzyme-AMP-PNP complex (21); R_p-cAMPs binds to protein kinase A and prevents activation by cAMP (22). PI(2)-MICS contained 5 mM AMP-PNP, and 50 μM DNP to block endogenous ATP production (23). PI(3)-MICS contained 5 mM AMP-PNP, 50 μM DNP, 10 mM ADP-β-S to inhibit adenylate cyclase activity (cf. ref. 24), and 100 μM cGMP to stimulate phosphodiesterase-mediated breakdown of cAMP (25). Fig. 2 summarizes how relative I_{Ca} amplitude (2 min value = 100%) changed with time in ventricular myocytes pulsed at 0.5 Hz and dialysed with MICS or one of the three PI-MICS

formulations. There was a considerable rundown of I_{Ca} in each group, but it was much more pronounced with the PI-MICS dialysates than with MICS dialysate. After 10 min dialysis, I_{Ca} ranged from 35 to 50% with PI-MICS dialysates compared to 62% with MICS.

The data in Fig. 2 indicate that the inclusion of phosphorylation pathway inhibitors aggravates the rundown of I_{Ca}. However, the experiments provided no indication of the extent to which the PI-MICS dialysates blocked the enzymatic pathways. One way of assessing the block of the adenyl cyclase/cAMP pathway was to test the response to external application of forskolin. This diterpene is a direct activator of adenyl cyclase (26), and markedly stimulates I_{Ca} in guinea pig ventricular cardiomyocytes by promoting Ca channel phosphorylation via cAMP-dependent protein kinase A (4).

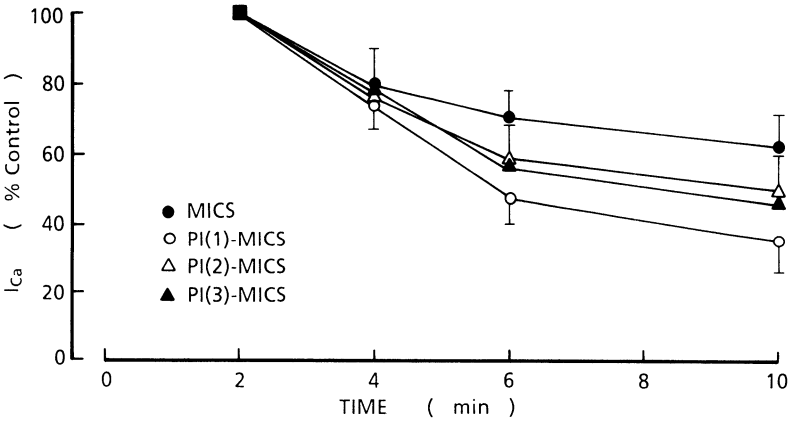

Figure 2. The rundown of I_{Ca} in myocytes dialysed with ATP-free MICS or with one of three MICS solutions containing phosphorylation pathway inhibitory agents (PI(1), PI(2), PI(3): see text for composition). In these single-pipette experiments, myocytes were pulsed from -60 to +10 mV for 160 ms at 0.5 Hz. Patch breakthrough was at 0 min, and the external solution was changed to Na, K-free solution at the same time. I_{Ca} amplitude is expressed as a percentage of the amplitude at 2 min post patch breakthrough. Mean ± SD, n = 3-8.

Fig. 3 shows the effect of 1 μM forskolin, a concentration that was maximally effective in augmenting I_{Ca} under control conditions (ATP-containing dialysates). When the drug was applied to myocytes dialysed with MICS containing 3 mM ATP (control) for 10-25 min, it caused an increase in I_{Ca} to about 210% of the pre-drug value (Fig. 3, left-most box).

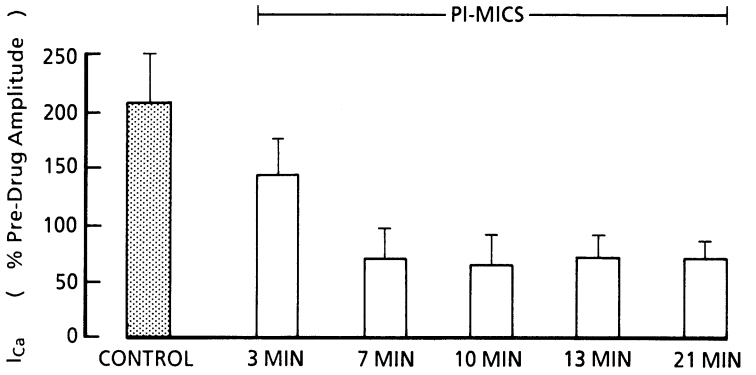

Figure 3. The availability of the adenyl cyclase system in myocytes dialysed with PI-MICS, as assessed with forskolin tests. In these single-pipette experiments, myocytes were superfused with Na, K-free solution and pulsed from -60 to +10 mV at 0.5 Hz. When myocytes were dialysed with ATP-containing MICS (control, left) for 10-25 min, forskolin (1-2 μM) produced a large stimulation of I_{Ca}. There was a much more subdued stimulation after short-term dialysis with PI-MICS, but no stimulation at all after dialysis lasting for 7 min or longer. I_{Ca} was measured after 4 min exposure to drug, and is expressed as percent of pre-drug amplitude. Vertical bars indicate SD, n = 3-4.

The response was first dampened and then completely abolished when myocytes were dialysed with PI(3)-MICS. When forskolin was applied after 3 min dialysis, I_{Ca} only increased to 140% of pre-drug value during the 4 min application. When the drug was applied after longer dialysis periods (7, 10, 13 and 21 min), there was no stimulation at all. I_{Ca} simply continued its rundown during the drug trials,

and this resulted in "responses" that were less than pre-drug 100% (Fig. 3, right hand boxes).

Agonist occupation of β-adrenergic receptors enhances the activation of the transducer protein G_s and, consequently, of adenyl cyclase and the cAMP-dependent phosphorylation pathway. Under normal conditions this leads to a robust increase in I_{Ca}, and we investigated whether this response was altered by dialysis with MICS and PI-MICS. Fig. 4 shows that 0.1 μM isoproterenol increased I_{Ca} to 280% of pre-drug amplitude when myocytes were dialysed with control ATP-containing solution. The stimulation was considerably weaker in myocytes dialysed with a PI-MICS. After 4 min PI-MICS, the stimulation was about 210%, and after 13 or 21 min it was about 140%. The blunting of the stimulation was somewhat less in myocytes dialysed with straight MICS; after 10-15 min dialysis, the stimulation with 0.1 μM isoproterenol was about the same as that observed after 4-6 min dialysis with PI-MICS (n = 3)(not shown).

Acetylcholine is a strong inhibitor of β-adrenergic mediated responses in cardiac cells (27), including the stimulation of I_{Ca} by isoproterenol (28,7,29). In cells dialysed with control solution, the external application of 10 μM acetylcholine had no effect on the amplitude of I_{Ca} (not shown). A similar outcome was obtained in myocytes dialysed with MICS (Fig. 5); 10 μM acetylcholine did not affect the course of I_{Ca} whether applied at 5 min (filled circles) or at 12 min (open circles). The MICS dialysate in the experiments detailed in Fig. 5 also contained 1 mM GTP to facilitate the activation of the two G-proteins coupled to muscarinic receptors, G_i and G_p. Thus, agonist-facilitated activation of G_i and G_p did not modify I_{Ca}.

It is well-established that it is the β-adrenergic agonist-facilitation of G_s activation that triggers the sequence of events leading to large stimulation of I_{Ca} (5,30,2). In theory, there are two ways in which activated G_s might act

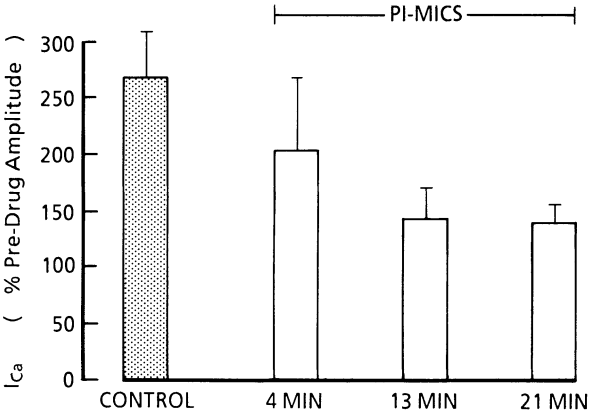

Figure 4. Effect of 0.1 μM isoproterenol on I_{Ca} in single-pipette experiments on guinea pig ventricular myocytes dialysed with either control ATP-containing solution or PI-MICS. The myocytes were bathed in Na, K-free solution, and pulsed from -60 to +10 mV for 160 ms at 0.5 Hz. The duration of the dialysis prior to the addition of isoproterenol is given below the boxes; the amplitude of I_{Ca} is expressed as a percentage of the pre-drug amplitude (mean ± SD, n = 3-4). The duration of the drug tests was 3-4 min for both control and PI-MICS experiments.

on Ca channels. The first is by an effector action on adenyl cyclase and the second by a direct action on the channels. As shown earlier, dialysis of guinea pig ventricular myocytes with PI-MICS results in complete suppression of the adenyl cyclase phosphorylation pathway as judged by forskolin's inability to stimulate I_{Ca}. However, under similar conditions, isoproterenol was still able to enhance I_{Ca}, albeit to a much smaller degree than was evident under control conditions. Provided that the adenyl cyclase system was as fully blocked in the isoproterenol-treated cells as in the forskolin-treated ones, the lingering stimulation by isoproterenol suggests that activated G_s may act directly on Ca channels.

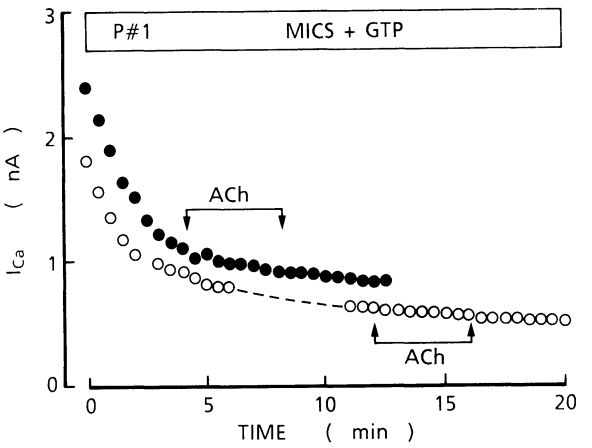

Figure 5. Stimulation of muscarinic receptors by 10 μM acetylcholine has no effect on I_{Ca} in myocytes dialysed with MICS. The two myocytes (open circles, filled circles) were superfused with Na, K-free solution and pulsed from -60 to +10 mV for 160 ms at 0.5 Hz. The MICS dialysate also contained 1 mM GTP.

As the first step in examining this hypothesis, myocytes dialysed with PI-MICS were first tested for their response to a maximally-effective concentration of forskolin, and subsequently for their response to 0.1 μM isoproterenol. Acetylcholine (10 μM) was added to the bathing solution for the duration of each test sequence (3-4 min forskolin, 1 min washout, 5-10 min isoproterenol). The purpose of the acetylcholine was to provide an additional brake on any possible stimulation of the adenyl cyclase system.

An example experiment is documented in Fig. 6. After 10 min dialysis with PI(2)-MICS that contained 1 mM GTP to facilitate receptor-mediated activation of G-proteins, 5 μM forskolin failed to stimulate I_{Ca}. However, the subsequent application of 0.1 μM isoproterenol clearly enhanced I_{Ca} amplitude. Similar results were obtained in two other myocytes subjected to this experimental protocol. Since the activation of G_i and G_p by the combined application of

external acetylcholine and internal GTP had no effect on I_{Ca} (Fig. 5), we could estimate the effect of G_s activation on whole-cell I_{Ca} by activating all three G-proteins with GTP-γ-S, a non-hydrolysable analogue of GTP. By carrying out this procedure at a time when the adenyl cyclase system was unavailable (see earlier forskolin tests), we could estimate the magnitude of any direct effect of activated G_s on I_{Ca}.

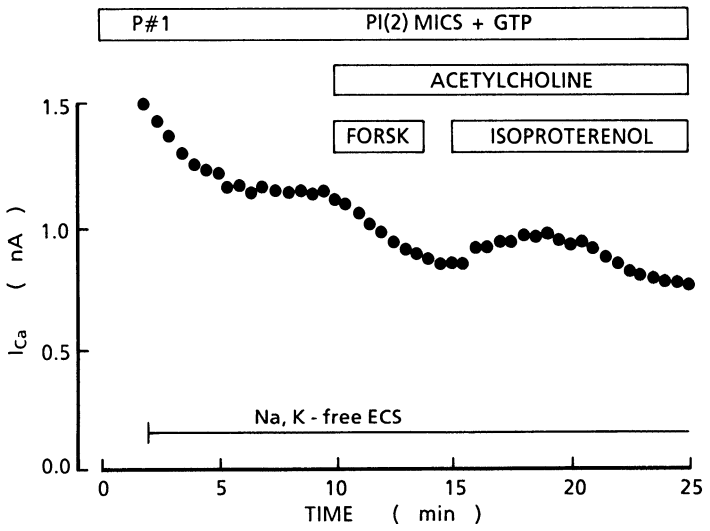

Figure 6. Small enhancement of I_{Ca} by 0.1 μM isoproterenol in a myocyte unresponsive to 5 μM forskolin after prolonged dialysis with PI(2)-MICS. Acetylcholine (10 μM) was present in the bathing solution (to suppress activation of adenyl cyclase), and the PI(2)-MICS contained 1 mM GTP. The myocyte was superfused with Na, K-free solution, and pulsed from -60 to +10 mV for 160 ms at 0.5 Hz.

The experiments were performed using the double-pipette method. A pre-dialysis with PI(1)-MICS from the first pipette was used to establish a block of the phosphorylation pathway, and the test was conducted by then switching to dialysis of PI(1)-MICS containing 100 μM GTP-γ-S from the second pipette.

The I_{Ca}-time plots in Fig. 7 begins after the 8-10 min pre-dialysis periods. In a control experiment (first pipette filled with PI(1)-MICS, and second pipette also filled with PI(1)-MICS), the downward trend in I_{Ca} was not greatly affected by dialysis from the second pipette (filled circles). However, when 100 μM GTP-γ-S was included in the dialysate of the second pipette, there was a short delay after the application of the pressure-assist, and then a quick 45% stimulation of I_{Ca} (open circles).

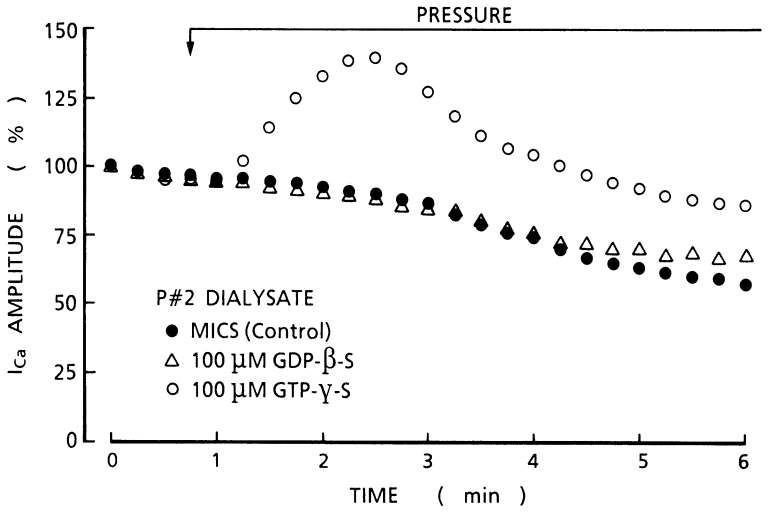

Figure 7. Stimulation of I_{Ca} by non-hydrolysable GTP analogue in myocytes voltage clamped and dialysed with the dual-pipette method. After 8-10 min pre-dialysis with PI(1)-MICS from the first pipette, dialysis with pressure-assist (arrow) was initiated from the second pipette. The second pipette contained PI(1)-MICS (control, filled circles), PI(1)-MICS plus 100 μM GDP-β-S (open triangles), or PI(1)-MICS plus 100 μM GTP-γ-S (open circles). The myocytes were superfused with Na, K-free solution and pulsed from -60 to +10 mV for 160 ms at 0.5 Hz. Zero time refers to time of patch breakthrough under the second pipette.

This effect was specific to GTP-γ-S, the non-hydrolysable analogue of GTP, since GDP-β-S, the non-hydrolysable analogue of GDP, was completely ineffective

when it was included at 100 μM concentration in the second pipette (open triangles). Although the dual-pipette experimental method has a very low success rate, we have nevertheless been able to verify each of these results in at least two other myocytes. The ineffectiveness of GDP-β-S makes it highly unlikely that the responses observed with GTP-γ-S were due to spurious formation of ATP-γ-S (i.e. formation of the latter could lead to Ca channel thiophosphorylation and stimulation of I_{Ca}). The reason is that a similar formation of ATP-γ-S might be expected from S contained in the GDP-β-S dialysate, and no stimulation was recorded with these dialysates. In addition, of course, the high concentrations of AMP-PNP and R_p-cAMPs that were present in the myocytes makes it highly unlikely that any ATP-γ-S formed could have been utilized by protein kinase.

DISCUSSION

Ca channel activity in dialysed mammalian ventricular cells is labile, and runs down unless there is adequate control of intracellular Ca concentration (e.g. 31). In the present study, 10 mM EGTA was included in the dialysate to buffer intracellular Ca but this proved to be an insufficient intervention, in the absence of ATP, to prevent marked rundown. When the latter condition (ATP-free MICS dialysate) was further modified to include phosphorylation pathway inhibitors, uncouplers of oxidative phosphorylation, and other inhibitors, the rate of I_{Ca} rundown was enhanced. Thus, there can be little doubt that active intracellular phosphorylation processes are a requirement for the maintenance of I_{Ca} in heart cells.

An additional consequence of an inhibition of intracellular phosphorylation pathways is a marked diminution of the response to agents that normally act by stimulating these enzyme systems. The enhancement of I_{Ca} by forskolin was reduced by nearly 50% in myocytes dialysed with MICS, and completely abolished in myocytes dialysed with PI-MICS for 7 min or more. I_{Ca} stimulation by

isoproterenol was also affected by dialysis with MICS and PI-MICS. However, unlike forskolin, the isoproterenol stimulation was reduced but not abolished after dialysis with PI-MICS.

The results with forskolin indicated that the phosphorylation pathway leading from adenyl cyclase was fully blocked after prolonged dialysis of myocytes with PI-MICS. However, the small stimulation of I_{Ca} by isoproterenol in forskolin-unresponsive cells suggests that the β-adrenergic agonist can act even when the adenyl cyclase cascade is blocked. The likely mediator of this action is the regulatory protein G_s. The Birnbaumer/Brown group has already postulated that activated G_s can cause a direct stimulation of Ca channels (24). In single Ca channel studies they found that activated G_s prolonged the survival time of channel activity in excised cardiac membrane patches (24), and enhanced the open-state probability of channels incorporated into lipid bilayers from cardiac membrane vesicles (32). In addition, the same group has shown that activated G_s increases the open-state probability of Ca channels incorporated into lipid bilayers from skeletal muscle T-tubular membrane (33).

In guinea pig ventricular myocytes dialysed with either MICS or PI-MICS, protocols designed to activate G_i and G_p (external acetylcholine, internal GTP) had no effect on I_{Ca}. This provided the opportunity to activate G_s, G_i and G_p with non-hydrolysable GTP analogues, and identify the outcome with the activation of G_s alone. In myocytes dialysed with PI-MICS, both GTP-γ-S and GMP-PNP stimulated I_{Ca}. The effect was specific since GDP-β-S was inactive. These results with non-hydrolysable GTP analogues complement those with isoproterenol in myocytes with blocked adenyl cyclase pathways in indicating a direct effect of activated G_s on cardiac Ca channels.

The physiological significance of a direct action of G_s on cardiac Ca channels is open to conjecture. As argued in a recent paper by Yatani and Brown (34), a direct action by G_s on Ca channels will have a much shorter latency to

effect than an indirect action via the multi-step adenyl cyclase/channel phosphorylation. In fact, the response time of the direct mechanism may be short enough to permit beat to beat changes in response to sympathetic nerve stimulation. There is an obvious counterpoint in the acetylcholine-sensitive K channel system in atrial cells (and probably in S-A and A-V nodal cells as well): these K channels are opened by a direct action of a G_i-like protein activated by agonist occupation of muscarinic receptors (cf. ref. 33). An additional role for the directly-acting G_s mechanism may be in the priming of Ca channels for further modification by other modulating agents.

The results in this study suggest that up-regulation by a direct action of G_s would become an increasingly important modulator of Ca channel activity when heart cells have a diminished capacity for up-regulation via the adenyl cyclase-cAMP-channel phosphorylation system. This situation may arise during cardiac dysfunction, and during periods of enhanced parasympathetic nervous activity.

SUMMARY

We have investigated the status of Ca channel activity in guinea pig ventricular myocytes dialysed with solutions designed to compromise enzymatic activity in pathways leading to channel phosphorylation. When myocytes were dialysed with an ATP-free, simple internal salt solution (MICS), there was significant rundown of I_{Ca} during relatively short (15-30 min) experiments with a single-pipette method. Rundown was more rapid when phosphorylation pathway inhibitors were added to MICS dialysate. Myocytes dialysed with the latter PI-MICS solutions quickly (<8 min) lost all responsiveness to forskolin, a drug that directly activates adenyl cyclase and normally enhances I_{Ca} by 2-3 fold in these myocytes. However, a significant (though reduced) increment in I_{Ca} was always elicited by isoproterenol in forskolin-unresponsive myocytes, indicating that this agonist has a mechanism of action that is independent of the adenyl cyclase/cAMP cascade. In dual-pipette experiments, the activation of the

stimulatory G-protein G_s by GTP-γ-S stimulated I_{Ca} in myocytes with blocked adenyl cyclase pathways. We conclude that the stimulation of I_{Ca} by isoproterenol in depressed myocytes is due to receptor-linked activation of G_s and a direct effect of the latter on Ca channels. This direct mechanism may be of importance in dysfunctional cells.

ACKNOWLEDGEMENTS

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3

HORMONAL AND NON-HORMONAL REGULATION OF Ca^{2+} CURRENT AND ADENYLATE CYCLASE IN CARDIAC CELLS

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INTRODUCTION

The influx of Ca^{2+} ions through transmembrane Ca^{2+} channels is fundamental in many aspects of cardiac function. Regulation of the heart beat by noradrenaline and acetylcholine (ACh) is in part mediated by the effects of these neurotransmitters on calcium current, I_{Ca} (1). β-adrenergic stimulation of I_{Ca} is mediated by a guanine-nucleotide binding protein, G_s (2), which triggers the activation of adenylate cyclase (AC) and in turn stimulates cAMP-dependent phosphorylation of Ca^{2+} channels (1,3). G_s has also been shown to directly activate Ca^{2+} channels (4). This latter mechanism, however, may play only a minor role in the physiological response to noradrenaline since the effects of β-adrenergic agonists on I_{Ca} were mimicked by external application of cAMP, its analogues or phosphodiesterase inhibitors (5), forskolin (6) [a direct activator of AC (7)], and by intracellular application of cAMP (8,9) or the catalytic subunit of cAMP-dependent protein kinase (PKA; ref. 8).

Inhibition of I_{Ca} by ACh takes place through a symmetrical but opposite mechanism to β-adrenergic stimulation (1). Binding of ACh to the muscarinic receptor activates a different guanine-nucleotide binding protein, G_i (2), which causes the inhibition of AC activity. Besides its effect on

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AC, ACh also stimulates cGMP production in heart (10-11) which may in turn reduce I_{Ca} through the activation of a phosphodiesterase (12-13) or a stimulation of a cGMP-dependent protein kinase (14).

It results that AC in cardiac cells is an essential target for the action of β-adrenergic and muscarinic agonists, as well as various hormones such as glucagon (15,16), VIP (16), secretin (16), etc.. Because AC is the first step in the cascade of events leading to phosphorylation of Ca^{2+} channels, its activity primarily regulates the degree of phosphorylation of Ca^{2+} channels. For this reason, it is difficult to determine whether other systems "down-stream" (such as cAMP phosphodiesterases, phosphatases, PKA, etc.) also play a role in the regulation of I_{Ca} by various neurotransmitters and hormones known to affect AC. In the present study, we have measured I_{Ca} and AC activity on the same cardiac preparations, to determine whether the effects of neurotransmitters (β-adrenergic and muscarinic agonists), hormones (glucagon) and drugs (forskolin) on I_{Ca} could be simply explained by their effects on AC activity. Part of these results have been reported in previous papers (17,18).

METHODS

Isolation of single cells

Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) or rat (male Wistar, 200-250g) according to methods previously described (13,19).

Composition of solutions

Control "frog" external solution contained (mM): 88.4 NaCl; 20 CsCl; 23.8 $NaHCO_3$; 0.6 NaH_2PO_4; 1.8 $MgCl_2$; 1.8 $CaCl_2$; 5 D-glucose; 5 Sodium pyruvate; $3x10^{-4}$ TTX; pH 7.4 maintained with 95% O_2, 5% CO_2. Control "rat" external solution was obtained by adding 20 mM NaCl and 50 µM TTX to the above solution. External solution could be changed by positioning the cell at the end of one out of eight capillary tubes (internal diameter = 250 µm) from which solution was flowing by gravity (≈15 µl/min) (9). Patch-electrodes (0.8-1.6 MΩ) were filled with standard internal solution. The solution in the patch-electrode could be changed continuously by a perfusion system previously described (12,13). The "frog" standard internal solution (MIG142) contained (mM): 119.8

CsCl; 5 K_2EGTA; 4 $MgCl_2$; 5 Na_2CP; 3.1 Na_2ATP; 0.42 Na_2GTP; 0.062 $CaCl_2$ (pCa 8.5); 10 HEPES; pH 7.1 adjusted with KOH. The "rat" standard internal solution was obtained by adding 20 mM CsCl to the above solution.

Voltage-clamp protocols

In all experiments, holding potential was -80 mV. The cell was depolarized every 8 s from -80 mV to 0 mV for 200 ms to elicit I_{Ca}, except during current-voltage relationships when the cell was depolarized to various potentials ranging from -50 to +50 mV. I_{Ca} was measured as the difference between peak inward current and the current at the end of the 200-ms pulse (9,12,13). All electrophysiological experiments were done at room temperature (21-24°C).

Preparation of membrane fractions

Membrane fractions were prepared according to ref. (20), with minor modifications. All preparative procedures were carried out at 4°C. Hearts were removed from frog or rat and placed in 10 ml of a Tris-HCl buffer containing 50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5. Ventricles, free of blood vessels and connective tissue, were then placed in 2 ml of fresh buffer and lacerated with scissors. The cardiac tissue was then homogenized with a glass-Teflon homogenizer. Membranes were collected by centrifugation at 14000xg for 3 min, resuspended in buffer at 2-3 mg protein/ml, and stored in liquid nitrogen 1-4 days prior to adenylate cyclase assays. Protein was determined using Bio-Rad assay (Bio-Rad Laboratories Gmbh, München, F.R.G.).

Adenylate cyclase assay

Adenylate cyclase activity was measured according to ref. 21. The assay medium contained, in a final volume of 60 µl: 3 mM [α-^{32}P]ATP ($3x10^6$ cpm); 5 mM $MgCl_2$; 50 µM Na_2GTP; 1 mM EDTA; 1 mM cyclic [8-^{3}H]AMP (15000 cpm); 0.2 mM methylisobutylxanthine (IBMX); 25 mM HEPES; pH 7.6; an ATP regenerating system consisting of 25 mM Na_2CP and 1 mg/ml creatine phosphokinase, and 40 to 60 µg of membrane protein. Incubation, which was initiated by addition of the proteins, was performed during 20 min at 37°C and was terminated thereafter by a modification of the procedure of White (22). Results, obtained from triplicate determinations, are expressed in % of basal activity (0.22 ± 0.07 and 0.16 ± 0.05 nmole cAMP/mg prot/20 min at 37°C in frog and rat, respectively).

Drugs

Drugs used in these experiments were: crystalized porcine glucagon (Novo Research Institute, Denmark), (-)isoprenaline (Sigma Chemical, Missouri, U.S.A.), acetylcholine (Sigma), forskolin (Sigma), IBMX (Sigma), and cAMP (Sigma). [α-^{32}P]ATP (10-50 Ci/mmoles) and cyclic [8-^{3}H]AMP (20-30 Ci/mmoles) were obtained from Amersham International (Amersham, U.K.). Forskolin was prepared as a stock solution of 10 mM in

anhydrous ethanol, and an appropriate amount of ethanol was added to each solution so that the same concentration of ethanol was present in all solutions tested. Crystalized porcine glucagon was made as a 1 mM stock solution in 0.01 N acetic acid for electrophysiological experiments or in 0.01 N HCl for adenylate cyclase assay, and dilutions were made respectively in external solutions or in 40 mM Tris-HCl, pH 7.6, containing 0.1% BSA. It should be noted that frog glucagon is identical to porcine glucagon except for substitution of Thr^{29} by homologous amino acid Ser (23).

RESULTS

Acetylcholine inhibition of isoprenaline-elevated I_{Ca} and adenylate cyclase

As shown in Fig. 1 (see also ref. 9), exposure of a frog ventricular cell to the β-adrenergic agonist isoprenaline (Iso) strongly enhances I_{Ca} and addition of acetylcholine (ACh) strongly depresses the stimulatory action of Iso. The distinguishing feature of the otherwise classical experiment

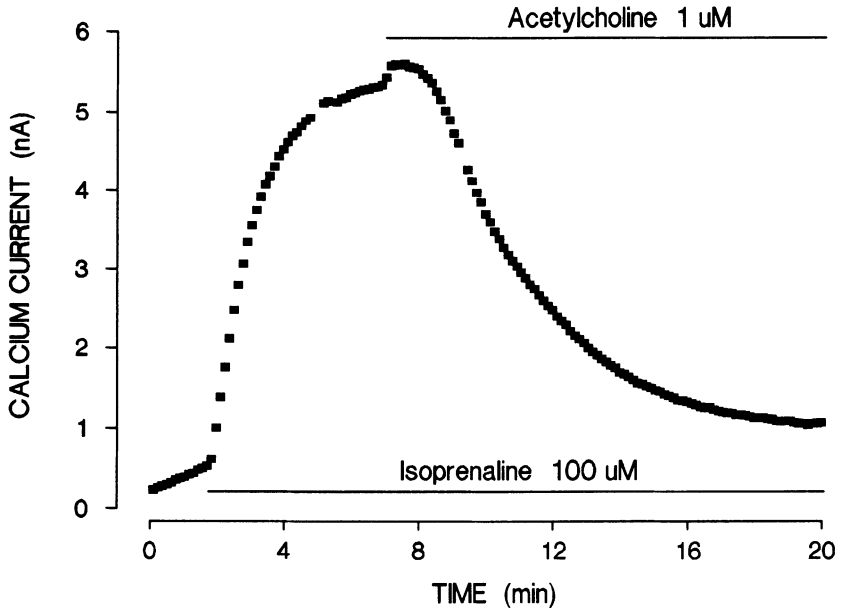

Figure 1. Inhibitory effect of acetylcholine (ACh) on isoprenaline (Iso)-stimulated I_{Ca}. I_{Ca} was recorded from an isolated frog ventricular cell as described in Methods. Each square represents net I_{Ca} recorded every 8 sec in response to 200 ms depolarizing pulses from -80 mV to 0 mV. The cell was initially superfused with control "frog" external solution. During the periods indicated, the cell was exposed to 100 μM Iso and then to Iso (100 μM) plus 1 μM ACh.

of Fig. 1 lies in the concentrations of Iso and ACh used. Only 1 µM ACh was capable of reducing, almost back to control level, I_{Ca} that had been elevated by 100 µM Iso, i.e. this is more than 10 times the Iso concentration required to produce maximal stimulation of I_{Ca}. This would tend to suggest that in frog, unlike in guinea pig ventricular cells (24), ACh antagonizes the effects of Iso in a non-competitive manner.

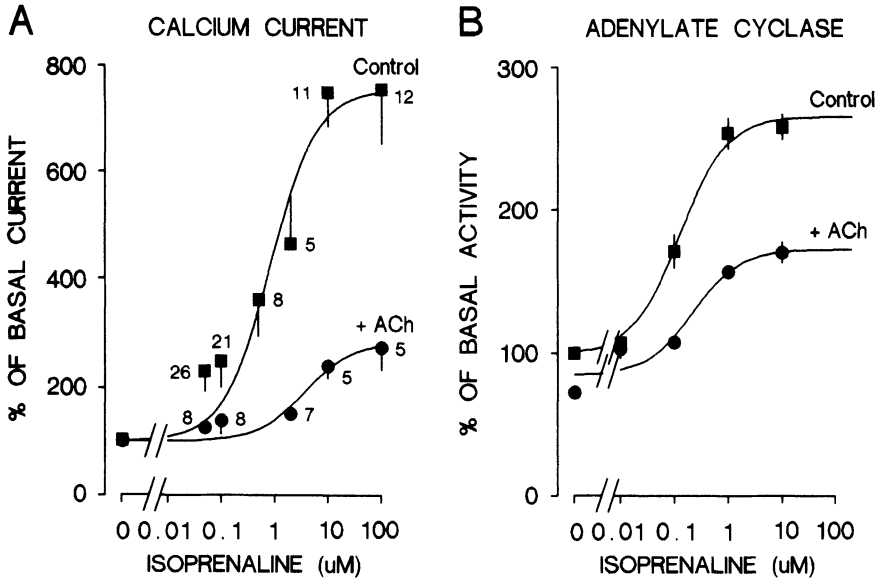

Figure 2. Dose-response curves for the effect of Iso on I_{Ca} (A) and AC activity (B) in the absence (squares) and presence (circles) of 10 µM ACh (modified from ref. 17). The continuous lines were derived from a non-linear least-mean-squares regression of the means to the Michaelis equation. The points in A) show the mean ± s.e.m. of the number of cells indicated near the symbols. The points in B) show the mean of triplicate determinations (for further details see Methods).

The capacity of ACh to reduce I_{Ca} even at maximal stimulation by Iso was further tested in a series of experiments where successively increasing concentrations of Iso were applied to frog ventricular cells in the presence or absence of 10 µM ACh. Fig. 2A summarizes the results of several such experiments. In the absence of ACh, Iso maximally stimulated I_{Ca} by 660% (E_{max}) on average, with half maximal effect occurring at ≈1 µM Iso (EC_{50}). 10 µM ACh, which did not, by itself, affect the basal level of I_{Ca} (see also refs.

9 & 24), strongly limited the stimulatory action of Iso. The inhibitory effects of ACh were characterized by a strong reduction of E_{max} from 660% to 185% stimulation (i.e. ≈70% reduction) and a slight increase in EC_{50} (from 1 to ≈4 µM). The effects of Iso and ACh on I_{Ca} were then compared with the effects of these compounds on adenylate cyclase (AC) activity measured in a membrane fraction of frog ventricle. Fig. 2B shows that Iso dose-dependently increased AC activity in frog heart (EC_{50}= 0.13 µM; E_{max}= 166%) and that the effects were strongly antagonized by ACh (10 µM). Here again, the main effect of ACh was a strong reduction of the maximal stimulation of AC by Iso (E_{max}=88%) accompanied by a small increase in EC_{50} (0.22 µM).

Acetylcholine inhibition of forskolin-elevated I_{Ca} and adenylate cyclase

Forskolin (Fo) has been shown recently to increase I_{Ca}, in frog ventricular cells, only when applied outside the cell

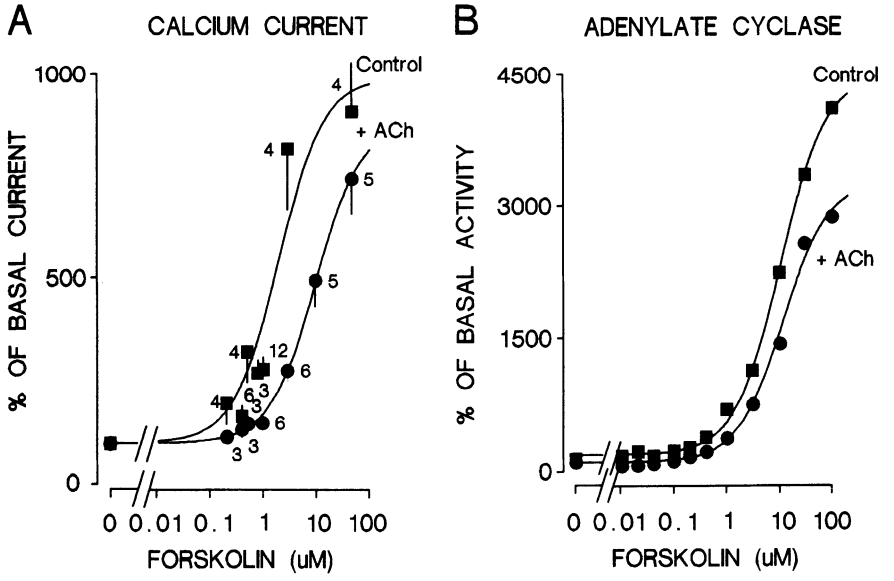

Figure 3. Dose-response curves for the effect of Fo on I_{Ca} (A) and AC activity (B) in the absence (squares) and presence (circles) of 10 µM ACh (modified from ref. 17). The continuous lines were derived from a non-linear least-mean-squares regression of the means to the Michaelis equation. The points in A) show the mean ± s.e.m. of the number of cells indicated near the symbols. The points in B) show the mean of triplicate determinations (for further details see Methods).

(6). Fo acts through a cAMP-dependent mechanism, in a manner qualitatively similar to Iso (6). Since activation of AC by Fo does not require the presence of a functional G_s (7,17), it was interesting to examine the stimulatory effects of Fo on AC and I_{Ca} with respect to the antagonistic effects of ACh. Fig. 3A shows that Fo dose-dependently increased I_{Ca} both in the absence and presence of 10 µM ACh. ACh antagonized the stimulatory effects of Fo in a different manner from those of Iso. Indeed, ACh only slightly reduced maximal stimulation achieved by Fo (from 890% to 780%) but significantly increased EC_{50} (from 2 to 10 µM). Therefore, the inhibitory effects of ACh on Fo-stimulated I_{Ca} appear to occur in an essentially competitive manner. We, then, investigated the modulation of AC activity by Fo and ACh. Fig. 3B shows that Fo strongly increased AC activity in a dose-dependent manner. The maximal stimulation of AC activity obtained with Fo was 10-15 times larger than that induced by a maximal concentration of Iso. ACh (10 µM) did not significantly modify the position of the dose-response curve (EC_{50} = 12 µM in ACh vs. 14 µM in control) but reduced E_{max} by ≈20% (from 2166% to 1790%). Therefore, the inhibitory effect of ACh on Fo-stimulated AC appeared essentially non-competitive as compared with the effects of ACh on Fo-stimulated I_{Ca}.

Regulation of I_{Ca} and adenylate cyclase by glucagon

Glucagon is known to produce positive chronotropic and inotropic effects in the heart (15,25). The cardiac effects of glucagon have often been correlated with a stimulation of AC activity (15,16,26). However, glucagon exerts physiological stimulatory effects also in cardiac preparations where it is ineffective in stimulating AC activity (16,27,28). Since the physiological effects of glucagon have been shown to depend on the presence of extracellular Ca^{2+} ions (29), we have investigated the effects of the peptide on I_{Ca} in frog and rat ventricular cells. Fig. 4 shows the effects of 1 and 10 µM glucagon on calcium current, I_{Ca}, recorded from a frog (Fig. 4A) or rat (Fig. 4B) ventricular cell. Glucagon increased I_{Ca} in both preparations in a reversible fashion. The effects of

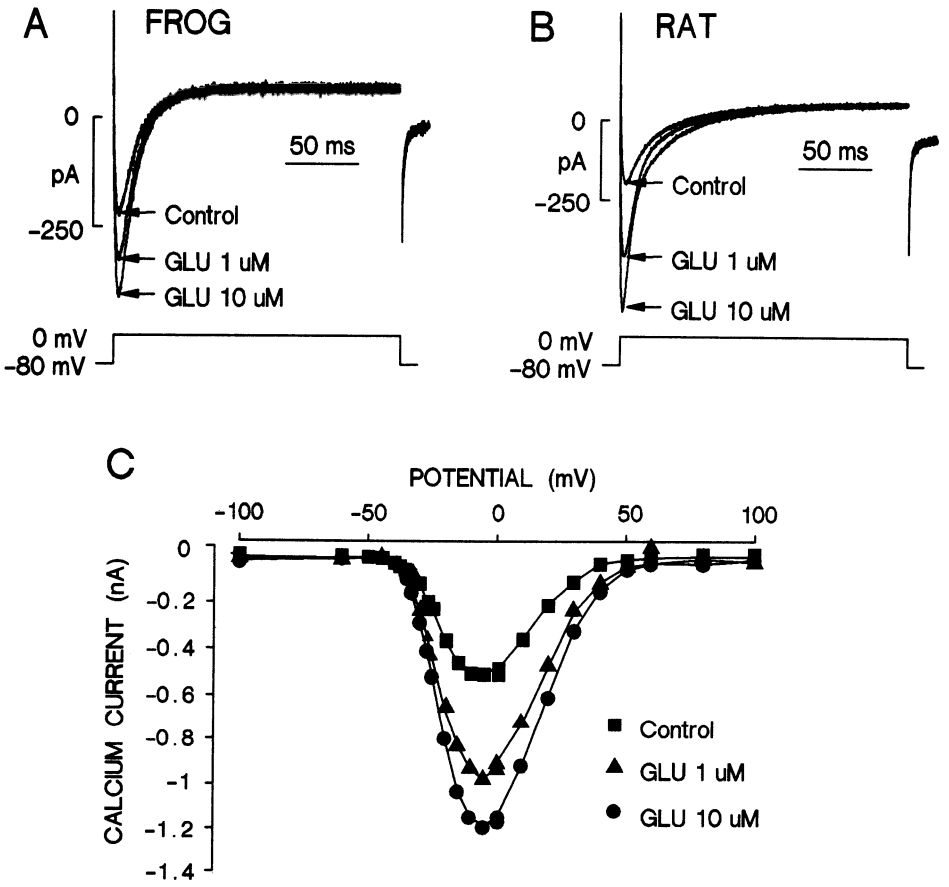

Figure 4. Effects of glucagon on I_{Ca}. A) & B) show individual current traces obtained in a frog (A) and a rat ventricular cell (B) in control external solutions, and after 5-6 min application of 1 and 10 μM glucagon (GLU). Current traces were digitized at 10 KHz (modified from ref. 18). C) shows current-voltage relationships for net I_{Ca} obtained in a frog ventricular cell under control conditions (squares), and in the presence of 1 μM (triangles) and 10 μM glucagon (circles).

glucagon on I_{Ca} were not voltage-dependent since the current-voltage relationships were not modified by the peptide either in frog (Fig. 4C) or in rat ventricular cells (data not shown).

Fig. 5 shows two experiments where successively increasing concentrations of glucagon were applied to a rat (Fig. 5A) or a frog ventricular cell (Fig. 5B). When the concentration of peptide was >0.1 μM, I_{Ca} increased in a dose-dependent manner

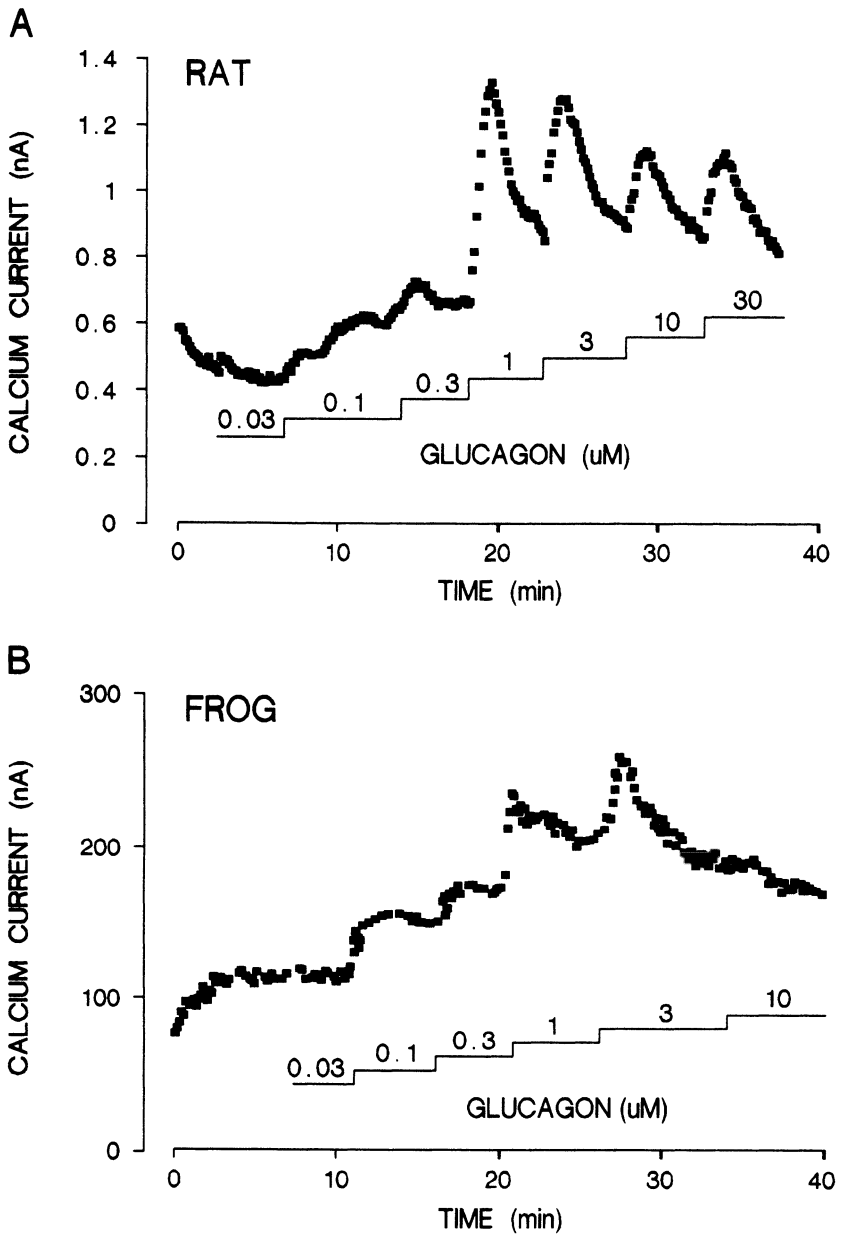

Figure 5. Effects of increasing concentrations of glucagon on I_{Ca} in a rat (A) and frog (B) ventricular cell. The cell was initially superfused with control "rat" (A) or "frog" (B) external solutions (see Methods). During the periods indicated, the cells were exposed to successively increasing concentrations of glucagon (30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM and/or 30 μM).

in both preparations. These experiments also illustrate that high concentrations of glucagon could produce a rapid

desensitization of the response of I_{Ca}, which was more pronounced in rat than in frog ventricular cells. Although a desensitization of the response of I_{Ca} to glucagon was not systematically observed in every cell, it should be noted, however, that the positive inotropic response to glucagon has also been shown to rapidly desensitize to the peptide (30). In frog, maximal stimulation of I_{Ca}, which was measured at 10 µM glucagon, averaged 42.1 ± 13.8% (mean ± s.e.m., n=7) and was much less than the stimulatory effects of Iso in the same cardiac preparation (see Fig. 2A). The stimulatory effect of glucagon on I_{Ca} was stronger in rat than in frog ventricular cells, where maximal stimulation of I_{Ca} induced by 30 µM glucagon (146.7 ± 38.7%, mean ± s.e.m., n=4) resembled the amplitude of the stimulation exerted by maximal concentrations (>50 nM) of Iso (i.e. 164.9 ± 29.8%, n=4). These results suggested a similarity of action between both agonists in rat, but not in frog ventricular cells.

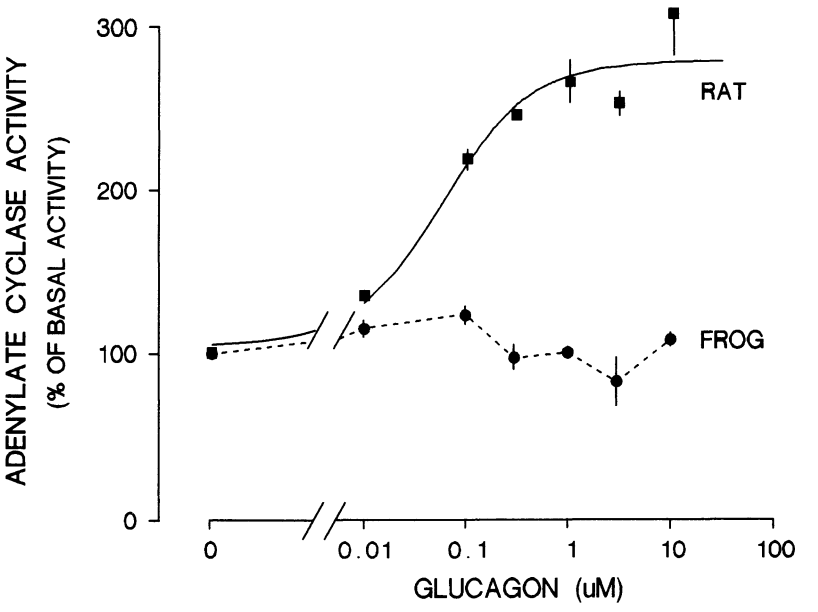

Figure 6. Dose-response curves of the effects of glucagon on adenylate cyclase activity measured in membrane fractions of rat (squares) and frog (circles) ventricles (modified from ref. 18). The points show the means of triplicate determinations. Values obtained in rat ventricle were fitted to the Michaelis equation (continuous line). EC_{50} and E_{max} were respectively 57 nM and 176% increase over basal activity (for further details, see Methods).

We have, therefore, investigated the effects of glucagon on AC by measuring AC activity in membrane fractions of rat and frog ventricles (Fig. 6). As already reported (15,16,26), we found that glucagon dose-dependently increased AC activity in rat ventricle. By contrast, we found that glucagon was unable to modify AC activity in frog ventricle (Fig. 6), while Iso dose-dependently enhanced AC activity in this preparation (Fig. 2B). Therefore, the stimulatory effect of glucagon on I_{Ca} in frog cardiac cells was unlikely to be due to activation of adenylate cyclase.

DISCUSSION

Comparing the regulation of Ca^{2+} current and adenylate cyclase activity in the same cardiac preparations brings some new insights into the mechanisms underlying the regulation of cardiac function by neurotransmitters, hormones and drugs. The present study allows us to draw three main conclusions.

1) The inhibitory effects of ACh on Iso-stimulated I_{Ca} resembled those observed on Iso-stimulated AC activity. This would suggest that ACh antagonizes β-adrenergic stimulation of I_{Ca} primarily by acting on cAMP synthesis, through an interaction between G_s and G_i subunits on AC (2,17). A difference exists, however, between the muscarinic inhibition of the two systems. While ACh was shown to be without effect on basal I_{Ca} (9,24), basal AC activity was reduced by ACh (Fig. 2B). One possible explanation for this discrepancy is that basal activity of AC is too low to induce a significant degree of phosphorylation of Ca^{2+} channels by cAMP-dependent protein kinase. Such assumption would imply that basal I_{Ca} is mainly due to non-phosphorylated Ca^{2+} channels, as already suggested (31).

2) The inhibitory effects of ACh on Fo-stimulated I_{Ca} were different from those observed on Fo-stimulated AC activity. Because the potency of Fo to increase AC activity was several times greater than that of Iso [as also observed for other stimulatory hormones in non-cardiac preparations (7)], AC activity stimulated by large concentrations of Fo in the

presence of 10 μM ACh remained much higher that AC activity stimulated by maximal concentrations of Iso in the absence of ACh (compare Fig. 2B and Fig. 3B). This makes it difficult to imagine that ACh antagonizes Fo-stimulated I_{Ca} by reducing the overall cAMP production. However, ACh could reduce cAMP production only in a small compartment (32) and, therefore, antagonize the physiological effects of Fo if only a small fraction of the cAMP produced in response to Fo was available for physiological processes (33). Such an hypothesis would be supported by the observation that, despite a considerably higher increase in cAMP production induced by Fo as compared to Iso (34; this study), the physiological responses induced by these agents in heart are equivalent [e.g. positive inotropic effect (35), cAMP-dependent protein phosphorylation (33), stimulation of I_{Ca} (6,24)].

ACh may also exert an additional action at a subsequent level in the cascade leading to phosphorylation of Ca^{2+} channels, which would provide an alternative mechanism to account for the differences in ACh inhibition of I_{Ca} and AC stimulated by Fo. The existence of such mechanism is supported by the fact that i) Fo activation of AC does not require activation of G_s (7,17), and ii) G_i subunits only weakly interact with AC in the absence of G_s activation (1,2,17). Therefore, this new mechanism would account for most of ACh inhibition of Fo-stimulated I_{Ca}, while ACh inhibition of Iso-stimulated I_{Ca} would take place mainly on AC (17). cGMP may participate in this mechanism, since ACh is known to stimulate cGMP production both in mammalian (10) and amphibian heart (11), and cGMP has been shown to strongly antagonize Iso-, cAMP- and Fo-stimulation of I_{Ca} (6,12,13) in frog ventricular cells by activation of a phosphodiesterase. If the turnover of cAMP hydrolysis was enhanced by cGMP upon ACh application, then a larger production of cAMP would be required to overcome this inhibition. Such a mechanism could explain the apparent competitivity observed in the inhibitory effect of ACh on Fo-elevated I_{Ca} (Fig. 3A).

3) Glucagon enhances I_{Ca} both in rat and frog ventricular cells, but stimulates AC activity in rat cells only. Whether

AC plays an essential role in the positive inotropic and chronotropic effects of glucagon has been a matter of debate (15,16,26-28,30). Here we found two cardiac preparations (frog and rat ventricles) where glucagon stimulates Ca^{2+} current. In rat, the effects of the peptide on I_{Ca} were qualitatively and quantitatively similar to the effects of isoprenaline. This suggested a similar mechanism of action for both agonists. This hypothesis was confirmed by measuring AC activity which was found to be enhanced by glucagon. In frog ventricular cells, however, the effects of glucagon on I_{Ca} were unlikely to be due to stimulation of AC activity since this system was found to be unaffected by the peptide. The exact mechanism of action of glucagon in frog heart remains to be elucidated. It seems clear, however, that glucagon has the ability to increase Ca^{2+} influx in some cardiac cells by a mechanism independent of adenylate cyclase activation.

SUMMARY

Ca^{2+} currents were measured in single cells isolated from frog and rat ventricles using the whole-cell patch-clamp technique. The dose-dependent stimulatory effects of isoprenaline (Iso), forskolin (Fo) and glucagon on I_{Ca} were determined in the presence or absence of acetylcholine (ACh), and were compared with the effects of these compounds on adenylate cyclase (AC) activity measured in membrane fractions from the same cardiac preparations. The inhibitory effects of ACh on Iso-elevated I_{Ca} were non-competitive and qualitatively resembled the effects of ACh on Iso-stimulated AC activity. The inhibitory effects of ACh on Fo-elevated I_{Ca}, however, occurred in an essentially competitive manner, and were different in nature from the effects of ACh on Fo-elevated AC activity. These results are discussed in terms of an ACh inhibition of AC and some additional action of ACh at a subsequent level in the cascade leading to phosphorylation of Ca^{2+} channels. Glucagon was found to increase I_{Ca} both in frog and rat ventricular cells, but enhanced AC activity in rat

ventricle only. Therefore, glucagon may also regulate I_{Ca} by a mechanism independent of adenylate cyclase.

ACKNOWLEDGEMENTS

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4

CALCIUM CURRENT IN NORMAL AND HYPERTROPHIED ISOLATED RAT VENTRICULAR MYOCYTES

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INTRODUCTION

Chronic overloading of the heart induces hypertrophy of the myocardium leading to structural, biochemical and functional alterations of the myocardial cells (1). Prolonged action potential duration is a general property of the hypertrophied hearts in different species (2,3,4). Action potentials (AP) recorded in cat papillary muscles, after pulmonary banding, shows marked alterations: a slow rate of rise associated with a slow conduction and an increase in duration (5). It was further described that AP prolongation and depressed plateau were more visible with larger hypertrophy and at moderate hypertrophy with higher stimulation frequency (6). Similar changes were reported in the hypertrophied right ventricle of the rabbit (7). In the left ventricle of the rat, prolonged AP (9) were also observed whatever hypertrophy was induced following renal hypertension (8) or abdominal aortic stenosis (9). Experimental hypertrophy is more marked in the epicardium than in the endocardium in the Goldblatt rats (10). This is associated with more durable AP in the ventricular trabeculum than in the papillary muscle (11). Among the many experimental conditions, only volumetric overloading which can induce up to 80% hypertrophy was not associated with prolonged AP (9). Natural hypertrophy, as during genetic cardiomyopathy,

also shows an increase in AP duration both in the Syrian hamster (12) and the human (13).

Prolongation of action potential duration has also been described in cardiomyocytes isolated from hypertrophied hearts (14-16). This provides good evidence that AP alterations are consequent to changes in the membrane properties of individual cells. Changes in syncytial interactions or in the external ionic environment following alterations in the extracellular spaces by accumulation of collagen during cardiac hypertrophy are thus not essential determinant of these AP alterations. These studies also show that the dissociation procedures did not destroy or alter the properties of the hypertrophied cells.

Action potential time course is under the control of more than 10 conductances. Alterations in the Ca conductance, both in its amplitude and kinetics (2,11,15,17) and in the late K outward conductance were reported (6,15). The present study, using the whole cell patch-clamp techniques, compared the characteristics of the pure Ca current, I_{Ca}, in single rat left ventricular cells isolated from normal and hypertrophied heart after aortic stenosis. Furthermore, the effects of β-adrenergic stimulation have been investigated in the two cell types.

METHODS

Preparation and solution

Surgery was performed on male Wistar rats (180-200 g). Abdominal aortic stenosis was induced using Weck forceps. The rats were sacrified for experiments 4 to 5 weeks after surgery. Myocytes were isolated according to the method of Wittenberg et al. (18). They were suspended in tissue culture Petri dish at

37°C and were used within 10 hours after isolation. The cells in a tissue culture dish were superfused by gravity with solution containing (mM): 20 CsCl, 117 NaCl, 1.8 $CaCl_2$, 1.7 $MgCl_2$, 1.5 NaH_2PO_4, 4.4 $NaHCO_3$, 10 glucose, 10 HEPES, pH 7.4 at 20°C bubbled with 100% O_2; 50 µM tetrodotoxin was added to block the Na current. The internal solution in the patch electrode (0.6-1.5 MΩ) contained (mM): 120 CsCl, 4 $MgCl_2$, 3 Na_2ATP, 5 Na_2-creatine phosphate, 0.4 Na_2GTP, 5 K_2EGTA, 0.062 $CaCl_2$ ($[Ca_i]$free = 10^{-8}M), 10 HEPES adjusted to pH 7.2 with KOH. During action potential recordings, external and internal CsCl was replaced respectively by 5.4 and 120 KCl.

Experimental arrangements and data analysis

The methods used for experiments with whole cell patch-clamp and for the analysis of data were derived from those developped on single frog cells (19). For monitoring I_{Ca}, the ventricular cell was depolarized from -70 mV holding potential to 0 mV for 200 ms every 4 s with a patch-clamp amplifier. To determine the membrane capacitance, pulses from -81 mV to -79 mV were applied to the cell. The exponential components of the decaying current were determined and analysed by a program which uses the Padé-Laplace method (20,21). Two time constants were obtained which correspond to the electrode (≤0.02 ms) and the cell membrane (≤1ms). The capacity of the membrane was calculated according to the equation:

$$Cm = \Gamma_c . I_0 / \Delta E_m \, (1- (I_\infty / I_0)) \quad (22)$$

where Γ_c is the time constant of the membrane capacitance, I_0 is the maximum capacitance current value, ΔE_m is the amplitude of voltage step, and I_∞ is the amplitude of steady state

current. The kinetics of I_{Ca} were analysed using the computer program EXCALC (21). Statistical analysis was performed with the computer program STATGRAPH (STSC, Inc., Rockville, U.S.A.). Values are expressed as mean ± S.D.

RESULTS

Heart weight of stenosed animals selected for this study was significantly higher (1.50 ± 0.19 g, n = 19) than that of sham-operated animal (1.01 ± 0.16 g, n = 17), although the body weight of both animal types were similar. The average size of cells isolated from the left ventricle was 11298 ± 4043 μm^2 (n = 32) in control and 16705 ± 4082 μm^2 (n = 27) in hypertrophied cells, assuming a cylindrical cell shape.

Action potentials

Resting potential and AP amplitude were not modified. However, repolarisation was clearly prolonged during hypertrophy and more particularly during the plateau phase (Fig. 1).

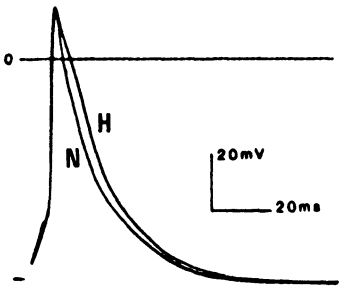


Fig.1: Superimposed action potentials recorded in single left ventricular cells isolated from normal (N) and hypertrophied (H) adult rat hearts. APD_{25} was 5.9 ± 1.2 and 12.3 ± 3.1 ms; APD_{75} was 22.2 ± 5.2 and 33.1 ± 6.9 ms for normal and hypertrophied cells respectively (n = 7, $p < 0.05$).

Ca current characteristics

The Ca current, I_{Ca}, in the left ventricular cells was estimated either as the difference between peak inward current and the current at the end of the 200 ms pulse or the current after addition of 2 mM cadmium (Fig.2A). The current-voltage relationships established on control and hypertrophied cells are shown in Fig.2B. Both display the same voltage dependence. From an holding potential of -70 mV, increasing depolarizations above -40 mV induced currents which increased smoothly to reach maximal activation around 0 mV typical of a L-type Ca current. There was no indication of a T-type Ca current.

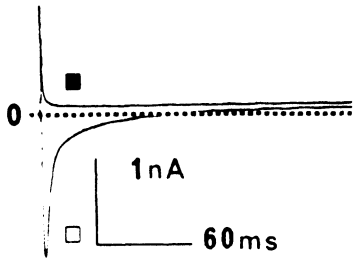
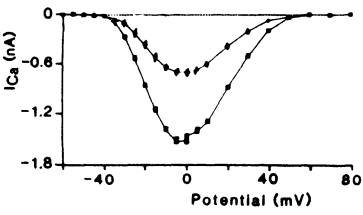


Fig.2: A) Currents elicited by depolarization from -70 mV to 0 mV in control solution (□) and in the presence of Cd^{2+} (■).



B) Current-voltage relationships established for the pure Ca current elicited by 200 ms depolarizations in normal (◆) and in hypertrophied cells (■).

The mean values of membrane capacity, calcium current amplitude and density obtained under similar conditions in

cells isolated from normal and hypertrophied rat heart are given in Table 1. The increase in membrane capacity (+78%) is in agreement with the hypertrophy of myocytes estimated from cell dimensions. I_{Ca} amplitude of hypertrophied cells was also significantly larger (+88%). Membrane capacity (Cm) and current amplitude increased in the same range; thus, the current density expressed by the ratio I_{Ca}/Cm was similar in both cell types.

Table 1: Density of peak I_{Ca} in isolated rat cells from normal and hypertrophied hearts.

	Cm (pF)	I_{Ca} (pA)	I_{Ca}/Cm (pA/pF)
N	148.1 ± 40.3	1197 ± 514	8.27 ± 2.15
H	264.1 ± 76.3 ***	2254 ± 651 ***	8.51 ± 2.47
% Increase	78.3	88.3	2.9

Data are mean values ± SD, 33 cells taken from 15 normal and 31 cells from 15 hypertrophied hearts; ***: $p < 0.001$. Cm: membrane capacity, I_{Ca} amplitude of calcium current.

A particular attention was paid to the inactivation of I_{Ca} since two recent studies reported that the slowest of the two components of inactivation was prolonged (15, 17). In our experimental conditions, Ca current decay was best fitted with two time constants whose values were τau_1 = 46.0 ± 7.0 ms and τau_2 = 5.7 ± 0.9 ms (n = 30) in control and τau_1 = 46.5 ± 4.5 ms and τau_2 = 6.6 ± 1.6 ms (n = 27) in hypertrophied cells; they were not significantly different.

β-adrenergic stimulation of the Ca current

The response to inotropic agents is altered in hypertrophied cells; this could be due to modification of the sarcolemmal receptors or of some subsequent steps. Contradictory results have been reported following β-adrenergic stimulation with differences related to the model of hypertrophy and the level of circulating catecholamines. In the hypertrophied rat heart following abdominal aortic constriction, as used in this study, a depressed responsiveness to isoproterenol has been reported (23). The difference in the responses elicited by β-adrenergic stimulation in the normal and hypertrophied cells could be due to a decrease in the number of β-adrenoceptors, in the intrinsic activity of the adenylate cyclase or its coupling to the receptor through the G-protein or/and in the phosphorylation of the Ca channel by the protein kinase A.

The maximal responsiveness of I_{Ca} to isoproterenol (1 μM), cAMP (50 μM) and forskolin (10 μM) is given in Table 2.

Table 2: % increase in I_{Ca} density by 1 μM isoproterenol, 50 μM cAMP and 10 μM forskolin in normal (N) and hypertrophied (H) rat cardiac cells.

	Iso	cAMP	forsk
N	120 ± 13 n = 12	140 ± 33 n = 7	135 ± 12 n = 6
H	78 ± 14.5 n = 8 *	132 ± 15 n = 8	135 ± 24 n = 9

Each experimental condition was performed on myocytes from at least on 3 different hearts. The % increase is given relative to current density before stimulation. * $p < 0.05$.

In normal myocytes, isoproterenol, cAMP anf forskolin led to about 130% increase in I_{Ca}. In hypertrophied cells, cAMP and forskolin had the same relative effect as in control cells;

they were significantly more efficient than isoproterenol stimulation. However, the β-stimulatory effect was obtained with the same apparent affinity (K_D = 4 nM) in the two cell types.

DISCUSSION

This study reports a prolongation of the action potential of isolated hypertrophied cells following aortic banding in rat. Previous studies on other models also showed prolongation of action potential of hypertrophied isolated myocytes (14-16). In our experimental conditions, recording of action potentials was made with a patch electrode containing EGTA which allowed control of the internal medium. Thus, it can be suggested that direct changes of ionic conductances may trigger the above effects.

We have also compared Ca currents recorded under whole cell patch-clamp in normal and hypertrophied cardiac rat cells and found no differences in Ca current densities and kinetics. In fact, I_{Ca} amplitude was larger in hypertrophied than in normal myocytes; this increase correlates with the larger membrane capacity. Thus, when peak I_{Ca} was normalized to the cellular membrane capacity estimated under voltage-clamp conditions, current density was not modified. To date, only two reports in isolated myocytes deal with the comparative analysis of I_{Ca} also normalized to membrane capacity. No change in peak I_{Ca} density was found in feline myocytes (15) while a 2.5 fold increase was reported in Goldblatt hypertrophied rat myocytes (17). Ca channel density was also estimated from nitrendipine

binding studies. Hypertension decreased the number of Ca channel receptor binding sites in the Goldblatt rat model (24), increased it in old SHR rats (25) and did not modified it in young SHR rats (25,26). On the other hand, similar densities are reported in the normal and hypertrophied rat after aortic stenosis (27). The lengthening of the action potential could also be due to alterations in the kinetics of I_{Ca}. Our study shows that the two time constants of I_{Ca} inactivation are identical in normal and hypertrophied cells in accordance with the results of Ten Eick et al (6) on hypertrophied right ventricle of cat but not with the results of Kleiman & Houser (15) and Keung (17) concerning the slow time constant.

Isoproterenol increases I_{Ca} two fold in control rat myocytes without altering its kinetics in agreement with previous results (28). In hypertrophied cells, isoproterenol was less effective. Our results showing no change in the sensitivity of I_{Ca} to isoproterenol agree with the lack of modification of receptor affinity in hypertrophied cells (29). Moreover, experiments with forskolin and cAMP suggest that adenylate cyclase activity and phosphorylation by protein kinase A are unaltered during hypertrophy. These results showing a reduced responsiveness to β-adrenergic agents correlate well with the lower number of β-adrenoceptors generally reported in such conditions (23).

In conclusion, the lengthening of the action potential and the depressed inotropic state in hypertrophied rat heart after aortic stenosis cannot be accounted by genuine modification of the Ca channel. Changes in AP duration should be more closely related to alterations in the K currents (30,31). Finally, our

results suggest that there was a coordinated synthesis of Ca channels following cell hypertrophy while the number of β-adrenergic receptors per cell did not change accordingly.

SUMMARY

Cardiac hypertrophy is one component of the physiological response to chronic pressure overload. It is associated with prolonged duration of the action potential among several other mechanical and electrophysiological alterations. This lengthening of the AP can be related to variations in the calcium current, I_{Ca}. The magnitude and kinetics of I_{ca} were compared in single rat ventricular cells isolated from normal or hypertrophied heart. Left ventricular hypertrophy was induced by stenosis of the abdominal aorta. The Ca^{++} current was larger in hypertrophied cells 2.2 ± 0.6 nA (n = 31), than in normal cells, 1.2 ± 0.5 nA (n = 33). However, if one relates I_{Ca} amplitude to the cell membrane area, as estimated by membrane capacity measurement, no significant difference was observed in current density : 8.5 ± 2.5 pA/pF, (n = 31) and 8.3 ± 2.1 pA/pF, (n = 33), in hypertrophied and in normal cells respectively. In both types of cells, I_{ca} displayed the same voltage and time dependence. Regulation of I_{Ca} by a β-adrenergic agonist was also analysed since β-induced positive inotropy is less marked in hypertrophied heart. When expressed as percentage, the maximal increase in I_{Ca} amplitude that was obtained with 1 µM isoproterenol was less in hypertrophied cells (Emax = 78%) than in normal cells (Emax = 120%). The sensitivity of I_{Ca} to β-adrenostimulation was not modified nor

were the effects of forskolin or of intracellularly applied cAMP. Consequently during hypertrophy, regulation of I_{Ca} by β-adrenergic agonist is decreased in agreement with the reduced number of binding sites of β-agonists.

ACKNOWLEDGEMENTS

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5

REGULATION OF CONTRACTILE ACTIVITY IN SINGLE ADULT CARDIOMYOCYTES ISOLATED FROM FOUR DIFFERENT SPECIES: THE EFFECT OF REDUCED SODIUM GRADIENT.

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INTRODUCTION

To study the basic regulatory mechanisms of cardiac contractile activity, we have analysed the shortenings and relengthenings that occur during the contraction-relaxation cycles of externally unloaded cardiac myocytes. Using an optical technique (1), we recorded the contractions of electrically stimulated single cells enzymatically isolated from rat, rabbit, dog and guinea pig ventricles. The aims of this study were *(a)* to establish the extent to which these unloaded shortenings resemble the behavior and responses of the respective intact tissues, *(b)* to investigate the relative importance of various steps of the e-c coupling process (Ca^{2+} entry, intracellular Ca^{2+} release, and (or) Ca^{2+} sequestration) and *(c)* to compare the contractile responses of ventricular myocytes from the four species in terms of their preferential source of Ca^{2+} for the activation of contraction.

To investigate the relative contributions of the various sources of activator calcium to the activation of contraction - specifically the role the Na^{+}-Ca^{2+} exchange - we have studied the effects of various drugs or experimental treatments affecting preferentially the e-c coupling process at the level of *(a)* the Ca^{2+} release by the sarcoplasmic reticulum (SR) stores (in presence or absence of ryanodine) and *(b)* the sarcolemmal Ca^{2+} entry through *(i)* the calcium channels (in presence or absence of nifedipine). or *(ii)* the Na^{+}-Ca^{2+} exchange (in presence or absence of veratridine or reduced $[Na]_o$).

Although the dependence of the contractile activation in cardiac muscle on the Ca^{2+} release by the SR became widely accepted, it remains to be further

experimentally supported, especially in the ventricular cells, where it seems to be species dependent (2, 3). Furthermore, although the role of the Na^+-Ca^{2+} exchange in the activation of contractile force has been rather firmly established in the frog cardiac muscle (4, 5, 6), the quantitative participation of Ca^+ entry via Na^+-Ca^{2+} exchange in the cardiac excitation-contraction coupling in various mammalian species remains to be determined. In this respect, our experimental model should prove very useful, since isolated myocytes from four species could be compared under identical experimental conditions, thus eliminating various possible problems related to the lack of control of the extracellular environment in the complex and heterogenous intact tissue preparations.

METHODS

Solutions

Ca^{2+}-free standard HEPES-buffered Tyrode's solution (used in all experiments unless stated otherwise) contained the following (in mM): NaCl, 120.5; KCl, 3.82; KH_2PO_4, 1.18; $MgSO_4$, 1.18; glucose, 11.1; and HEPES, 10; pH was adjusted to 7.4 with NaOH (increasing $[Na^+]_o$ to 126mM) and the solution was gassed with 100% O_2.$CaCl_2$ was later added as specified.

The collagenase was obtained from BMC; trypsin, veratridine and nifedipine were from Sigma (St. Louis, MO, U.S.A.); and ryanodine was from Peninck Corp. (Lynhurst, NJ, U.S.A.). The solutions of nifedipine were prepared in darkness and were protected from light exposure during the entire experimental procedure. In the solutions where the $[Na]_o$ was reduced, the NaCl was replaced isotonically by LiCl.

Isolation of cardiac myocytes

The perfusion methods used for the enzymatic isolation of rat, guinea pig

and rabbit myocytes are similar to these described elsewhere (1, 7). Briefly, male Sprague-Dawley rats (275-325 g) and male guinea pigs (275-325 g) or New Zeland rabbits (1-1.5 kg) were injected i.p. with heparin (1000 IU/mL; 1 mL/300 g) 30 min before being injected i.p. with sodium pentobarbital (65 mg/mL;80 mg/kg body weight). The aorta of the anesthetized animal was cannulated and subjected to retrograde perfusion with Ca^{2+}-free standard HEPES-buffered solution while the heart was removed from the body. This was followed by a 5-min retrograde perfusion of the isolated hearts (Langendorf perfusion) with Ca^{2+}-free solution. The following was then added to this solution: 0.06% to 0.25% collagenase, 0.004% trypsin (Sigma type III), 0.4% type F albumin (essentially fatty acid free), and 25 μM $CaCl_2$. This perfusate was recirculated for 5-35 min, after which the ventricles were cut into small pieces and incubated at 35°C for 2 min in the perfusate. The cell suspension was filtered (through a 250-μm nylon mesh), gently centrifuged (at 50 x g for 2 min), and resuspended in HEPES - Tyrode containing 0.1 mM Ca^{2+}. After 2 min in the perfusate, the tissue was further incubated at 35°C for 10-min intervals in Tyrode containing 50 μM Ca^{2+}. After four to five collections, the cells were pooled and resuspended in Tyrode containing 1 mM Ca^{2+}. Dog ventricular myocytes were isolated as described in (1). The cell concentration and the percentage of rod-shaped cells in the cell population were determined by counting them in a special cytometer (Graticules Ltd., Kent, England). Nonmyocytes comprised <1% of all cells. The viability of myocytes (>85%) was determined by Trypan blue exclusion, which is a good indicator of their functional integrity. The electrical and contractile behavior of the freshly isolated cells was determined as described below.

Recording of electrical activity and contractility

The isolated myocytes were placed into a small experimental chamber (0.5 mL), superfused (2 mL/min) with Tyrode's solution (pH 7.4; 36 ± 0.5°C, as recorded continuously by a thermoprobe in the chamber), and gassed with 100% O_2. The concentration of $CaCl_2$ was 2.5 mM. The membrane resting and action potentials were recorded with conventional microelectrodes (R_{el}=40-60 MΩ)by a WPI KS-707 microprobe system, as described earlier (1). The quiescent cells were intracellularly stimulated through the recording microelectrode by current pulses 1 ms in duration and 1-5 nA in amplitude at a frequency of 0.7 Hz.

The contractility of an isolated myocyte induced by the electrical stimulation was recorded by a TV camera attached to an inverted microscope (Zeiss IM 35), and the cell image was continuously displayed on a TV monitor (final magnification x 2300) and recorded on videotape. The contractions of the isolated myocytes were recorded in a manner similar to that described previously (1). The shortening and relengthening of the externally unloaded myocytes were determined by an array of 25 photodiodes placed on the TV monitor over the end of the stimulated cell. The usual diastolic length (DSL) of the myocytes was 1.90-1.95 μm, and myocytes that exhibited shorter DSL (at the beginning or during the experiment) were not included in this study. Each figure shows a representative example of at least three to five experiments.

RESULTS

The effects of ryanodine

Figure 1A shows a representative example of the electrical activities (action potentials) and the corresponding isotonic contractions (shortenings and relengthenings) elicited by electrical stimulation of the externally unloaded single myocyte from the rat, dog, rabbit and guinea pig. In spite of some

variability in the contractile behavior of the individual myocytes within each group, they generally exhibited very similar responses to exposure to ryanodine; the representative responses of rat, dog, rabbit and guinea pig myocytes (10-15 myocytes investigated in each group) are shown in Fig. 1B. Consistent with the results reported from intact tissue preparations (3), the effect depended on the species. The fastest response was observed in rat myocytes; 1 μM ryanodine completely inhibited the unloaded contractions in these myocytes within 4-5 min after it was applied (Fig.1*b*), while in dog myocytes, full inhibition of contractions occurred after 10-15 min (Fig. 1*d*). The contractions of the guinea pig and especially the rabbit myocytes were affected by this drug much less; 1 μM ryanodine decreased the extent of the shortenings within 15-25 min to a steady level, reaching >60% of the controls in both species (Fig. 1*f*, *h*).

Figure 1

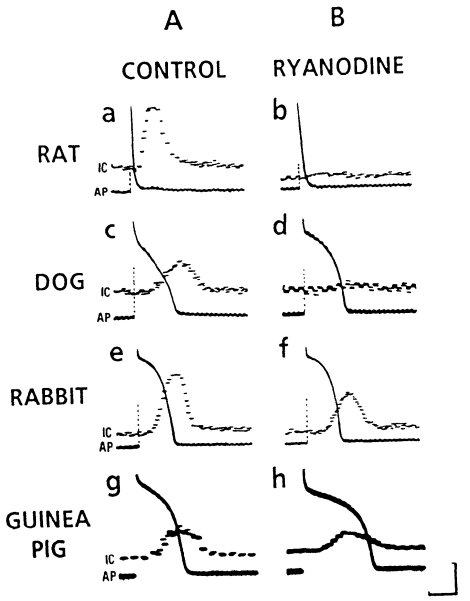

Figure 2

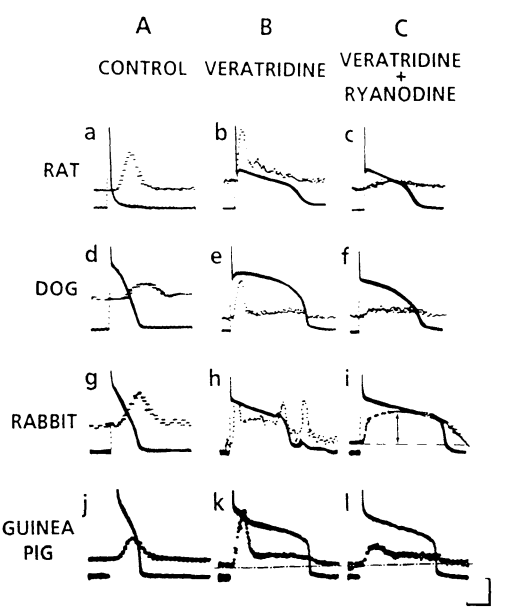

Fig. 1 The effect of ryanodine on the action potentials (AP) and the isotonic contractions (IC) of isolated cardiac myocytes from rat, dog, rabbit and guinea pig. The upward deflections represent the isotonic shortenings of the externally unloaded isolated myocytes elicited by electrical stimulation. (A) The controls were recorded in standard Tyrode's containing 3 mM $CaCl_2$. (B) The effect of 1 μM ryanodine was recorded in the rat myocyte after 4 min (*b*), in the dog myocyte after 10 min (*d*), and in the rabbit and guinea pig myocytes after 20 min (*f, h*) of the drug's application. The stimulation fequency was 0.7 Hz. Vertical scales: AP amplitude: 60 mV; sarcomere shortening: 0.15 μm (rat), 0.3 μm (dog), 0.15 μm (rabbit and guinea pig). The average diastolic length of the sarcomere was in all four preparations 1.9-1.95 μm. Horizontal scale: 150 ms. The resting membrane potentials (mV) were -82 (*a*), -85 (*b, c, d*), -90 (*e*), -92 (*f*), -94 (*g, h*).

Fig. 2(A): The controls in Tyrode's containing 3 mM $CaCl_2$. (B): The effect of 1.5 μM veratridine on the action potentials (lower traces) and contractions (upper traces) of isolated cardiac myocytes from rat, dog, rabbit and guinea pig, 2-3 min after application of the drug. (C): The effect of 1 μM ryanodine added to 1.5 μM veratridine-containing solution is shown 3 min (*c*) and 10 min (*f, l*) after the addition, and the effect of 10 μM ryanodine is shown 15 min after its application (*i*); the arrow represents the extent of the shortening. The stimulation frequency was 0.3 Hz (*a, k*) and 0.1 Hz (*i*). Vertical scales: AP amplitude, 60 mV; sarcomere shortening, 0.2 μm (rat, rabbit and guinea pig) and 0.3 μm (dog). The average diastolic length of sarcomeres was 1.9-1.95 μm in all recordings. Horizontal scale: 150 ms in controls (A) and 600 ms in presence of veratridine (B, C). The resting membrane potentials (mV) were: -90 (*a, d, g*), -94 (*b*), -96 (*c*), -95 (*e, f*), -92 (*h, i*).

The effects of veratridine and reduced $[Na]_o$

We investigated the effects of veratridine which presumably increases Ca^{2+} entry via the Na^+-Ca^{2+} exchange mechanism by enhancing Ca^{2+} influx linked to Na^+ efflux; this exchange of the intracellular Na^+ for the extracellular Ca^{2+} is expected to be facilitated by the increase of $[Na^+]_i$ produced by this drug (see Discussion). Figure 2 shows representative results of the effects of 1.5 μM veratridine (8-10 myocytes from each species were investigated). The action potentials were greatly prolonged (consistent with the expected prolonged increase in the Na^+ conductance), the APD reaching 1-2 s in myocytes from all four species. The APD was related to the fequency of stimulation, being longer at lower frequencies and shorter at higher frequencies

(e.g. Fig. 2*h* and 2*i*). The contractile responses of myocytes were also greatly affected by veratridine, and they consisted of two components: the initial shortening and relengthening, followed by a second component of a sustained shortening that relaxed only upon the repolarization (Fig. 2B). These two components closely resemble the twitch (phasic) component and the sustained (tonic) component of contractile activity usually observed in intact cardiac tissue preparations under conditions of increased $[Na^+]_i$ (see Discussion). The initial (phasic) component of contractions was increased by 1.5 μM veratridine in myocytes from all four species to 145.1 ± 9% in rat (n=9), to 172 ± 8.2% (n=8) in dog, and to 195 ± 20% (n=5) in guinea pig and to 187 ± 8% (n=10) in rabbit myocytes ($P<0.01$ in all four groups). Higher concentrations of veratridine increased the contractions even further, but they were usually accompanied by a development of spontaneous activity, which interfered with our investigations. The second, sustained (tonic) component of the shortenings was present in all four species (Fig. 2B) but was most enhanced in rabbit myocytes (Fig. 2*h*). The regular action potentials and contractions elicited by stimulation in the presence of veratridine were often followed by spontaneous aftercontractions and after-depolarizations (Fig. 2*h*), which were completely abolished by ryanodine. The effects of ryanodine on the veratridine-induced changes are demonstrated in Fig. 2C; while the APD remained greatly prolonged, the initial (fast) component of shortening was abolished by 1 μM ryanodine within a few minutes in the rat and dog myocytes. However, the slow, sustained components were not inhibited by ryanodine, even after long (60 min) exposures (2*c*, *f*, *l*). The veratridine-induced shortenings of the rabbit myocytes were very little affected even by 10 μM ryanodine (Fig. 2*i*); except for reducing the initial (fast) phase, these shortenings, reaching 104 ± 3% (n=10) of the controls, were maintained at a

more or less constant level throughout the action potential (e.g., about 2 s in Fig. 2*i*). Thus, these results suggest that in rabbit ventricular cells strong contractions could be activated by long depolarizations in the presence of an increased Na^+ entry probably independently of an additional Ca^{2+} release by the SR, as indicated by their insensitivity to ryanodine. The ryanodine-insensitive shortenings did not appear to be significantly affected by 10-15 min exposures to 1 μM D600; however, in the control myocytes, such exposures diminished the shortenings to <20% of the original values (M. Horackova, unpublished observations). The exposure of the rat myocyte to a reduced $[Na^+]_o$ generated a PIE (Fig. 3*b*) which, like that of veratridine, was rather insenstive to ryanodine (Fig. 3*d*). The application of nifedipine did not exhibit very significant effects in the rat ventricular myocytes on the shortening in control Tyrode's solution (*not shown*) or the presence of veratridine (Fig. 3*e*), supporting the notion that Ca^{2+} entry through the Ca^{2+} channels plays a less important role in the generation of contractile force in rat than in guinea pig

Figure 3

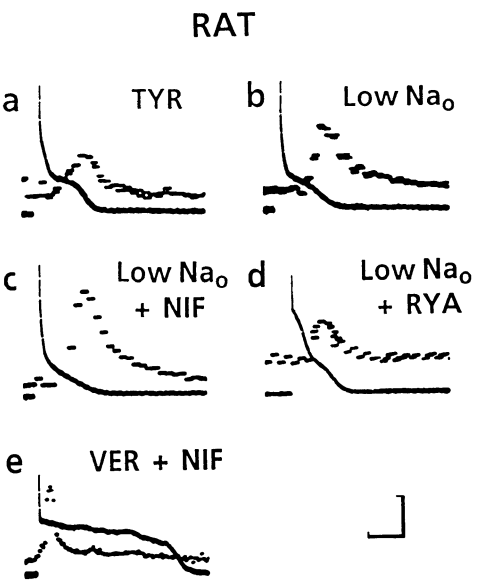

Fig. 3 Action potentials (lower traces) and contractions (upper traces) recorded from an adult rat ventricular myocyte in control Tyrode's solution (TYR); applying 1 μM veratridine (VER) elicited a positive inotropic effect, which remained unaffected by 0.2 μM nifedipine NIF (*e*). The positive inotropic effect of reduced $[Na]_o$ (40 mM) (*b*) seems even enhanced by 0.2 μM nifedipine (*c*). Horizontal scales: 50 ms (*a, b, d, e*), 100 ms (*e*) and it is only slightly reduced by 0.1 μM ryanodine (RYA). Vertical scales: 40 mV and 0.2 μm for electrical and contractile activities.

cardiac cells. Furthermore, the PIE elicited in the rat myocytes by reduced $[Na^+]_o$ (Fig. 3*b*) remained unaffected (or sometimes was even enhanced) by nifedipine (Fig. 3*c*). However, it should be noted that these effects of a decreased Na^+ gradient could also modify (increase) the SR Ca^{2+} load by changing the Na^+-Ca^{2+} exchange (i.e., by increasing Ca^{2+} influx and decreasing Ca^{2+} efflux) and thus possibly compensating for the inhibitory effect of nifedipine on the Ca^{2+} entry via Ca^{2+} channels. In isolated adult guinea pig myocytes, reducing NaCl to 40 mM (replacing it with LiCl) abbreviated the APD and increased their shortening by approximately 30-50% (Fig. 4*d*). The subsequent addition of 0.5 μM nifedipine to the low Na^+_o solution decreased the plateau (although the overall APD did not change significantly) and fully inhibited all the contractile activity within a few minutes after the drug's application (Fig. 4*f*). This inhibitory effect of nifedipine indicates the importance of Ca^{2+} entry through the Ca^{2+} channels for the activation of contractility in the guinea pig myocytes. The effect of nifedipine was fully reversible: the AP configuration as well as the contraction returned to the original steady-state level within a few minutes after nifedipine was removed from the superfusing solution. In contrast to inhibitory effect of nifedipine in presence of reduced $[Na]_o$, the PIE of veratridine (4*b*) in guinea pig myocytes was only slightly reduced by nifedipine (4*c*). The application of 1 μM ryanodine to the reduced $[Na]_o$ Tyrode's solution did not affect the contractile amplitude

significantly, but it slightly prolonged APD and also the contraction (Fig. 4*e*). While control Tyrode's solution plus ryanodine decreased the shortening by approximately 20-30%, the PIE of low $[Na^+]_o$ was not affected by 1 μM ryanodine; this result indicated that the Na^+-Ca^{2+} exchange determines the level of Ca^{2+} stored in SR and this level increases at reduced $[Na^+]_o$, as suggested by other investigators (8-10).

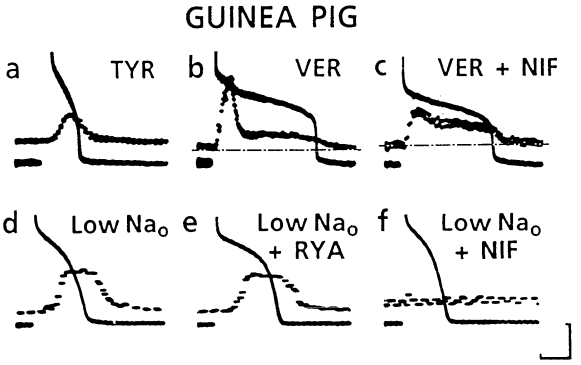

Fig. 4 Action potentials (lower traces) and contractions (upper traces) recorded from an adult guine pig ventricular myocyte, in the control Tyrode's solution (TYR) and after the application of 1 μM veratridine (VER) in the absence (*b*) or presence of 0.2 μM nifedipine (*c*). Veratridine elicited large sustained (tonic) contractions (as indicated above the dashed lines) even in the presence of nifedipine. An exposure to reduced $[Na]_o$ (40 mM) elicited a positive inotropic effect (*d*) that was unchanged by 1 μM ryanodine (RYA) (*e*) but it was completely inhibited by a subsequent application of 0.5 μM nifedipine (NIF) (*f*); this effect was fully reversible, the contractions returning to the original level (observed in low $[Na]_o$) after removal of nifedipine (*not shown*).

DISCUSSION

Our results indicate that the electrical and contractile behavior of externally

unloaded isolated cells from rat, dog, rabbit and guinea pig hearts approximates (at least qualitatively) the behavior of the respective multicellular tissue preparations and that this experimental model could be used in studies of e-c coupling in cardiac muscle.

It has been suggested that the Ca^{2+}-induced release of Ca^{2+} from the SR is most developed in rat cells and least developed in rabbit cells, while dog and guinea pig cells represent an intermediate type of these various ventricular myocytes (2). On the other hand, the contribution of sarcolemmal Ca^{2+} entry to the activation of the contractile response seems to be more pronounced in the rabbit, guinea pig and the dog ventricular tissue, while the adult rat cardiac tissue appears to be least sensitive to this source of Ca^{2+} activator. Consistent with the order of dependence of the force development in these species on intracellular Ca^{2+} release are the data of Sutko and Willerson (3) indicating that ryanodine (10^{-9} to 10^{-4} M) was most effective in inhibiting the contractile force in the papillary muscles of rat, was less effective in dog, and had relatively little effect on rabbit papillary muscles. Our results (Fig. 1) demonstrate a similar order of sensitivity to ryanodine in the isolated ventricular cells from these species, where the guinea pig cardiocytes exhibit relative insensitivity to ryanodine similar to that of rabbit cells. However, ryanodine (1 μM) appears to be 10-30% more effective in isolated ventricular cells (23 and this study) than in the corresponding multicellular preparations (3). The lower sensitivity of the multicellular ventricular preparations to ryanodine may be due (at least partially) to the prolonged (1-2h) preequilibration of these preparations routinely employed in this study. During this period, the cells probably accumulate Na_i^+ and this in turn could lead to an increased Ca^{2+} entry via the ryanodine-insensitive Na^+-Ca^{2+} exchange.

It has also been suggested that in addition to the two sources of Ca^{2+} for contractile activation (the Ca^{2+} entry via Ca^{2+} channels and the Ca^{2+}-induced release of Ca^{2+} from the SR) the -Na^{+}-Ca^{2+} exchange as an electrogenic mechanism may also contribute to contractile activation, especially when the gradient is decreased (e.g., 4, 5, 11, 12). The electrogenicity of this system is supported by several studies on intact isolated myocytes (10, 13-18). To decrease the Na^{+} gradient, we used veratridine, which slows the inactivation of the fast Na^{+} channel (4) increasing $[Na]_i$ (19), which in turn leads to the PIE in a variety of cardiac preparations, presumably via an enhanced $[Ca^{+}]_i$ by Na^{+}-Ca^{2+} exchange (1, 4, 19). In this study, we have observed the PIE elicited by applying 1 μM veratridine in ventricular myocytes isolated from all four species. While the APD was greatly increased, the regular twitch contractions were enhanced in their amplitudes and, moreover, were followed by second, sustained (tonic) contractions lasting as long as the action potential (up to several seconds). In spite of the obvious differences in the regulartory contractile mechanisms between the various myocytes under control conditions, as described above, in the presence of veratridine the two components of contractions (the phasic and the tonic) exhibited similar characteristics towards the specific inhibitors: the phasic components were decreased in all four species by ryanodine, while the tonic components in both species remained without any significant changes (Fig. 2). This clearly indicated that different mechanisms are involved in the generation of these two components. Similarly, the enhancement of the veratridine's effect on tonic tension was insensitive to application of nifedipine in the rat and guinea pig myocytes (Figs. 3*e*, 4*e*). On the other hand, the PIE elicited by reduced $[Na^{+}]_o$ was not affected by ryanodine in either type of myocytes (Figs. 3*d* and 4*e*), but, although it was not

affected by nifedipine in rat myocytes (Fig. 3c), surprisingly it was usually fully suppressed by this inhibitor in the guinea pig cells (Fig. 4f). This indicated that the mechanisms of the PIEs due to the decreased Na^+ gradient could be somewhat different depending on whether the gradient was changed by a reduction of $[Na^+]_o$ or by a veratridine-induced increase in $[Na^+]_i$. Indeed, the possibility that Na^+-Ca^{2+} exchange may depend directly on the level of intracellular Na^+ activity (a^i_{Na}) has been recently suggested (11). Thus, it is likely that an increased a^i_{Na} by veratridine might stimulate an additional nifedipine-insensitive Ca^{2+} entry via Na^+-Ca^{2+} exchange which is lacking when the Na gradient is decreased by reducing $[Na]_o$; it seems that this additional Ca^{2+} influx is necessary for the activation of contraction in guinea pig cardiomyocytes. Our data are consistent with those of Fabiato (20, 21), indicating that the effectiveness of sarcolemmal Ca^{2+} entry at rising $[Ca]_i$ (and thus contractility) could be highly dependent on the state of internal stores and the Ca^{2+} uptake of these stores would be, in turn, greatly dependent on the rate of Ca^{2+} entry. Additional experimental evidence from the voltage-clamp study of the nifedipine's effect is needed to determine the direct effects of this drug in both species under these experimental conditions.

SUMMARY

(A) Our data indicate that the contractile activity in isolated ventricular myocytes from rat, dog, rabbit and guinea pig is primarily regulated by Ca^{2+} entering the cells via the slow Ca^{2+} channel and by Ca^{2+} released by SR (the relative contributions of these two Ca^{2+} sources being different in these species). (B) Our results are in agreement with data of Bers et al. (12) from rabbit papillary muscles, indicating that Na^+-Ca^{2+} exchange does not seem to be of any special importance for activation of contractile activity under control

conditions in either type of cardiomyocytes; however, it becomes important as a source of Ca^{2+} when the Na^{+} gradient is decreased, especially when this is due to an increase in $[Na^{+}]_i$. This conclusion appears also in agreement with recent studies (10, 22) suggesting that the main function of Na^{+}-Ca^{2+} exchange during normal cardiac electrical activity is Ca^{2+} extrusion out of the cell.

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6

INVOLVEMENT OF SODIUM-CALCIUM EXCHANGE IN CARDIAC PATHOLOGY

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Introduction

Transsarcolemmal Ca^{2+} movements are crucial for force development in the heart. One transsarcolemmal Ca^{2+} transport pathway exchanges Ca^{2+} for Na^{+}. Under physiological conditions, this exchange appears to occur primarily in the direction of calcium leaving the cell for sodium entering the cell (1). Thus, sodium-calcium exchange may play a role in the relaxation phase of excitation-contraction coupling in the heart under normal conditions. However, the involvement of sodium-calcium exchange in cardiac pathology is not as well defined. The purpose of the present treatise is to review the current literature on this subject from two scientific methodological approaches: i)pharmacological studies which employ drugs aimed at altering appropriate cellular ion transport pathways, and ii)biochemical studies using isolated sarcolemmal membranes. In addition, the mechanisms which may be responsible for the change in sodium-calcium exchange will be discussed.

Pharmacological Evidence

Several studies have examined the possible involvement of sodium-calcium exchange in two experimental models of cardiac pathology: ischemia/reperfusion damage and the calcium paradox. Both models have a common pathology in that the necrotic process is closely associated with excessive calcium entry and overload (2). If this calcium entry is through the sodium-calcium exchanger, then drugs which directly or indirectly affect the sodium-calcium exchange

pathway will ultimately alter the necrotic process in the heart. Amiloride is a drug which can inhibit sodium-calcium exchange (3). If the drug was present during low-flow ischemia and reperfusion, it reduced the release of creatine kinase from the heart and improved force, +dF/dt and -dF/dt during recovery (4). However, because of the non-specificity of amiloride's actions (3), it was difficult to ascribe with any degree of certainty the involvement of a specific ion transport pathway in the results. The results are encouraging, however, and suggest further work in the ischemia field with better drugs may be worthwhile.

If a heart is perfused with a calcium-free solution followed by normal calcium-containing medium, massive contracture develops along with contractile dysfunction. This has been termed the calcium paradox (2). It is associated with elevated calcium entry (2), possibly through the sodium-calcium exchanger. If sodium-calcium exchange is involved in this damage, interventions which elevate intracellular Na^+ during the paradox should stimulate the exchanger and augment cardiac damage whereas lowering Na^+_i should preserve cardiac function. Work from our laboratory has shown this to be the case (5). Typical force recordings from an isolated right ventricular wall during the calcium paradox protocol are shown in Figure 1A. Note the contracture development and poor force generation during the Ca^{2+} repletion phase. If the perfusate [Na^+] during the Ca^{2+}-free perfusion was lowered to 25 mM (thus lowering [Na^+_i]), then cardiac function was better perserved during reperfusion with the Ca^{2+}-containing solution (Figure 1B). Conversely, cardiac function was worse and contracture greater if 2 mM ouabain was included for 1 minute prior to initiation of Ca^{2+}-free perfusate (Figure 1C). If 5 mM amiloride was included in the Ca^{2+}-free perfusate, force development was significantly protected (Figure 1D). All of these

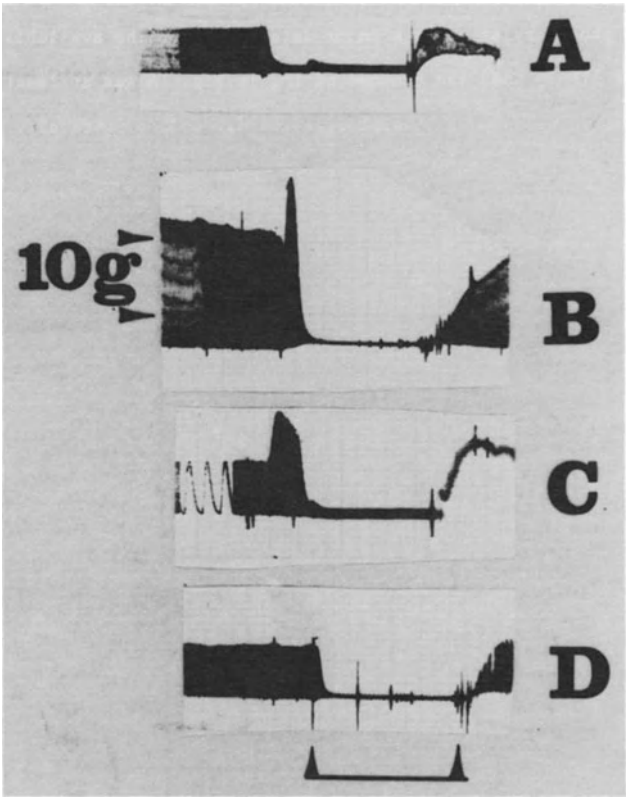

Figure 1. Typical tension recordings from an isolated, perfused right ventricular wall before, during and after a 4 minute period of Ca^{2+}-free perfusion (between arrows). A. Control; B. Perfusate [Na^+] was lowered to 25 mM during the Ca^{2+}-free period; C. 2 mM ouabain was included 1 minute prior to and during the Ca^{2+}-free period; D. 5 mM amiloride was included in the Ca^{2+}-free perfusate. Further experimental details can be found in reference.

observations support the contention that the transsarcolemmal Na^+ gradient is extremely important in determining force recovery during the paradox. Benzamil, an analogue of amiloride which is a more potent blocker of sodium-calcium exchange (3), also demonstrates significant protection against calcium paradox-induced cardiac damage (5). As shown in Figure 2, if as little as 10-50 uM benzamil is included in the Ca^{2+}-free perfusate, a significant

recovery in -dF/dt is observed (5). Significant protection by benzamil against tissue enzyme release and ionic content changes have also been reported (5). Taken together, these data strongly suggest an involvement for the sodium-calcium exchanger in the calcium paradox-induced cardiac damage. It is likely predicated by an abnormal increase in $[Na^+_i]$ which then stimulates the exchanger to work in a Na_i-dependent Ca^{2+} uptake mode. The initiating pathology, the excessive $[Na^+_i]$, may occur through a passive leak of Na^+ down its concentration gradient into the cell (6). In summary then, the pharmacological data would suggest that sodium-calcium exchange is stimulated in these two models of experimental cardiac pathology.

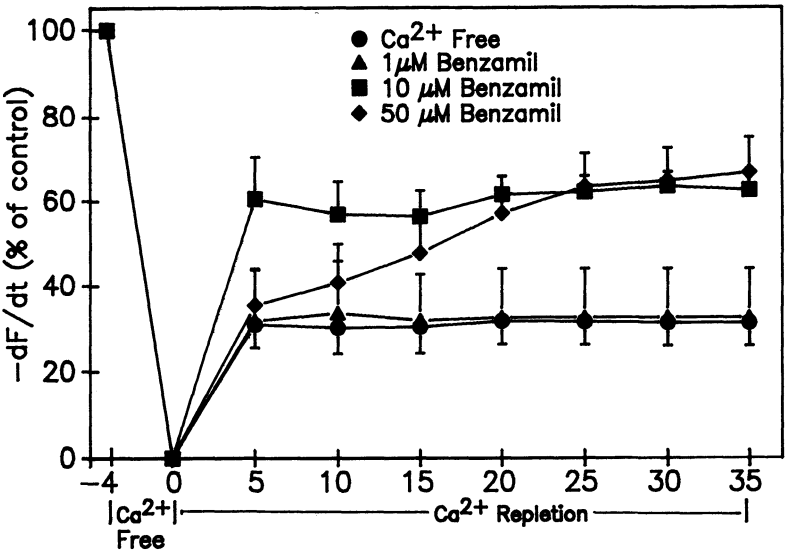

Figure 2. Rate of relaxation (-dF/dt) of untreated and benzamil-treated (2 minutes prior to and during the Ca^{2+}-free perfusion) right ventricular muscles. For further experimental details see reference 5.

Biochemical Evidence

A number of independent laboratories have investigated the involvement of sodium-calcium exchange in cardiac pathologies by studying isolated, cardiac sarcolemmal membrane vesicles (7-16). A wide variety of disease conditions have been examined including several cardiomyopathies, cardiac hypertrophy and various experimental models of cardiac pathology. The findings of these investigations are summarized in Table 1. Two salient features are of interest. First, with two exceptions (8,12), all of the studies observed a

Table 1. Biochemical evidence supporting an involvement of sodium-calcium exchange in cardiac pathology.

a) Cardiomyopathies		
i) Genetic (UM-X7.1 hamsters)	Makino et al (7)	55%
(B10 14.6 hamsters)	Wagner et al (8)	400%
ii) Catecholamine-induced	Makino et al (9)	49%
iii) Diabetic	Makino et al (10)	83%
	Pierce et al (11)	53%
b) Cardiac Hypertrophy	Heyliger et al (12)	No change
c) Ischemia	Bersohn et al (15)	50%
d) Hypoxia	Daly et al (14)	42%
e) Calcium Paradox	Makino et al (16)	36%

depression in sarcolemmal sodium-calcium exchange during the different pathologies. One exception, the work of Wagner and colleagues (8), showed a stimulation in sodium-calcium exchange during the early stages of

cardiomyopathy (30 day old) which reversed to a 50% depression during the later stages of the disease (360 day old). The work of Heyliger and colleagues (12) was done using a stable model of cardiac hypertrophy which did not demonstrate significant functional changes. This may explain the lack of sarcolemmal changes as well. Secondly, the vast majority of studies which observed a depression in sarcolemmal sodium-calcium exchange reported a decline of ~50%. Most of these studies controlled for non-specific permeability changes, therefore, the results depict the direct effects of the pathological state on the sodium-calcium exchange process. Thus, the observations of depressed sodium-calcium exchange in biochemical studies contrasts sharply with the pharmacological data which showed a stimulation of sodium-calcium exchange in the two different pathologies.

Mechanisms Responsible for Altered Sodium-Calcium Exchange

The mechanisms which may be responsible for the observed depression in

Table 2. Possible mechanisms responsible for the alteration in sodium-calcium exchange in pathological conditions.

a) Membrane Modification
i) Protease activation
ii) Phospholipase activation
iii) Cholesterol changes
iv) Endogenous amphiphiles
v) Free radicals
b) Changes in Intracelluar [Na]
i) Glycoside cardiotoxicity
ii) Calcium paradox

sarcolemmal sodium-calcium exchange may be divided into two general categories: those which are localized to the membrane structure itself and those which involve modifications to the external ionic environment surrounding the exchanger. The various possible mechanisms within these two general headings are summarized in Table 2.

The sodium-calcium exchanger is known to be sensitive to its membrane environment. Is it possible then that modification of the membrane during the pathological condition can explain the depressed activity exhibited in the sarcolemmal studies cited above? It is well recognized that ischemic insult to the heart will activate various hydrolytic enzyme processes like proteases or phospholipases . However, protease (17), phospholipase D (18), phospholipase C (19) or phosphatidylinositol-specific phospholipase C (20) treatment of cardiac sarcolemmal membrane vesicles all result in a stimulation of sodium-calcium exchange. It was only after extensive modification of the membrane that the exchanger became inhibited (17,19). In some disease states like the diabetic cardiomyopathy, membrane cholesterol content increases (21). Could this lead to depressed sodium-calcium exchange? Again, this possibility is unlikely. Increasing sarcolemmal cholesterol content in situ has been shown to stimulate sodium-calcium exchange (22). Alternatively, it is possible that the release of endogenous amphiphiles may modify sodium-calcium exchange by inserting into the sarcolemmal membrane and thereby altering the membrane structure. Platelet-activating factor (PAF) is released during ischemia (23) and has the capacity to inhibit sodium-calcium exchange (24). However, the concentration of PAF required to inhibit sodium-calcium exchange (20 uM) may exceed that actually present during the ischemic insult (24). Free radicals are also generated during ischemia (25). However, depending upon the species of radical generated, sodium-calcium exchange may be

stimulated (26,27) or inhibited (22,27). Thus, it is difficult to assess its involvement in the modification of sarcolemmal sodium-calcium exchange. In summary, the mechanism responsible for inhibiting sodium-calcium exchange after pathological challenge is unclear from a membrane biochemistry approach. The most likely candidate is extensive attack from the various hydrolytic enzymes. However, further examination of the problem is warranted.

The stimulation of sodium-calcium exchange observed in the pharmacological studies is more easily answered. It is almost certainly due to the altered ionic environment. The elevation in $[Na^+_i]$ during the Ca^{2+}-free perfusion stimulates the exchanger to work in a Na^+_i-dependent Ca^{2+} uptake mode. The mechanism here is similar to that observed in glycoside cardiotoxicity where glycosidic inhibition of the Na^+ pump will elevate $[Na^+_i]$, stimulate sodium-calcium exchange and ultimately cause excessive Ca^{2+} entry from the extracellular space into the myocardial cell (28).

Conclusions

In conclusion, the data are consistent with the hypothesis that the sodium-calcium exchanger is involved in cardiac pathology depending upon the nature of the disease. Increased sodium-calcium exchange, as was evident in the perfused muscle studies, is likely due to an elevated intracellular $[Na^+]$. Drugs which inhibit sodium-calcium exchange represent a viable approach for treatment. Decreased sodium-calcium exchange, as shown in studies of isolated sarcolemmal membranes, may be due to free radical involvement or more extensive structural modification of the membrane protein/lipid environment.

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7

MODIFICATIONS IN SARCOLEMMAL REGULATION OF Ca^{2+} WITH AGING

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INTRODUCTION

Age-related changes in myocardial function have been reported both in humans (1) and in various animal species (2,3). Patient studies revealed a decline in peak diastolic filling rate with age without the impairment of systolic function (1,4). Many of the animal studies have employed rat model, in which the changes in myocardial function and metabolism occur as early as during adult maturation (2,5,6) and progress through senescence (3). At about 12 months of age, there begins an alteration in mechanical activity, which continues to change through aging process to 24 months and older (3,7). These changes are characterized in left ventricular papillary muscle by an increase in time to peak isometric developed tension associated with an increase in time to half maximal relaxation. Neither the twitch force nor the maximum rate of force production are age-related. These alterations have also been demonstrated in other animal species such as guinea-pigs, dogs and rabbits (8-10).

Prolonged contraction duration with aging in rats has been partly attributed to the reduced myosin ATPase activity due to a switch in isomyosin composition (5,11). However, as indicated earlier, these changes have also been observed in other species, where the switch in isomyosin composition is not seen (12). Hence the prolonged duration of contraction may be contributed to a large extent by the rate of Ca^{2+} delivery to cytoplasm and the rate of subsequent Ca^{2+} removal from the cytoplasm. In the heart, contraction and relaxation-related Ca^{2+}

translocation rest mainly on the sarcotubular as well as sarcolemmal membrane systems (13). Significantly reduced ATP-supported (oxalate-facilitated) Ca^{2+} uptake by sarcoplasmic reticulum (SR) enriched fraction has been shown in aging heart (14). Sarcolemmal fraction from aged heart on the other hand was found to have two fold higher rates of ATP-supported Ca^{2+} accumulating activity (14). These reports demonstrate that differential alterations in Ca^{2+} transport activities of SR and sarcolemma occur with aging. Thus, while reduction in the rate of net Ca^{2+} uptake into the SR seems most attractive explanation of the prolonged Ca^{2+} transient in the senescent heart (3,14), any mechanism that can alter the flux of Ca^{2+} into or out of the myoplasmic space might affect the duration of myoplasmic Ca^{2+} transient. Furthermore, the transmembrane action potential (TAP) that excites the cell and the myoplasmic Ca^{2+} transient are both prolonged in senescent (3,15,16) and indicate that the trigger for Ca^{2+} release or Ca^{2+} influx may be greater in aged.

Contraction of cardiac muscle is critically dependent upon the external Ca^{2+} concentration and it has been proposed that entry of Ca^{2+} through the early plateau phase of action potential provides a trigger for release of larger amounts of Ca^{2+} from internal stores, SR (17). However, since SR Ca^{2+} uptake has been shown to be reduced with senescence (6,14) the release of Ca^{2+} from SR with each excitation should also be lower. If this was true, then the maintenance of developed tension in aged (7) would require additional influx of Ca^{2+} from extracellular sources. The observed prolonged duration of contraction in senescent heart muscle indicates that this additional influx of Ca^{2+} from

extracellular source may occur over a longer period of time. Alternatively, efflux of Ca^{2+} through sarcolemmal Ca^{2+} pump and Na^{+}-Ca^{2+} exchange is limited. That sarcolemmal Ca^{2+} influx may increase with aging is further supported by observations with skinned ventricular fragments (18). In a preliminary setting, Fabiato (1982) has shown that senescent heart muscle preparation requires a greater Ca^{2+} trigger for the release of SR Ca^{2+} than in preparation from younger animals. Furthermore, senescent heart muscle has been shown to be more sensitive to increased extracellular Ca^{2+} concentration and produces longer duration of contraction than in young adult rat heart papillary muscle (19).

The above reports clearly suggest that the changes in trans-sarcolemmal Ca^{2+} influx with aging may be responsible for the altered contraction duration and also be able to maintain the developed tension inspite of defective SR. The bulk of Ca^{2+} that enters the cell during excitation, results in the early part of plateau phase of the action potential (13). The influx of this Ca^{2+} is modulated by opening and closing of the voltage-dependent "slow" Ca^{2+} channels, which are localized in sarcolemmal membrane. This increased sarcolemmal Ca^{2+} influx in senescent heart (as discussed above) should be a reflection of increased Ca^{2+} channel activity.

In this study we have obtained evidence to suggest that Ca^{2+} fluxes through Na^{+}-Ca^{2+} exchange may decrease with aging. We have also shown that the density of myocardial calcium channels increases significantly during aging. These channels may be the main gateway to the Ca^{2+} influx for the maintenance of contractile force in aging animal heart.

METHODS

Male Sprague-Dawley rats of 2,12 and 24 months of age and guinea-pigs of 3,6 and 18 months of age were used in the present investigation. These animals reach sexual maturity around 2-3 months of age, adult maturation at 6-12 months and reach senescence at around 18-24 months of age.

Isolation of sarcolemmal membrane:

Relatively pure sarcolemmal membrane vesicles were prepared as described by us earlier (20). Briefly, the rat heart left ventricles were homogenized in 0.6 M sucrose, 10 mM imidazole/HCl (pH - 7.4) and centrifuged at 10,000 g for 20 min. The supernatent was diluted (2 fold) with 160 mM KCl/20 mM MOPS (pH - 7.4) and centrifuged at 96,000 g for 60 min. The pellet was resuspended in 2 ml of KCl/MOPS and layered over 15 ml of 30% sucrose solution containing 0.3 m KCl, 50 mM sodium pyrophosphate and 0.1 M Tris-HCl (pH - 8.3) and centrifuged at 95,000 g for 90 min. The white band at sample sucrose interface was recovered, diluted with 3 vol of KCl/MOPS and centrifuged at 100,000 g for 30 min. The pellet was resuspended in Tris-HCl to a final protein concentration of approximately 1 mg/ml. The protein concentration was determined by the method of Lowry et al. (21).

Specific [^{3}H]Nitrendipine binding:

About 80-100 ug of the sarcolemmal fraction, isolated by the above described procedure, was incubated in the medium containing 50 mM Tris-HCl buffer (pH - 7.4). After 10 minutes of pre-incubation, the reaction was started by the addition of a given concentration (0.05 - 1.0 nM) of [^{3}H]Nitrendipine (New England Nuclear Corp; specific activity 71 Ci mmol^{-1}) and incubated for 60 min at 25^{o}C. Non-specific binding was

determined in the presence of 0.1 uM non-labelled Nitrendipine in the medium and subtracted from the total [^{3}H]Nitrendipine bound to obtain specific binding. At the end of the incubation period, the samples were immediately filtered through GF/B whatman (Fisher Scientific) filters, using constant vacuum suction system. The filters were washed three times with 3 ml of each ice-cold 50 mM Tris-HCl buffer (pH - 7.4), dried under vacuum, and then placed in 10 ml of scintillation cocktail (Fisher Scientific) and counted using Beckman LS8100 liquid scintillation counter. Correction for quenching was performed by external channel ratio method.

Characterization of [^{3}H]BAY K 8644 binding:

Sarcolemmal protein of 80 to 100 ug was incubated in a total volume of 5 ml containing 50 mM Tris-HCl (pH - 7.4), at 25^{o}C, with varying concentrations (1-50 nM) of [^{3}H]BAY K 8644. After 60 minutes of incubation, samples were filtered through Whatman GF/B filters with the aid of a vacuum pump. The filters were washed three times with 3 ml of ice-cold 50 mM Tris-HCl, dried under vacuum, placed into scintillation vials and 10 ml of a scintillation cocktail (Fisher Scientific) was added. The radioactivity was counted in a Beckman LS 8100 liquid scintillation counter at 45-50% efficiency. Binding of [^{3}H]BAY K 8644 in the presence of 1 uM non-labelled Nitrendipine was defined as non-specific binding and was subtracted from the total binding to obtain specific binding.

Na^+-dependent $^{45}Ca^{2+}$ Uptake:

Vesicles were preloaded with Na^+ by incubating them in 160 mM NaCl/20 mM MOPS (pH 7.4) for 60 min at 37^{o}C. To initiate Na^+-Ca^{2+} exchange, aliquots of 20 ul (equivalent to 20 ug protein) of Na^+-loaded vesicles were added to a series of tubes containing an incubation mixture

(160 mM KCl/20 mM MOPS, pH 7.4 at 37°C) plus 50 uM $^{45}CaCl_2$ (100 cpm/pmol) in a volume of 500 ul. The exchange was terminated by adding the "termination solution" [2 ml ice cold 160 mM KCl/20 mM MOPS/1 $LaCl_3$, pH 7.4] at desired times followed by filtration through Millipore filters (0.45 uM) under vacuum. Tubes and filters were rinsed 4 times with 2 ml of "termination solution". The filters were placed into scintillation vials, 10 ml of scintillation cocktail was added and the radioactivity was counted in a Beckman LS 8100 liquid scintillation counter. In all experiments, non-specific $^{45}Ca^{2+}$ uptake was determined in the vesicles which were loaded with potassium [160 mM KCl/20 mM MOPS, pH 7.4) instead of sodium.

Sarcolemmal vesicles isolated from 3 and 18 month old guinea-pigs were first loaded with sodium and then incubated in the incubation mixture (160 mM KCl/20 mM MOPS, pH 7.4 at 37°C) with various concentrations of $^{45}Ca^{2+}$, ranging from 20 to 80 uM. The Na^+-dependent Ca^{2+} uptake was terminated after 5 seconds of incubation. The filtration and counting of $^{45}Ca^{2+}$ on the filter were carried out as described above.

Na^+-dependent $^{45}Ca^{2+}$ Efflux:

Na^+-loaded vesicles were allowed to accumulate $^{45}Ca^{2+}$ for 1 minute in 160 mM KCl/20 mM MOPS (pH 7.4) plus 50 uM $^{45}Ca^{2+}$ in a volume of 500 ul. Calcium efflux was then initiated by increasing the Na^+ concentration of the medium to 90 mM. The exchange was terminated at desired times and filtered through the millipore filters as in $^{45}Ca^{2+}$ uptake study and the quantity of $^{45}Ca^{2+}$ was counted by liquid scintillation.

BAY K 8644 inotropy in 2 and 12 month old rat:

These studies were carried out in a whole animal rat model. Animal

preparation and measurement of cardiovascular parameters were carried out as described earlier (23). BAY K 8644 (10 ug/kg/min) was administered by slow infusion through external jugular vein. Lead II EKG, left ventricular pressure and the rate of its rise (dp/dt) were recorded using a Hewlett Packard recorder (model 1308A).

Reagents:

Analytical grade chemicals dissolved in deionized glass-distilled water were used throughout. ^{3}H-BAY K 8644 and ^{3}H-Nitrendipine were obtained from New England Nuclear. Non-labelled nitrendipine and BAY K 8644 was supplied by Miles Pharmaceuticals, USA. courtesy of Dr. Alexander Scriabine.

Statistical Analysis:

The data are expressed as the mean $\pm$ S.E. The student's t test or the analysis of variance was used wherever appropriate for statistical analysis, taking $P < 0.05$ as the level of significance.

RESULTS

Na^{+}-Dependent $^{45}Ca^{2+}$ Uptake:

The uptake of $^{45}Ca^{2+}$ was studied using Na^{+}-loaded vesicles isolated from 3 months and 18 months old guinea-pigs. $^{45}Ca^{2+}$ uptake in K^{+}-loaded vesicles was taken as non-specific uptake and subtracted from the respective values of Na^{+}-loaded vesicles to obtain net Na^{+}-dependent calcium uptake. The vesicles from 3 month old guinea-pig hearts accumulated 6.4 $\pm$ 0.34 n mol mg^{-1} protein within 15-30 sec of initiation of influx and reached steady state in 2 min. The uptake was however slower in older animal heart vesicles which accumulated a maximum of 4.63 $\pm$ 0.05 n mol mg^{-1} protein in 45 sec and reached a steady state in 5 min. Analysis of Eadie-Hofstee plot of $^{45}Ca^{2+}$ uptake (Table 1) demonstrated

significant differences in kinetic parameters in 3 months (Km 35.78 ± 1.14 uM, Vmax 54.2 ± 1.7 nmol mg^{-1} min^{-1}) and 15-18 month old guinea-pigs (Km 58.4 ± 6.7 um, Vmax 20.0 ± 0.82 nmol mg^{-1}. min-1).

Table 1. Analysis of Eadie-Hofstee Plot of Na^+-dependent Ca^{2+} uptake.

	Young Adults (3 month old)	18 month old
Apparent Km (uM)	35.78 ± 1.14	*58.4 ± 6.7
Apparent Vmax (nmol $mg^{-1}min^{-1}$)	54.2 ± 1.7	*20.0 ± 0.82

Values are mean ± S.E. of 8 experiments.
* significantly ($P < 0.05$) different from 3 month olds.

Na^+-dependent Ca^{2+} Efflux:

Calcium efflux was initiated after 1 minute of Na^+-dependent $^{45}Ca^{2+}$ uptake by increasing Na^+ concentration in the incubation medium. The initial rate of Ca^{2+} efflux during first 5 to 10 seconds was substantially lower in 18 month olds than 3 month old animals (0.11 ± 0.03 as compared to 0.25 ± 0.02 nmol mg^{-1} protein $second^{-1}$, $p < 0.05$). At the end of 120-180 seconds of incubation, the vesicles from the older animal hearts retained 40% of the basal calcium, which was significantly higher than the vesicles isolated from 3 month old animals, which retained only 21% of the basal $^{45}Ca^{2+}$, indicating that the Na^+ dependent $^{45}Ca^{2+}$ efflux is reduced in older animals.

BAY K 8644-induced Inotropy:

Infusion of BAY K 8644 at 10 ug/kg/min in 2 and 12 month old rat produced significant increases in +dp/dt (Fig. 1). However the maximum increase in +dp/dt 32% in 12 month olds was significantly higher than +dp/dt (18%) produced in 2 month olds. Furthermore the doses of Bay K 8644 required to produce maximum changes in +dp/dt in 12 month olds was

only 1/3rd (10 ug/kg) of that required in 2 month olds. Perfusion of Bay K 8644 (10^{-7} M) in isolated guinea-pig hearts also demonstrated significant differences in developed tension in young and older guinea-pigs. Perfusion in 2 month olds thus caused 35-40% increase in developed tension within 5 min. On the other hand there was an increase of 80-90% in developed tension in 12-24 old guinea-pig hearts during the same time period. Effective dose for 50% response (ED 50) was 20 nM in 12-24 months and 65 nM in 2 month old guinea-pig hearts.

Calcium channel agonist ^{3}H-BAY K 8644 and antagonist ^{3}H-Nitrendipine binding:

Calcium channel agonist ^{3}H-BAY K 8644 and ^{3}H-Nitrendipine binding were examined in sarcolemmal membranes from 2 month and 12 month old rat hearts.

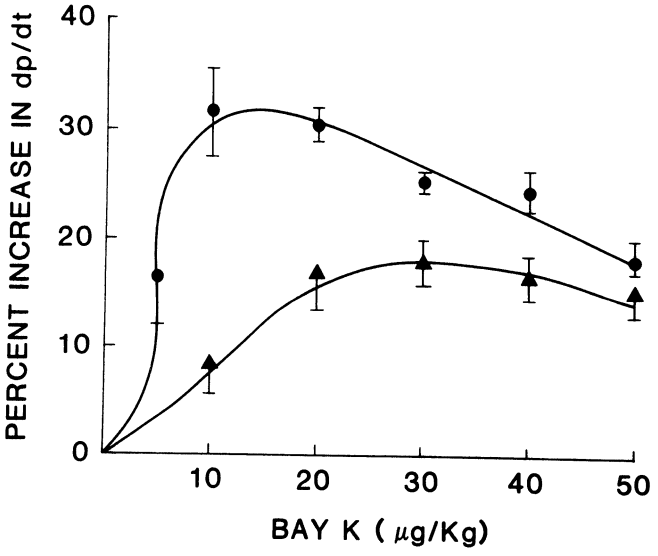

Figure 1. The figure shows percent change in +dp/dt with infusion of BAY K 8644 in 2 (▲—▲) and 12 (●—●) month old rats. Values are mean ± S.E. of 6 experiments in each age group.

Table 2 Scatchard plot analysis of specific ^{3}H-BAY K 8644 and ^{3}H-Nitrendipine binding

Age (Months)	Bmax (pmol. mg^{-1} prot)		KD (nM)	
	^{3}H-NIT	^{3}H-BAY	^{3}H-NIT	^{3}H-BAY
2	0.27 ± 0.03	1.7 ± 0.2	0.27 ± 0.04	4.8 ± 0.3
12	0.45 ± 0.06*	2.4 ± 0.1*	0.31 ± 0.06	14.5 ± 0.8*

The values are mean ± S.E. of 6 experiments.
* significantly different ($P < 0.05$) from 2 month olds.

As shown in Table 2, the specific binding of both agonist and antagonist were significantly higher in older rats (Bmax 2.4 ± 0.1 and 0.45 ± 0.06 p mol mg^{-1} prot.) than 2 month old rats (Bmax 1.7 ± 0.2 and 0.27 ± 0.03 p mol. mg^{-1} prot.). There was no significant differences in KD for ^{3}H-Nitrendipine binding in two age groups. The agonist ^{3}H-BAY K 8644 binding analysis however showed significantly higher KD values in 12 month olds as compared to 2 month old rats.

DISCUSSION

In the present study Na^{+}-dependent uptake of Ca^{2+} was substantially reduced in vesicles of the older animal hearts. The apparent maximal initial rate of Ca^{2+} (Vmax) declined by 70% with a significant reduction in the affinity for calcium. The passive diffusion of $^{45}Ca^{2+}$ from the vesicles was not determined in the different age groups. However, the substantial reduction in the rate of $^{45}Ca^{2+}$ uptake is unlikely to be due to the differences in the passive diffusion. The observed reduction of Na^{+}-Ca^{2+} exchange in the older animal hearts thus demonstrates an impairment of the Na^{+}-dependent calcium transport in and out of the cell.

In our studies on binding of ^{3}H-Nitrendipine and ^{3}H-BAY K 8644 to purified sarcolemmal membranes, a single class of saturable binding sites was demonstrated. The number of ^{3}H-Nitrendipine binding sites was found increased by 70% during adult maturation of rat from 2 to 12 months, which was maintained at this level during further aging to 24 months. The affinity of these sites for binding of ^{3}H-Nitrendipine remained unaltered during adult maturation and aging. The binding of calcium channel agonist ^{3}H-BAY K 8644 also increased significantly (41%) with aging of rat to 12 months. This increasing in number of binding sites for ^{3}H-BAY K 8644 was however associated with significant increases in KD or reduction in the affinity of ^{3}H-BAY K 8644 for the receptor sites. Although the significance of reduced affinity for BAY K 8644 with aging is unclear, it has been suggested earlier that activator interaction cause confirmational changes in the receptor protein (22) which might be responsible for the reduced affinity. The increases in the number of ^{3}H-dihydropyridine binding sites in 12 month old rats was also found to be associated with a substantially higher positive inotropic response to BAY K 8644 infusion. The dose of BAY K 8644 required for 50% of maximal increases in +dp/dt (ED 50) was only 5 ug/kg in older rats as compared to 13 ug/kg of BAY K 8644 required in 2 month olds. This age-related change in positive inotropy to BAY K 8644 was not limited to rat species as isolated perfused guinea pig hearts also responded in a similar manner. Perfusion of isolated guinea pig hearts with BAY K 8644 resulted in 80-90% increase in developed tension with ED 50 of 20 nM in 12-24 month old as compared to 35-40% increase in 2 month olds with ED 50 of 65 nM. These observations in our laboratory thus demonstrate that an increase in the density of Ca^{2+} channels with adult maturation and aging of animals

is associated with alteration in Ca^{2+} channel function and conceivably, consequent changes in intracellular Ca^{2+} metabolism.

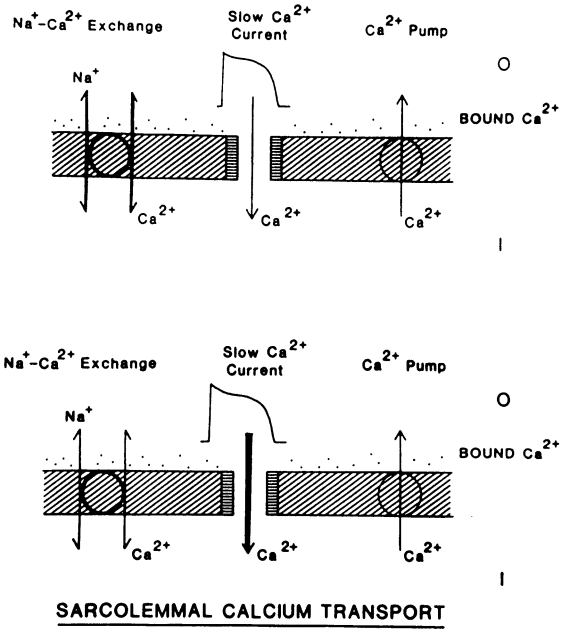

Figure 2 The figure shows a model diagram of sarcolemmal regulation of Ca^{2+} fluxes. The upper panel shows normal activities of bidirectional Na^{+}-Ca^{2+} exchange, Ca^{2+} influx through Ca^{2+} channel and Ca^{2+} efflux through Ca^{2+} pump. The lower panel shows reduced activities of Na^{+}-Ca^{2+} exchange and enhanced activity of Ca^{2+} channel in senescent.

The bulk of Ca^{2+} that enters the cell during excitation enters in the early part of plateau phase of action potential (13). The influx of this Ca^{2+} is modulated by opening and closing of the voltage-dependent "slow" Ca^{2+} channels localized in sarcolemmal membrane (see Fig. 2). An increased Ca^{2+} channel activity in senescent heart may thus reflect additional Ca^{2+} influx through these channels in the wake of reduced Na^{+}-Ca^{2+} exchanger activity. This hypothesis is supported by the fact that the duration of action potential is prolonged in older animals (3) and patient's (1). Our observation of increased +dp/dt with BAY K 8644 also supports the contention that senescent heart depend more heavily on Ca^{2+} through calcium channels for the maintenance of contractile tension. These observations may have clinical implications in treatment of cardiac disease in geriatric population.

SUMMARY

In this study we have shown that the Na^{+}-dependent Ca^{2+} exchange is significantly depressed in senescent heart whereas the myocardial density of calcium channels is significantly increased. Greater increase in +dp/dt with BAY K 8644 further confirmed the increased activity of calcium channel in senescent heart as compared to young adults. A model is presented to show that senescent heart may depend more heavily on Ca^{2+} through calcium channel for the maintenance of contractile tension than young adults.

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II

CARDIAC HYPERTROPHY AND CARDIOMYOPATHIES

8

ALTERATIONS OF MEMBRANE PROTEINS IN CARDIAC HYPERTROPHY

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INTRODUCTION

Hypertrophy of the heart is an adaptive mechanism to an increase in hemodynamic work. During this process, development without multiplication of the cardiac myocytes occurs and new sarcomeres are added to improve the contractility. As shown by Anversa et al. in compensatory hypertrophy of the rat (1,2), the overall result of the membrane development is an increase of the surface area parallel to the degree of hypertrophy which maintains a constant surface/volume ratio. However, some specialized membrane structures undergo a preferential development like the T-tubules (a 107 % increase for the T-tubules as compared to a 33 % for the sarcolemma (SL)) and the sarcoplasmic reticulum (SR) (a two fold increase). In this review on the membrane proteins of the hypertrophied myocyte, we shall take this into account to estimate their density of the receptors and their total number per myocyte or per left ventricle. We report that three types of regulation occur with enhanced, unchanged or decreased density leading to increased or unchanged total number of membrane proteins.

Together with these quantitative modifications, we shall consider if a qualitative alteration of the expression of the genes coding for the cardiac membrane proteins might be assumed. In fact, the expression of new isoforms of proteins during cardiac hypertrophy is well documented particularly for myosin (3). In the rat heart, the myosin isoform shift from the fast V1 to the slow V3 is, at least in part, responsible for the decrease in Vmax and the improvement in the wall stiffness. However, transcriptional or pre-translational modifications of the expression of a gene coding for a membrane protein have not yet been clearly demonstrated in the hypertrophied heart but might not be totally discarded since the properties of the Na^+/K^+ ATPase (4,5) or the coupling of the ß-receptors are altered (6).

This review is focused on the quantitative and/or qualitative alterations of Ca^{2+} channels, ß-adrenergic system, Na^+/K^+ ATPase and Ca^{2+}-ATPase from SR. These modifications which seem to develop with the severity and the stage of the heart failure account for the alteration in the inotropic response of the hypertrophied heart. Hence the use of inotropic agents in the treatment of cardiac failure has to adapt to these new parameters of the hypertrophied myocyte.

Ca^{2+} CHANNEL

In cardiac muscle, where Ca^{2+} influx is essential for contraction, the voltage-dependent L type calcium channel plays a key role in excitation-contraction coupling. Moreover, as described in skeletal muscle (7) it might also act as a voltage-sensor to induce the release of calcium from the SR. The $\alpha 1$ subunit of the cardiac dihydropyridine (DHP) receptor has a molecular mass of 243,000 D. Its primary structure has recently been elucitated by cloning and sequencing its cDNA (8). Injection of the $\alpha 1$ subunit mRNA into Xenopus oocytes led to the production of a functional calcium channel. However the $\alpha 2$ subunit might cooperate with $\alpha 1$ since the co-injection of $\alpha 1$ and $\alpha 2$ mRNA enhanced the Ca^{2+} -current activity. Regulation of the Ca^{2+} channel function also occurs through phosphorylation by cAMP dependent kinase (9) and coupling to α Gs protein (10, 11). Binding of Ca^{2+} agonists or antagonists lenghtens or shortens the opentime of the Ca^{2+} channel (12).

Ca^{2+} channels in the hypertrophied heart

One of the ordinary features of the hypertrophied heart is a prolongation of the duration of action potential (AP), particularly of the phase II which is observed in ventricles or isolated myocytes (13). This occurs whatever the model (hypertrophy secondary to hypertension or mechanical overload) or the species. It has been suspected that the Ca^{2+} channel might be responsible for this increased duration of the AP and numerous studies have appeared over the past few years. The studies have been performed on isolated membranes to determine the characteristics of the DHP receptors and on isolated myocytes to measure the current.

DHP receptors

The affinity constant of the DHP receptors is unchanged in the hypertrophied heart. The number of DHP receptors (table 1), expressed as fmol/mg protein remains the same in the young spontaneously hypertensive rat (SHR) model (14, 15) and in compensatory hypertrophy induced by mechanical overload in both the rat (16) and the guinea pig (17) whereas it is increased in the old SHR rat (18, 19) and in the cardiomyopathic hamster (20). In one study concerning the renal hypertensive rat (RHR) this number is decreased (21).

Table I: Number of DHP receptors in the hypertrophied heart

	Model	Bmax (fmol/mg)	Density*	Total number per heart **	Ref
Rat	SHR (9 w)	102 (103)	=	↑	(14)
	(10 w)	218 (187)	=	↑	(15)
	(16 w)	1960 (1538)	↑	↑	(18)
	(24 w)	141 (98)	↑	↑	(14)
	RHR	334 (549)		↓	(21)
	AS	310 (286)	=	↑	(16)
Guinea pig	AS	168 (195)	=	↑	(17)
Hamster	CM (4 w)	355 (187)	↑	↑	(21)

*( ): control values. * calculated assuming that the surface/volume ratio of the myocytes and the yields of the membrane preparations of hypertrophied and control hearts were identical and ** according to the % of hypertrophy. SHR: spontaneously hypertensive rat; RHR: renal hypertensive rat; AS: aortic stenosis; CM: cardiomyopathy.*

Ca^{2+} current

The Ca^{2+} current has been measured using the patch-clamp technique on isolated myocytes from the right ventricles of cats with pulmonary arteria stenosis (22) and from left ventricles of rats with renal hypertension (23) or aortic stenosis (24). The current amplitude is

enhanced in these models. The current density of hypertrophied myocytes from cat (22) and rat (24) is not different from that of control myocytes whereas it has been shown to be increased in RHR (23), due to the membrane capacitance calculated in this study.

Adaptation to hypertrophy

During compensatory hypertrophy of the rat heart, we have shown (16, 24) that 1) the total number of DHP receptors per left ventricle and the Ca^{2+} current amplitude per myocyte is proportional to the degree of hypertrophy ; 2) the density of DHP receptors calculated according to the increase in sarcolemmal and T-tubule surface area measured by Anversa (1, 2) and the density of Ca^{2+} current calculated by normalization of the amplitude by the membrane capactitance were indentical in normal and hypertrophied myocytes. They result in a similar inotropic effect of external calcium and of dihydropyridines on hypertrophied and control hearts (25) and represent an adaptational process of the calcium channel to pressure overload. It seems likely that the same conclusions are valid of the young SHR (14, 15) and the A.S. guinea pig (17) whereas the increase in density in the old SHR (14) and particularly in the CM hamster (21) impaired might induced an increased Ca^{2+} influx and Ca^{2+} overload.

However, regulation of the Ca^{2+} channel activity by functions which might be altered in the hypertrophied heart has not yet been investigated. It might be assumed that the decreased number of β receptors in the rat compensatory hypertrophy would lead to a decrease in the stimulatory effect of β-agonist. Moreover if the G regulatory protein of the Ca^{2+} channel is modified it would affect the Ca^{2+} current of the hypertrophied myocyte.

ß-ADRENERGIC SYSTEM

Compared to the Ca^{2+} channels or the Na^{+}/K^{+} ATPase, the ß-adrenergic receptors are scarce in cardiac myocytes. Nevertheless, according to the amplification mechanism of the ß-adrenergic system through coupling to G proteins then activation of the catalytic subunit, the binding of ß-agonists induces a high inotropic response. As a consequence of the increase in the intracellular levels of cAMP, protein kinases are activated promoting the phosphorylation of

various proteins within the myocardial cell. Phosphorylation of the Ca^{2+}channels increases the Ca^{2+}influx during each action potential plateau (9) ; at the SR level, that of phospholamban stimulates the Ca^{2+}-ATPase activity, and hence the relaxation and contraction (26) ; at the myofibrillar level, phosphorylation of TnI enhances the Ca^{2+} affinity of TnC. By these ways the ß-adrenergic system participates in the regulation of the inotropic response of many other systems.

Two subtypes of ß-receptors have been characterized first by their pharmacological properties then by cloning the cDNA of their mRNA (27). ß1 and ß2 receptors are transcribed from two genes. They are glycoproteins with a molecular mass of 64,000 D. The two subtypes are expressed in human heart (28) while only the ß1 subtype is expressed in rat myocytes (29).

Expression of these cardiac receptors is regulated through : 1) the action of circulating catecholamines (homologous regulation). Desensitization and internalization of the ß-receptors occur after binding of an agonist and rapidly result in a reduction in the number of ß-receptors. Down-regulation observed after a long period of stimulation by ß-agonist is maintained by regulation of the gene expression. On the other hand, up-regulation was observed after binding of an antagonist. 2) presence of a cAMP responsive element (30) on the gene. This down-regulation of transcription is observed after ß-agonist action and results in a direct increase in cAMP levels. 3) action of thyroid hormone. There is general agreement that the density of ß-adrenergic receptors does increase during hyperthyroidism, and the opposite effect occurs during hypothyroidism. The presence of a glucocorticoid responsive element (31) on the gene is involved in this regulation.

ß-adrenergic receptors in the hypertrophied heart.

In the hypertrophied heart, the regulation of the number (table II) and the function of ß-receptors is well documented in man and in experimental animal models. It is often associated with a reduction of the inotropic responsiveness to catecholamines (32).

In man

In the human heart (28, 33), there is a decrease in the number of ß-receptors which is more pronounced at the terminal stage of heart failure . This down-regulation is correlated with

an elevated level of circulating catecholamines and is mainly carried on by the ß1-subtype which is the predominant subtype in the normal left ventricle (B1/B2 =65/35) (33). Hence the inotropic and chronotropic effect of the ß2 subtype which in the hypertrophied heart might become the predominant subtype, has to be considered during the therapy of the failing heart. In fact, the treatment of cardiac insufficiency by a ß-agonist which would have been beneficial during the compensatory phase of hypertrophy appeared to be deleterious at the end stage of heart failure by increasing the cAMP level and cousing cardiac arhytmia (34). In order to prevent a deleterious down- regulation of the receptors and still get an inotropic effect, the use of selectif ß1 agonists with partial antagonist activity has been tested (35). Another way to get an inotropic effect without down-regulation of the β-receptors by using PDE inhibitors and in particular PDEIII. The positif inotropic effect of amrinone and milrinone is proportional to the inhibition of PDEIII and is accompanied by a very slight chronotropic effect and a vasodilatator effect of the arterial and veinous circulation (36). Although these effects are beneficial for a short period, it seems that the PDE inhibitors also increase the rate and severity of ventricular arythmias in severe heart failure(37).

Table II: Number of β -receptors in the hypertrophied heart.

	Model	Bmax (fmol/mg)	Density*	Total number**	Ref
Man	FH	24 (52)	↓		(28)
		32 (59)	↓		(33)
Rat	SHR	34 (48)	↓		(38,39)
		188 (480)	↓	↓	(85)
	RHR	22 (37)	↓	=	(40)
		75 (110)	↓		(41)
		51 (38)	↑	↑	(38)
	AS	25 (31)	↓	=	(42)
		160 (480)	↓	↓	(85)
Dog	AS	111 (61)	↑	↑	(44)
Guinea pig		54 (28)	↑		(45)

*( ): control values. * calculated assuming that the surface/volume ratio of the myocytes and the yields of the membrane preparations of hypertrophied and control hearts were identical and ** according to the % of hypertrophy. FH: failing heart; SHR: spontaneously hypertensive rats; RHR: renal hypertensive rats; AS: aortic stenosis.*

In rat

In the hypertrophied rat heart, the regulation of ß-adrenergic receptor density has been studied in SHR (38, 39), RHR (38, 40, 41), and pressure overload induced hypertrophy (42). Despite a normal content of circulating catecholamines (40), the density of the ß-receptors is decreased in the pressure overload model. It is also decreased in all the studies concerning the SHR model where an elevated level of catecholamines has been described but the results in the RHR model are contracdictory (table II). However, most of these studies have been conducted without any distinction of the ß1 and ß2 subtypes although the ß1 subtype is the only one present on the rat myocyte (29). The ß2-subtype which represented 15 % of the receptors in the normal heart is localised on the non-myocardial cells. One report (43) which takes into account this repartition, shows that the 30 % decrease observed in compensatory hypertrophy concerns only the ß1 subtype. Moreover, a new low-affinity ß1 subtype has been characterized. In fact, the decrease in the number is related to a decrease in the density of receptors whereas the total number of receptors calculated per left ventricle is unchanged. This can be applied to most of the models of the hypertrophied rat heart .

In other species

In other species, contradictory results have been published and this might depend of on the stage of the disease. In the dog with cardiac failure the number of receptors is increased but their affinity is reduced (44). Whereas an increase in receptor density has been reported in guinea-pigs (45).

Regulation of the inotropic response

There is no obvious correlation between the density of the β receptors and the inotropic response (32). The latter is often diminished, rarely unchanged whatever the density of receptors. These data which do not discard the role of the density of the β receptors in the inotropic response argue in favor of additional levels of regulator.

A very recent paper (6) on the human failing heart deals with a direct relationship between the reduced inotropic effect and the enhanced level of G_i protein. In the hypertrophied

heart, the role of G protein has been suspected in RHR (38) and dogs (44) but there are still few results concerning their precise regulation during hypertrophy. In compensatory hypertrophy of the rat heart (43), the new low affinity β1-subtype argue for a defect in the coupling of the receptor and the Gs protein. It recently appears that the level of the αs subunit of the G protein and of its mRNA correlate rather well to the inotropic response during chronic dynamic exercise or in volume overload pigs independently of the of ß-receptors density (46).

Measurements of the adenylate cyclase activity in various species and models indicate an unchanged activity except in RHR (38) and dogs (44).

Consequences of a treatment with ß-agonist will only be fully understood and previsible after further investigation on the characteristics of the three components of the adrenergic system will be completed at each stage of the disease.

Ca^{2+}-ATPase FROM SARCOPLASMIC RETICULUM

The sarcoplasmic reticulum (SR) is a highly organized intracellular membrane system which plays a critical role in the contraction-relaxation mechanisms. The junctional SR where the ryanodine receptor is localised (47, 48) is responsible for the Ca^{2+} efflux needed for the contraction of myofibrils (49). The longitudinal SR contains the Ca^{2+}-ATPase which is responsible for the uptake of Ca^{2+} liberated during relaxation and participates in the low $[Ca^{2+}]_i$ level during diastole with the Na^+/Ca^{2+} exchange and the Ca^{2+}-ATPase from SL(50).The Ca^{2+}-ATPase is a membrane protein with a molecular mass of 110,000 D that constitutes the major part of the proteins in cardiac SR. Recently, Mc Lennan and co-workers (51-54) have cloned and sequenced the cDNA encoding four distinct Ca^{2+}-ATPase isoforms. Two isoforms are the products of one gene in fast-twitch muscle. The other two are producted by alternative splicing of a second gene and one of them is the slow-twitch/cardiac isoform. One major difference between the fast-twitch and cardiac Ca^{2+}-ATPase is the lower activity and the regulation by phospholamban of the latter one (26). Phospholamban is a low molecular mass protein (22,000 D) which is also present in the longitudinal SR and whose phosphorylation by cAMP-dependent protein kinase activates the Ca^{2+} uptake.

Ca^{2+}-ATPase from SR in the hypertrophied heart

In cardiac hypertrophy due to pressure overload, alteration of the SR properties have been suspected because 1) the duration of isometric contraction and relaxation were prolonged (55, 56) ; 2) the Ca^{2+} transient, measured by aequorin, was also prolonged in human heart failure and in hypertrophied ferret hearts (57, 58); 3) the tension independent heat (TIH) which can be related to the movements of $[Ca^{2+}]_i$ was decreased and the time to reach the TIH peak was delayed (59) ; 4) very recently, Ca^{2+} reuptake has been shown to be slower in partially skinned cardiac fibers from mechanical overload rat hearts (60). These properties were thought to be due to impaired Ca^{2+} handling and to a decreased activity of the SR Ca^{2+} pumping system.

In fact, Ca^{2+} transport was shown to be reduced in isolated SR from hypertrophied hearts of man (61), rabbit (62) calf (63) and rat (SHR (64), RHR (21) and pressure overload models (65)). The decrease in Ca^{2+}-ATPase (21, 62-65) activity was observed despite differences in the purification procedure and the yield in SR proteins between hypertrophied and normal myocardium. It might be related to changes in the turn-over rate of the enzyme and/or the density of the Ca^{2+}-ATPase. These two possibilities have been carefully investigated in a recent paper that also examined the regulation of expression of the gene coding for the Ca^{2+}-ATPase (65).The authors show that oxalate-stimulated Ca^{2+} uptake/mg protein was decreased in mild and severe compensatory hypertrophy induced by pressure overload in the rat. It was related to a parallel decrease in the density of functionally active Ca^{2+}-ATPase in severe hypertrophy. Moreover the total amount of Ca^{2+}-ATPase estimated by immuno-blot analysis was the same in LV'S from both hypertrophied and control hearts. By S1 nuclease mapping, they demonstrated that the same Ca^{2+}-ATPase mRNA was expressed in the two groups and by dot-blot analysis they quantified the level of this mRNA. Their results which show a decrease in the mRNA concentration confirm those of Komuro et al.(66) and Nagai et al. (67). However, it must be pointed out that the total content of Ca^{2+}-ATPase mRNA per left ventricle remains unchanged in normal and hypertrophied hearts. Taking into account their results on the activity, number and gene expression of the Ca^{2+}-ATPase and also the results from the morphometric studies of Anversa (1, 2) on the increase of SR area ($\alpha 2$) in the hypertrophied myocyte, the authors

conclude that the decrease in Ca^{2+} pumping activity observed in hypertrophied heart was not due to a qualitative alteration of the Ca^{2+}-ATPase gene expression but rather to a relative diminution of the density of the Ca^{2+}-ATPase which might be explained by a maintained level of expression of the Ca^{2+}-ATPase gene. However another regulatory mechanism might occur in cases of mild hypertrophy since there is no significant decrease in the density of the Ca^{2+}-ATPase whereas the Ca^{2+} uptake was decreased.

Regulation of Ca^{2+}-ATPase function

The role of phospholamban whose phosphorylation activates the Ca^{2+}-ATPase is as yet poorly documented in the hypertrophied heart. However, the first indication of a possible role was found by Nagai et al. (67). In hypertrophied rabbit heart they observed a decrease in the level of phospholamban mRNA which is parallel to the decrease in the level of Ca^{2+}-ATPase mRNA . Regulation of SR functions also occured through β-adrenergic system whose activity is often impaired during hypertrophy. The implication of such an alteration has not been extensively studied. In SHR (68), a strong correlation between the decreased Ca2+ accumulation and the reduced cAMP-dependent protein kinase activity argue in favor of such a regulatory role. Finally, other factors such as calsequestrin may also regulate the SR activity but their role is still unknown in the hypertrophied heart.

Na^+/K^+ ATPase ISOFORMS

The Na^+/K^+ ATPase is an oligomeric functional unit of two α catalytic subunits with a molecular mass of 112,000 D and two β subunits with a molecular mass of 45,000 D. The Na^+ pump extrudes 3 Na^+ ions against 2 K^+ ions and thus participates in the intracellular homeostasis of Na^+ and K^+. Its inhibition by specific cardiac digitalis such as ouabain enhances the $[Na^+]_i$ which in turn leads to an increase in $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchange and to a positive inotropic effect. Until recently, the only way to identify the Na^+/K^+ ATPase isoforms was their high or low sensitivity to ouabain as measured by inhibition of activity, ouabain binding, and inotropic effect and their separation after electrophoresis.

The molecular basis of these effects have been clarified by the molecular cloning of three mRNA's coding for three isoforms of the α catalytic subunit (69). The α1 mRNA codes for the ubiquitous low-affinity α1 isoform, the α2 mRNA for the high-affinity isoform which is highly represented in brain; the α3 mRNA is expressed in brain and in neonatal rat heart and corresponds to a high-affinity isoform (70, 71).

Na^+/K^+ ATPase properties in the hypertrophied heart.

The properties of the Na^+/K^+ ATPase from hypertophied heart have been studied in various species.

Table III: Na^+/K^+ ATPase activity or ouabain binding site number in the hypertrophied heart.

	Model	Bmax (pmol/mg)	Activity (µmol Pi/mg/h)	Density*	Total number**	Ref
Man	CM	331 (559)		↓		(72)
Cat	PAS	3,9 (5,2)		↓		(75)
Pig	AS		3,9 (5,6)	↓		(73)
Hamster	CM		2,4 (4,5)	↓		(73)
			9,1 (20)	↓		(74)
Rat	SHR	4,7 (8,9)	87 (133)	↓	↓	(76)
			62 (144)	↓	↓	(18)
	RHR		8 (14)	↓	↓	(79)
			140 (133)	=	↑	(21)
	AS (HA)	3,8 (2,1)		↑	↑	(4)
	AS (LA)	80 (148)		↓	=	(4)

*( ): control values. * calculated assuming that the surface/volume ratio of the myocytes and the yields of the membrane preparations of hypertrophied and control hearts were identical and ** according to the % of hypertrophy. CM: cardiomyopathy; PAS: pulmonary aortic stenosis; AS: aortic stenosis; SHR: spontaneously hypertensive rats; RHR: renal hypertensive rats.*

As shown in table III, a decrease in the number of ouabain binding sites and/or in the Na^+/K^+ ATPase activity has been observed in human (72) and syrian hamster CM (73, 74), in

pig (73), cat (75) and hypertrophied hearts. In rat, the pattern is more complex, depending of which model is being studied. In SHR, there is a decrease in the number of sites (18, 76, 77); in aortic stenosis model, the number of low affinity binding sites is reduced whereas the number of high affinity binding sites increases two fold (4) ; in RHR the total activity is unchanged (21). By measuring the dissociation constant for ouabain or the EC50 of the inotropic effect the sensitivity to ouabain of the hypertrophied heart has been shown to be unchanged (41) or increased (78) in man, increased in cat (75) dog (78) hamster (73) SHR (76, 77) unchanged in RHR. The inotropic effect is prolonged in rat aortic stenosis (5).

According to these quantitative or/and qualitative modifications the inotropic responsiveness to ouabain must be modified. For example, we have shown in rat heart hypertrophied by pressure overload that a five to seven fold increase in the dissociation rate constants of the high-and low- affinity sites is responsible for the prolonged inotropic effect of the drug. Moreover, the decrease in the toxic effect might be due to a decreased number of low affinity sites. However, it is worthy to note that the correlation between changes in the number or affinity of the Na^+ pump and the inotropic response is not clearly established.

Regulation of the Na^+/K^+ ATPase isoforms in the hypertrophied heart.

Although most of these results did not take into account the presence of the various isoforms of the catalytic subunit of the Na^+/K^+ ATPase, we might assume that during hypertrophy, there is a regulation of the genes coding for these isoforms. Indeed, this regulation has been observed during the ontogenic development of the rat heart (70, 71) and under the influence of thyroid hormone (80). It has been shown that the level of $\alpha 1$ mRNA which is the predominant Na^+/K^+ ATPase subunit in the rat myocardium does not vary either under the influence of age or triiodothyronine (T3) whereas the $\alpha 2$ mRNA, absent in the fetal and neonatal heart is up regulated after birth and by T3. The $\alpha 3$ mRNA present in fetal and neonatal hearts, disappears in the young animal but is still up regulated by T3. Hence, a highly complex pattern of regulation of at least three isoforms may occur during hypertrophy : 1) a quantitative

up- or down-regulation of the isoforms already present 2) a qualitative regulation of the expression of an isoform absent from the control or the expression of mutated genes.

Gene expression of the catalytic subunits of the Na^+/K^+ ATPase has been studied in two genetic hypertensive rat models (81, 82). In these models, the mRNA level of the predominant $\alpha 1$ isoform is unchanged and the $\alpha 2$ is reduced. In fact, the important factor appears to be a single mutation of the $\alpha 1$ gene which codes for an $\alpha 1$ isoform with reduced Na^+/K^+ ATPase activity (See Table III).

To explain the increased density and total number of the high-affinity binding sites in rat compensatory hypertrophy (4) an increased accumulation of mRNA coding for a high-affinity form might have been suspected. In fact, preliminary results (83) indicate that 1) the $\alpha 3$ isoform which is expressed in the neonatal heart is not detected using the $\alpha 3$ specific probe in the hypertrophied rat heart although the enzymatic properties of the neonatal Na^+/K^+ ATPase are similar to those of the hypertrophied heart. 2) the expression of the $\alpha 2$ is decreased . According to these results, it appears that other regulatory factors might be involved either at the level of the gene (another yet undetected isoform ?) or of the membrane (phospholipids, regulatory proteins).

In man since the affinity is increased and the density is decreased quantitative and qualitative regulation of the gene expression might occur.

Among the heterologous factors which might regulate the inotropic effect of cardiac digitalis, the Na^+/Ca^{2+} exchange might be a good candidate. In two recent studies (21, 84), the activity and the Ca^{2+} affinity of the Na^+/Ca^{2+} exchange was depressed in the RHR and the compensatory hypertrophy of the rat heart was decreased. Thus the reduced efflux of Ca^{2+} during relaxation might participate into the positive inotropic responsiveness to digitalis.

CONCLUSION

The data reported in this review suggest that the adaptive mechanism of the membrane proteins to an increase in hemodynamic work is not homogeneous. Moreover, they emphasize the necessity to estimate not only the density of the receptors but their total number per myocyte

or per heart. Three different types of adaptation have been observed in compensatory hypertrophy of the rat heart and are discussed for other models.

1) The density of the membrane proteins is unchanged whereas their total number is increased. The Ca^{2+}-channels estimated by the number of DHP receptors represent an example of this regulatory pathway (16). These conclusions are also supported by the measurement of the Ca^{2+} current in hypertrophied myocytes (24). They suggest that the increase in total number which is proportional to the hypertrophy is related to activation of the gene coding for the Ca^{2+}-channel. This adaptational process might be overdeveloped in aged SHR and in the hamster cardiomyopathy since the receptor density is increased.

2) The density of the membrane proteins is reduced but their total number per myocyte or per left ventricle remains unchanged. In compensatory hypertrophy of the rat heart, this is the case for the ß1-receptors (43), the α1 low-affinity form of the Na^+/K^+ ATPase (4) and the Ca^{2+}-ATPase from SR (65). Moreover, analysis of the accumulation of the Ca^{2+}-ATPase mRNA strongly suggests that the expression of the cardiac Ca^{2+}-ATPase gene is neither up- nor down-regulated. The unchanged total number of ß1-receptors also suggests that the expression of the genes coding for these proteins remain unchanged. Hence, the decreased density of these receptors observed during compensatory hypertrophy does not seem to be related to a down-regulation as often assumed in similar models. In the human heart however the constant decrease of the ß1-receptors is associated with the severity of the disease and argues in favor of a down-regulation.

3) the density and the number are increased together with a modification of the properties of the receptors. This occured for the high-affinity binding sites of the Na^+/K^+ ATPase (4) but actually we don't know whether a new receptor isoform is expressed or whether adaptation comes from other factors (83). It seems neither related to the mRNA accumulation of the α2 isoform nor to the expression of the α3 mRNA. However, the regulation of the two types of ouabain binding sites is totally different . The increase in ouabain sensitivity observed in other species and in heart failure in man might also depend more on the regulation of the high-affinity form than on that of the low-affinity form.

It appears that the most numerous studies have been performed on the mechanisms involved in the inotropic response of therapeutic drugs, particularly ß-agonists and cardiac digitalis. However modification of other receptors even scarecely represented in the membrane might play a role in the contraction regulation of the hypertrophied heart and would deserve complementary studies. In this context, studies on α1- and muscarinic receptors (45,43,86) and on Na^+/Ca^{2+} exchange (21,84) are still poorly developed in the hypertrophied heart. Their adaptation mechanism is similar to that of the β-adrenergic mechanism. Studies on angiotensin II, histaminic, purinergic receptors are almost absent. Characterization of the G proteins involved in the coupling mechanism of the adrenergic system is in constant progress (6). Among the channels, there is no extensive report on the Na^+ and the K^+ channels although the latter might be involved in the lengthening of the action potential. The Ca^{2+} release channel from SR is another example of a yet unstudied channel. This is certainly due to its very recent characterization in the normal cardiac SR (87) . There is no doubt that investigation of the properties of this receptor in the hypertrophied myocyte would help to solve the problem of Ca^{2+} handling and of E-C coupling.

Finally, the regulation of expression of the genes coding for the membrane receptors of the hypertrophied myocyte is the next step necessary to enable us to understand this inhomogeneous regulation. The development and therapeutic use of new inotropic agents depend on the precise knowledge and evolution of their targets during hypertrophy.

SUMMARY

We report and compare the qualitative and quantitative modifications of membrane receptors in the hypertrophied heart of different species. In the hypertrophied rat heart, according to the morphometric results of Anversa on the increase in surface area of the myocyte, we suggest that three types of adaptational mechanism occur. The first type which is observed for the Ca^{2+} channels is responsible for an increased number of receptors with an unchanged density. This adaptational process maintains a similar Ca^{2+} current density in the normal and the hypertrophied myocyte. In the second mechanism the number of receptors remains constant which results in a decreased density. This was observed for the ß-receptors and Ca^{2+}-ATPase from sarcoplasmic reticulum. In the case of the Ca^{2+}-ATPase, the study of the mRNA coding for the cardiac isoform confirmed that the same gene was expressed without up- or down-regulation. In the third mechanism, observed for the high-affinity form of the Na^+/K^+ ATPase, both the number and density of receptors were increased, and the properties of the low- and high-affinity sites of the Na^+/K^+ ATPase were modified. In other species, the same types of adaptation may occur during compensatory hypertrophy. However, it seems likely that in hypertrophied heart from aged rats or during severe hypertrophy or cardiac failure in man, these regulations are overpassed. For example, the decrease in ß1-receptors at the terminal stage of heart failure is probably due to a down-regulation of the gene which was not observed during compensatory hypertrophy. Expression of the results both in density and total number is essential for a valid interpretation of the qualitative and quantitative modifications occuring at the membrane level. The search for new inotropic drugs must take into account these modifications of their targets.

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9

SIGNAL TRANSDUCTION IN MYOCARDIAL HYPERTROPHY AND MYOSIN EXPRESSION

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Myocardial hypertrophy is associated with a number of heart diseases and is of great clinical relevance. Despite many research efforts, the signals involved in the hypertrophy process are still poorly understood (1-4). Recently, it was found that the subcellular structure of the myocyte differs in various forms of hypertrophy. Great emphasis has been placed on gene expression of the myosin heavy chains (MHC) which determine the isoenzyme population of myosin. The myosin isoenzymes influence the ATPase activity (5,6), the energetics of the cross bridge cycle (7,8), oxygen consumption of the whole heart (9,10) and the mechanics of heart muscle (11-13). Although changes in the MHC expression are less relevant for the heart of large mammals with predominantly V_3, they nonetheless provide the unique possibility of tracing an altered mechanical performance of the heart to an altered gene expression. Because there is increasing evidence that in a number of functional states the MHC expression is associated with coordinated changes in the activity of the Ca^{2+}-stimulated ATPase of sarcoplasmic reticulum (14,15), a better understanding of the regulation of gene expression of myosin should help also in understanding the regulation of the activity of the sarcoplasmic reticulum Ca^{2+}-pump. At present, our knowledge of the

signals involved in myocardial hypertrophy and altered MHC expression is still very fragmentary and a unifying concept has not yet evolved. The situation is particularly complex because not all interventions which cause cardiac hypertrophy influence MHC expression. It was, therefore, considered important to compare the possible signals involved in the induction of hypertrophy and in MHC expression, taking into account also interventions which result in reversal of cardiac hypertrophy.

Thyroid-dependent growth of the heart

The effect of thyroid hormones on myocardial hypertrophy and MHC expression is well documented. Most of the evidence is based on pharmacological interventions which involve either surgical or chemical thyroidectomy or the injection of high doses of thyroid hormones. The changes seen in myosin isoenzyme distribution are regulated at the nuclear level because the concentrations of the respective proteins and mRNAs are directly correlated (16). It is typical for the effect of thyroid hormones that the genes encoding for alpha-MHC and beta-MHC are regulated in an antithetic manner, whereby the expression of the alpha-MHC gene is favoured by thyroid hormones. Because the promoter region of the myosin gene contains a T_3 receptor binding site, thyroid hormones seem to influence directly myosin gene expression (17,18). The comparison of different pharmacological doses of thyroid hormones shows that at thyroid hormone concentrations where a nearly complete switch in myosin expression occurs, the maximum stimulus for cardiac growth has not been reached. Thus, at a daily dose of

0.2 mg/kg T_4, a nearly homogeneous V_1 was induced, whereas with 1 mg/kg T_4 no further effect on myosin expression was observed but the degree of hypertrophy still increased (unpublished). The effect of thyroid hormones on cardiac growth is particularly pronounced when T_4 was injected into rats which have previously been chemically thyroidectomized. The ventricular weight increased by 70% within 3 weeks which was associated with a transition of myosin expression from V_3 to V_1 (unpublished). The cardiac hypertrophy seems not to be mediated by alpha- or beta-receptors (19). Because the increased cardiac output of hyperthyroid rats is not affected by beta-receptor blockade, the mechanical activity could provide a signal for cardiac growth (19). This would be in accordance with the finding that thyroid hormones did not prevent atrophy of heterotopically transplanted hearts with a greatly reduced cardiac output (20,21). One should, however, take also into account that T_3 has direct anabolic effects in cultured cardiac myocytes (22). Compared with the effect of thyroid hormones, much less is known on the signals involved in clinically more relevant types of myocardial hypertrophy.

Pressure overload of the heart

Pressure overload of the heart results in myocardial hypertrophy and changes myosin expression in favour of beta-MHC (23). Any theory which tries to explain these events has to take into account the much less studied process of reversal of cardiac hypertrophy. In the case of pressure overload arising from constriction of the renal artery, the hypertrophy

and the increased expression of beta-MHC are normalized after removing of the load by unclipping of the renal artery (24) (Fig.1).

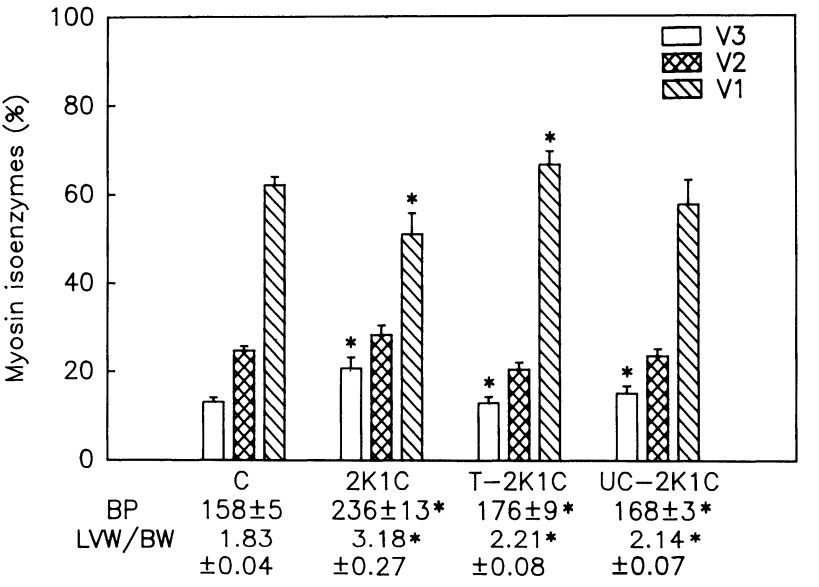

Fig.1 Myosin isoenzyme populations of rat left ventricles of normotensive Wistar control rats (C), renal hypertensive rats (two-kidney, one-clip Goldblatt; 2K1C), renal hypertensive rats treated with felodipine (0.5 mg/g food) and metoprolol (6 mg/g food) (T-2K1C) and renal hypertensive rats in which the renal artery constriction was removed (UC-2K1C). The values of blood pressure (mm Hg) and of the ratio of left ventricular weight (mg) to body weight (g) are means ± S.D.; * statistically significant ($P < 0.05$) from C in the case of 2K1C or from 2K1C in the case of T-2K1C or UC-2K1C; data from ref. 24.

Nephrectomy of the ischemic kidney also normalized myocardial hypertrophy and MHC expression (25). A reversal of hypertrophy and MHC expression was also observed when the high blood pressure was normalized by a combined treatment with felodipine and metoprolol (Fig.1) or with a converting enzyme

inhibitor (26,27). A treatment with clonidine, hydralazine and furosemide had, however, no effect on myocardial hypertrophy and myosin expression although blood pressure was reduced from 215 mm Hg to 173 mm Hg which was, however, still higher than that of the control rats (26).

If the increased wall stress were the decisive signal in the pressure overloaded heart for cardiac growth, one could assume that stretch-sensitive systems were involved which could trigger a second messenger cascade affecting growth and myosin expression. Possible signals which are sensitive to stretch could involve the adenylate-cyclic AMP system (28) or ion channels (29). The identity of the initiating signal remains to be shown also for the proto-oncogene concept which assumes a coordinated action of growth factors, growth factor receptors, intracellular transducers and transcription factors (30,31).

There is general agreement that the signals responsible for the increased beta-MHC expression in the heart with a defined pressure overload are not likely to arise from altered circulating thyroid hormones (32). Because in models of pressure overload where the overload is restricted to one chamber, the unaffected chamber exhibited only a slight increase in V_3 (32) or beta-MHC mRNA (33), the stimulus for growth and myosin expression seems to be closely linked to the increased load. The increase in V_3 of the apparently unloaded ventricles of rats with aortic stenosis can most probably not be attributed to a reduced thyroid influence (32). In the case of right

ventricular hypertrophy induced by ingestion of *Crotalaria spectabilis* seeds, the proportion of V3 was markedly increased in the non-hypertrophied left ventricle (34). Because in the *Crotalaria spectabilis* treated rats the blood thyroxine concentration was reduced, a reduced thyroid influence could be responsible for the increased beta-MHC expression. In the aldosterone-salt hypertensive rats, the non-hypertrophied ventricle exhibited the same increase in V_3 as the overloaded left ventricle (35). Although blood thyroxine was reduced, it cannot solely explain the biventricular change in MHC expression because one would nonetheless expect to see the effect of the left ventricular overload (35). Whether a volume overload of the right ventricle contributes to the MHC expression, remains to be shown.

Compared with the models of pressure overload where the overload is clearly defined, the signals for hypertrophy and MHC expression are less clear in the case of spontaneous hypertension. Although the high blood pressure of spontaneously hypertensive rats (SHR) results in myocardial hypertrophy and a switch in myosin expression in favour of beta-MHC (36), no complete reversal of these processes has yet been achieved. Physical exercise in the form of swimming or spontaneous running lowered the high blood pressure of SHR but did not result in a ventricular weight typical of exercised normotensive rats (37). Both swimming exercise and spontaneous running lowered blood pressure to a similar extent, the proportion of V_3 was reduced, however, only by swimming (37) (Fig.2).

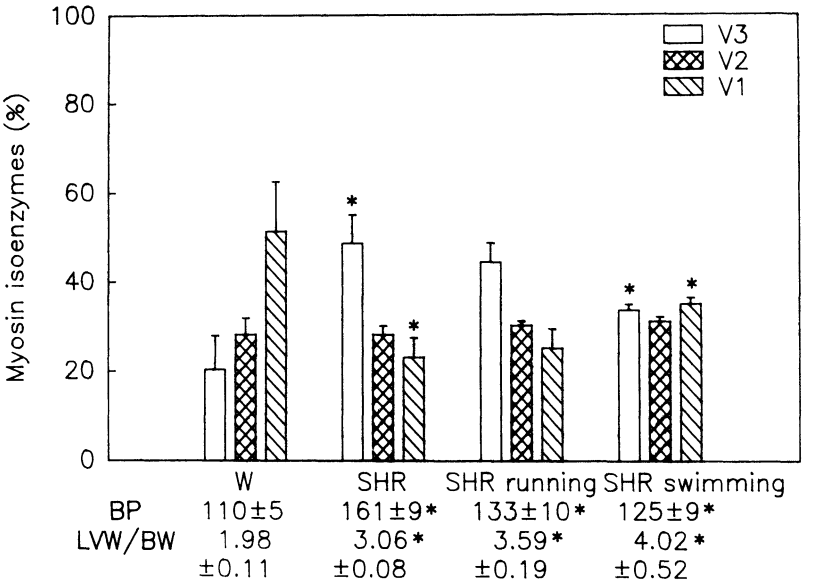

Figure 2. Myosin isoenzyme populations of rat left ventricles of sedentary Wistar rats (W), sedentary SHR (SHR), SHR running spontaneously (SHR running) and swim exercised SHR (SHR swimming). The values of blood pressure (mm Hg) and of the ratio of left ventricular weight (mg) to body weight (g) are means ± S.D.; * statistically significant ($P < 0.05$) from W in the case of SHR and from SHR in the case of running SHR or swimming SHR; data from ref. 37.

Because swimming had a comparable effect in normotensive rats (36-39), the switch in myosin expression is most likely not a result of blood pressure reduction, but is a characteristic feature of swimming.

Normalization of blood pressure by pharmacological treatment is also not necessarily associated with a reversal of hypertrophy or normalization of MHC expression in SHR. Thus, treatment of SHR with the vasodilator hydralazine for 12 weeks normalized blood pressure but reduced hypertrophy only

slightly and had no significant effect on MHC expression (Fig.3).

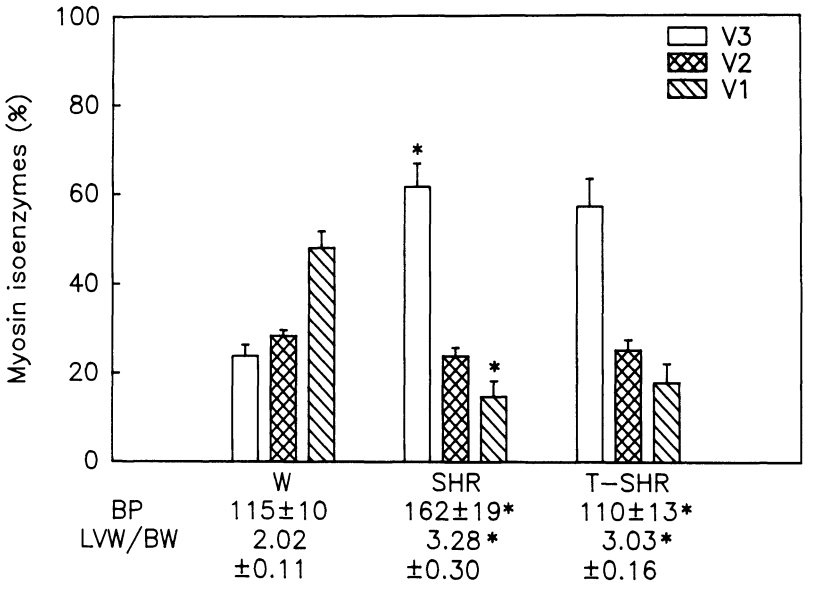

Fig.3 Myosin isoenzyme populations of left ventricles of Wistar rats (W), SHR and SHR treated with hydralazine (80 mg/l drinking water) (T-SHR). The values of blood pressure (mm Hg) and of the ratio of left ventricular weight (mg) to body weight (g) are means ± S.D.; * statistically significant ($P < 0.05$) from W in the case of SHR or from SHR in the case of T-SHR.

In the study of Nagano (40), the proportion of V_3 was slightly reduced in hydralazine-treated SHR but did not reach the level seen in normotensive rats although blood pressure was again normalized. One might argue that the incomplete reversal of MHC expression could arise from the reflex stimulation of the sympathetic nervous system which is typical for a vasodilator therapy. Because in isolated myocytes, where the

effect of catecholamines can precisely be controlled, catecholamines resulted in an increased proportion of V_1 (41), it is unlikely that the high proportion of V_3 arises from an enhanced adrenergic influence. Captopril which also normalized blood pressure and reduced hypertrophy to a greater extent than hydralazine had only a small effect on V_3 (40). In SHR blood pressure control on its own is thus not sufficient for normalization of hypertrophy or MHC expression. These findings are intriguing because hypertrophy is present at normal blood pressure and the wall stress is expected to be lower than in the heart of normotensive rats.

Alpha-methyldopa had only a slight effect on blood pressure, but nonetheless reduced hypertrophy to the same extent as hydralazine (40). Myosin expression was not changed. When the beta-adrenergic blocking drug bunitrolol or the Ca^{2+} antagonist verapamil were given to SHR, blood pressure was not lowered, but hypertrophy was reduced. Myosin V_3 was again not affected. Because methyl-dopa, bunitrolol and verapamil which all reduce the influence of the adrenergic system lowered the degree of hypertrophy irrespective of the pressure overload, the adrenergic system seems to have a modulatory effect on cardiac growth (42). Taken together, the data show that the mechanism by which the overload is removed is critical and that not all treatments which normalize the cardiac load normalize also the cellular signals which stimulate cardiac growth and increase the beta-MHC expression. Thus, stretch sensitive systems are either not of primary importance for

inducing hypertrophy and changing MHC expression or the intracellular messenger cascade can be a target for alterations.

Compared with the effect of short-term pressure overload typically not exceeding 3-4 months, our understanding of the processes involved in long-standing pressure overload is still limited. Of particular interest are the signals which are responsible for the transition of the compensated heart with concentric hypertrophy to the dilated failing heart. In the case of old SHR, the dilated ventricles exhibited a higher proportion of V_3 compared with ventricles with compensated hypertrophy (43). In accordance, cardiac norepinephrine depletion which most probably precedes dilatation was observed in ventricles with the highest proportion of V_3 (43).

Volume overload of the heart

Volume overload induces also myocardial hypertrophy and a switch in gene expression in favour of beta-MHC (44,45). A comparison of the effect of pressure overload due to renal hypertension and volume overload due to an abdominal arteriovenous shunt demonstrates that both the degree of hypertrophy and the redirection of MHC expression are greater in the volume overloaded hearts (Table 1). When pressure overload and volume overload were combined, systolic wall stress was greatly increased and high output failure occurred (46). Thus, rats with combined renal hypertension and arteriovenous shunt exhibited after 6 months typical symptoms of congestive heart failure, such as systemic oedema (46). The myocardial working capacity was reduced and the time-dependent parameters were

decreased. Also in this model, myocardial hypertrophy and MHC expression are more markedly affected than in pressure overloaded hearts (Table 1).

Table 1. Effect of pressure overload due to renal hypertension (2K1C) and volume overload due to abdominal arteriovenous shunt (AV) and a combination of both overloads (2K1C + AV) on absolute left ventricular hypertrophy and myosin isoenzyme populations.

	Hypertrophy	V_3 (%)	V_2 (%)	V_1 (%)
Control		26.3±4.5	27.6±1.5	46.1±6.0
2K1C (8 wk)	+24.7%	30.1±4.7	32.3±1.0*	37.6±5.3
Control		10.8±1.9	22.3±2.0	66.9±2.7
AV (10 wk)	+43.1%	36.0±9.1*	35.7±2.8*	28.3±9.7*
Control		33.5±2.0	33.0±0.6	33.5±2.1
2K1C (24 wk)	+31.5%	43.2±1.5*	32.8±4.0	24.0±1.7*
Control		29.9±4.1	30.2±1.1	39.9±5.3
2K1C + AV (24wk)	+57.0%	59.7±4.7*	26.6±3.0	13.7±2.1*

Values are means ± S.D; * significantly ($P < 0.05$) different from control. The operation techniques used for 2K1C and AV shunt rats were the same in all groups. The duration of the overload is given in brackets. Data from refs. 9, 45, 46.

Based on the data of rats with high output failure and SHR with low-output failure it can be concluded that in the stage of heart failure the expression of beta-MHC persists or is even increased. Whether the neuroendocrine mechanisms operating during cardiac failure provide additional signals for the expression of beta-MHC remains to be shown.

Physical exercise

Compared with pressure overload or volume overload where myocardial hypertrophy seems to be associated invariably with a switch in myosin expression in favour of beta-MHC, physical

exercise represents a more heterogeneous type of load. Spontaneous running of rats in activity wheels for 10-12 h/day resulting in up to 15 km/day induced absolute left ventricular hypertrophy but did not significantly affect MHC expression (37). This finding is in accordance with previous reports showing that enforced running routines provided a sufficient load for inducing cardiac hypertrophy but had either no or only a slight effect on myosin ATPase activity (47). The effects of enforced running routines are, however, more complex because an enforced routine is expected to be associated with ill-defined effects of stressors. The fact that a dissociation occurs between the signals resulting in cardiac hypertrophy and those affecting MHC expression is important because it demonstrates that the signals resulting in hypertrophy do not necessarily affect MHC expression.

The unique effect of swimming exercise on alpha-MHC expression cannot be attributed to a higher exercise intensity. Swimming exercise is considered to be mild, corresponding to 50-65% of the aerobic capacity (48). The effect of swimming exercise on myocardial hypertrophy seems also not to be greater than that of spontaneous running (37). The signal for the increased alpha-MHC expression in the swim exercised rat is most likely associated with the marked adrenergic drive of swimming (37). Although one could in principal assume that thyroid hormones contribute to the higher proportion of V_1, the experimental evidence is not in favour of a significant role of blood thyroid hormones (unpublished).

Because myocardial hypertrophy is not necessarily associated with an altered MHC expression, the classification of cardiac hypertrophy in terms of "physiological" or "pathological" based on the switch of MHC expression or the related myosin activities (39,49) seems not sufficient for describing the complex reactions operating in the heart. This concept nonetheless points out the intriguing fact that in the pressure or volume overloaded heart the hypertrophy process seems invariably linked to a reduced expression of beta-MHC.

Cardiac hypertrophy due to drugs

Myocardial hypertrophy can be induced by a number of drugs which increase the influence of the sympathetic nervous system on the heart (1,4). The interpretation of these in vivo experiments is complicated by the fact that the drugs could either have a direct effect or act indirectly via an altered hemodynamic load. In a cell culture model for cardiac hypertrophy, norepinephrine stimulated cardiac myocyte hypertrophy by an $alpha_1$-receptor mechanism (50). If the $beta_1$-adrenergic receptor was activated along with the $alpha_1$-receptor, the myocytes developed spontaneous contractile activity and the hypertrophy was augmented (50).

Both isoproterenol and dobutamine increased the expression of alpha-MHC in the intact rat (51-53). This does, however, not necessarily indicate a direct effect on the myocyte. In studies on isolated neonatal myocytes cultured for 4-5 days in the presence of isoproterenol and prazosin, the proportion of V1 was increased (41). Phenylephrine in the presence of

propranolol had a significant but smaller effect (41). Adrenergic agonists affect, therefore, MHC expression in culture essentially in the same manner as in the intact rat.

Hypotrophy of the heterotransplanted heart

The influence of the hemodynamic load on growth and myosin expression in the normal heart can be deduced also from experiments involving transplantation of a heart into the abdominal cavity of the rat (20,21). The heart chambers are under these conditions essentially unloaded and the size of the heart is reduced (20,21). The signals resulting in hypotrophy are difficult to separate because not only the hemodynamic load but also the neural influences are reduced in the transplanted heart. This approach demonstrates, however, that the normal heart represents a state within a continuum ranging from hypotrophy to hypertrophy. If one extrapolates from the effect of pressure and volume overload, one might expect that a reduction of workload leads to a reduced expression of beta-MHC. The proportion of V3 was, however, increased (20,21). Circulating thyroid hormones are not expected to play a major role in this switch because the heart is influenced also in the abdominal cavity by thyroid hormones. The reduced expression of alpha-MHC could be attributed to a smaller influence of catecholamines. Although one might assume an upregulation of adrenergic receptors in the transplanted denervated organ, it seems unlikely that the adrenergic drive is as strong as in the intact organ. Because beta-adrenergic blockade with atenolol or chemical sympathectomy induced also an increase in V_3

in the absence of alterations in circulating thyroid hormones (unpublished), the surgical denervation could contribute to the switch in MHC expression in the transplanted heart. Whether additional signals related to the reduced hemodynamic load are involved, remains to be shown.

Remodeling of the heart with pressure overload

It can be concluded that myocardial hypertrophy represents a heterogeneous response of the heart to a number of different functional loads. MHC expression does not only differ quantitatively but also qualitatively depending on the hemodynamic load or neuroendocrine status. It appears that myocardial hypertrophy can be associated with either no change in MHC expression, an increased or a decreased expression of alpha-MHC. In functional states which result in concentric hypertrophy and finally in impaired heart performance, the expression of alpha-MHC is reduced. If one focussed only on the consequences of an altered MHC expression for mechanical performance and energetics one could consider the altered MHC expression as an adaptational event. Because there is increasing evidence that also subcellular structures involved in Ca^{2+} handling are affected, the consequences of the overall changes in the subcellular structure of the myocyte for heart performance are more complex and require further evaluation.

Irrespective of the functional consequences of an altered MHC expression, it was attempted to develop interventions which affect the subcellular structure of the myocyte of the pressure loaded heart without having appreciable side effects.

Based on extensive screening experiments, it was found that feeding sucrose in the drinking water in a low concentration can greatly prevent the changes seen in MHC expression and also in Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum (54). Because this approach has no appreciable side effects, it is considered being superior to previous attempts based on swimming exercise (36,39), sodium-deficient diet (27) or dobutamine treatment (53). Although at present the functional consequences of such a "remodeling" of the subcellular structures of the myocyte are not known, this approach should open the way of manipulating the subcellular structure of the heart in a defined manner.

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10

MOLECULAR AND SUBCELLULAR MECHANISMS OF THYROID HORMONE INDUCED CARDIAC ALTERATIONS

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Introduction

The cardiovascular effects of excess thyroid hormone were first described by Parry 200 years ago (1), and the effects of thyroid deficiency have similarly been long recognized (2). Since the cardiac effects of thyroid hormone excess and deficiency are prominent, considerable research has focused on the mechanism by which thyroid hormone induces these changes. A number of reviews have been published which summarize the clinical and experimental literature regarding thyroid hormone effects on the heart (3-5). The intent of this chapter is to present the principal areas of investigation concerned with the effects of thyroid hormone action on the heart. Clinical and experimental consequences of thyroid disease on the heart are briefly presented, followed by a review of molecular and subcellular effects of thyroid hormone. Research directed towards understanding the effects of thyroid hormone on these latter two aspects of the heart has led to considerable insight into mechanisms responsible for the behavior of the heart in thyroid disease. Throughout this chapter the hormones of the thyroid gland, 3,5,3',5'-tetraiodothyronine (thyroxine) and 3,5,3'-triiodothyronine, will be referred to as T_4 and T_3, respectively, or nondistinctively as thyroid hormone.

Clinical descriptions of hypothyroidism and hyperthyroidism include alterations of the cardiovascular system. Cardiac signs of hyperthyroidism are amongst the most profound physiological alterations occurring in the disease (3-5). Typically, there is a hyperdynamic cardiac state: positive inotropy and chronotropy, palpitations and an increased susceptibility to arrhythmias are present. There is also reversible cardiac hypertrophy (6). In addition to these alterations of cardiac function in hyperthyroidism, when the disease is chronic and untreated the patient can develop congestive heart failure (7), although this is a rare complication of the disease and more frequently results from concomitant cardiac disease (i.e. atherosclerosis). In man, hyperthyroidism has also been associated with hyperthyroid cardiac dysfunction. During dynamic exercise, when euthyroid left ventricular ejection fraction (LVEF) increased, the elevated (above the euthyroid level) LVEF of hyperthyroid patients paradoxically declined (8), in addition, under the same conditions, the pre-ejection period of the hyperthyroid patients increased significantly beyond that predicted for euthyroid subjects and the velocity of circumferential fiber shortening declined by 30% (9). These changes in the cardiac response to exercise may be indicative of early manifestations of heart failure in hyperthyroid patients. The cardiac signs of hypothyroidism are generally opposite those in hyperthyroidism: a negative inotropic and chronotropic state is observed (10). Non-invasive studies have shown left ventricular systolic function to be impaired in hypothyroidism (11). A prolonged pre-ejection period and reduced LVEF have also been described (12). Impaired diastolic function, assessed by a significantly prolonged rate of thinning of the left ventricular posterior wall, has been described in the hypothyroid patient (13). Thyroid hormone deficiency has also been found to be the cause of essential hypertension in 1.2% of 618 hypertensive patients (14). Clearly the heart is affected by both an excess and a deficiency of thyroid hormone.

Experimental thyroid disease is readily reproduced in a laboratory with cardiac changes resembling clinical observations. Hypothyroidism can be induced by the antithyroid drugs propylthiouracil (15) or methimazole (16), by surgical removal of the thyroid gland (17) or radioiodine treatment (18). Hyperthyroidism can be induced by treatment with exogenous T_4 (18), T_3 (19) or desiccated thyroid tissue powder (20). The cardiac manifestations of thyroid disease have been investigated experimentally *in vitro* using isolated ventricular and atrial tissues and isolated

hearts, and *in vivo* with whole animal preparations. The *in vitro* work of Buccino *et al* (21) using isolated cat ventricular papillary muscles is demonstrative of the effects of hypo- and hyperthyroidism on isolated cardiac tissue. Compared to the euthyroid tissue, papillary muscle from hyperthyroid cat has an increased rate of tension development and a decreased time to peak tension, whereas papillary muscle from hypothyroid cat has a decreased rate and time to peak developed tension. That these are characteristic alterations of the thyroid state is supported by the recent work of MacKinnon *et al* (22), which essentially confirmed the experiments of Buccino *et al* (21) using hyper- and hypothyroid ferret ventricular papillary muscle. Isolated working rat heart experiments have shown peak left ventricular developed pressure (23) and the rate of left ventricular pressure development to be increased in hyperthyroidism (23,24), whereas spontaneous heart rate and the maximal rate of rise of left ventricular pressure are reduced in hypothyroidism (25). *In vivo* studies of hyperthyroidism in the dog (26) and pig (27) have shown increased heart rate, left ventricular systolic pressure and cardiac output. Thus, as the above *in vitro* and *in vivo* studies show, experimentally induced thyroid disease provides a suitable model system in which to study the effects of thyroid hormone on the heart. Experimental models of thyroid disease have been utilized to explore the molecular and subcellular effects of thyroid hormone on the heart.

Thyroid Hormone and Cardiac Myosin

The observation that experimental hyperthyroidism is associated with an increase and hypothyroidism with a decrease in the intrinsic velocity of cardiac contraction (21,22) has resulted in the investigation of changes in the contractile proteins of the heart. Cardiac myosin, one of the most thoroughly investigated contractile proteins, is a major component of the contractile apparatus directly involved in the contractile process. Myosin is a hexameric protein consisting of two heavy chains (200,000 kDa), two phosphorylatable light chains (18,000-22,000 kDa) and two non-phosphorylatable light chains (16,000-27,000 kDa) (28). There are two different types of myosin heavy chain (MHC) in the heart, alpha and beta, which together with the four light chains, combine to form three different myosin proteins (isozymes), termed V_1, V_2 and V_3. The different MHC composition of the three different myosin isozymes confers uniqueness: the isozymes have been characterized on the basis of their intrinsic calcium-ATPase activity, and the protein composition of

their myosin heavy chains. The V_1 isozyme consists of two alpha chains and expresses the highest intrinsic myosin calcium-ATPase activity, the V_3 isozyme consists of two beta chains and expresses the lowest intrinsic myosin calcium-ATPase activity and the V_2 isozyme consists of a single alpha and a single beta chain and expresses an intermediate intrinsic calcium-ATPase activity. Intrinsic calcium-ATPase activity of myosin is physiologically relevant since cardiac myosin calcium-ATPase activity is directly proportional to the intrinsic velocity of muscle contraction (29). The differences in myosin calcium-ATPase activity were initially determined in myosin extracts of hypo- and hyperthyroid rat heart (30). These studies also demonstrated that different myosin ATPase activities were the consequence of isoenzymes with different protein composition, conclusions which were based on the different electrophoretic mobilities in nondenaturing gels of myosin extracted from hyper-, eu- and hypothyroid rat hearts (30). Significant differences in single and two dimensional gel electrophoretic peptide maps of cyanogen bromide digests of myosin extracted from euthyroid and hyperthyroid rabbit heart further supports the argument that thyroid hormone alters the primary structure of myosin present in the heart (31). Subsequently it has been shown that thyroid hormone controls the expression of the alpha and beta MHC genes antithetically (28). In the hypothyroid rat, beta-MHC mRNA is the sole detectable MHC transcript (28). Restitution of euthyroid status by exogenous thyroid hormone results in a rapid increase in the cardiac alpha-MHC mRNA level and a simultaneous reduction in the beta-MHC mRNA. The total amount of MHC mRNA was not affected by the thyroid state, only the relative amount of the individual mRNAs. The quantity of the alpha- and beta-mRNA products, alpha-MHC and beta-MHC, respectively, lagged behind the changes in the respective mRNAs. The apparent half-lifes of either of the MHC mRNAs (approximately 3.5 days) were not different during either the induction phase nor the deinduction phase. This indirectly suggests that the rate of mRNA synthesis was affected by thyroid hormone and not the stability of the individual mRNAs. The data represented in *Figure 1* clearly demonstrate the effect of T_4 on the relative amounts of ventricular alpha- and beta-MHC mRNA and MHC isoenzymes in rat heart.

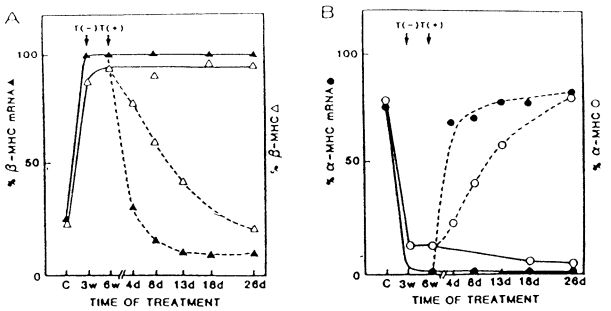

Figure 1. Effect of thyroid hormone on the relative levels of ventricular alpha- and beta- MHC mRNAs and isozymes. The relative amounts of beta-MHC mRNA (▲) and isoenzyme (△) (A) and alpha-MHC mRNA (●) and isoenzyme (o) (B). *Solid line*, thyroidectomized animals; *dashed line*, thyroidectomized animals injected daily with L-thyroxine. *From* J. Biol. Chem. 259: 6437-6446, 1984. Used with authors' permission.

That thyroid hormone alters the structure of cardiac myosin has been confirmed using synthetic oligonucleotide probes complementary to the unique 3' untranslated end of skeletal muscle mRNAs (32). These studies have demonstrated that daily T_3 injection of methimazole induced hypothyroid rats, resulted in a 500% increase in alpha-MHC mRNA and a 65% decrease in beta-MHC mRNA within 48 hours. Thyroid hormone control over MHC synthesis has also been investigated using cultured fetal rat heart cells (33). This approach afforded the opportunity to study the effect of thyroid hormone in the absence of extraneous and potentially confounding factors, such as increased basal metabolic rate, which may influence cardiac gene expression. In cultured fetal heart cells in the absence of thyroid hormone, only the beta-MHC mRNA is expressed. Addition of T_3 to the culture media resulted in a 10 to 15 fold increase in the alpha-MHC mRNA after 24 - 48 hours, and after 48 hours of T_3 exposure the level of beta-MHC mRNA was decreased to 10 - 20% of the level in the absence of thyroid hormone.

Since thyroid hormone influences the expression of growth hormone from anterior pituitary somatotrophs (34), it was of interest to determine the effect of thyroid hormone on cardiac MHC levels *in vivo* under conditions where growth hormone is not present. The genetically hypothyroid

dwarf mutant mouse (carrying an autosomal recessive mutation resulting in the inability of pituitary cells to produce prolactin, thyroid stimulating hormone or growth hormone) confers these conditions (35). A single 1 ug injection of thyroxine resulted in a shift from the exclusively present beta-MHC to a MHC profile where the alpha-MHC accounted for 55% of the total cardiac myosin. The relative amount of beta-MHC decreased in proportion to the increase in alpha-MHC. The MHC shift in the genetically hypothyroid *dwarf* mouse was dependent on the continued presence of thyroid hormone since the amount of alpha-MHC declined after peaking at four days post-injection. These results demonstrate that the effect of thyroid hormone on the expression of alpha- and beta-MHC protein is independent of growth hormone. That the effect of thyroid hormone on the myocardial expression of MHC is a direct cardiac effect and independent of secondary influences of the hormone, such as increased cardiac work or intact innervation, is demonstrated by studies using heterotopically isotransplanted hearts. The transplanted hearts are perfused and spontaneously contract, although they perform little if any external work. A donor rat heart isotransplanted via the aortic stump to the abdominal aorta of a euthyroid recipient rat atrophies, and the predominant V_1 myosin isoform is replaced by the V_3 isoform (36,37). When a recipient animal is subjected to exogenous thyroid hormone treatment, the *in situ* heart typically hypertrophies however the transplanted heart atrophies. Myosin isoenzyme expression in the *in situ* heart of the thyroid hormone treated rat does not change, remaining approximately 95% of total myosin. Interestingly, and supporting a direct cardiac effect of thyroid hormone independent of thyroid hormone mediated increases in cardiac work, is that the myosin shift seen in transplanted hearts of euthyroid rats, is prevented by the thyroid hormone treatment: the V_1 isoenzyme predominates (approx. 95%) in the transplanted heart when the animal is rendered hyperthyroid.

The effect of thyroid hormone on the expression of cardiac alpha- and beta-MHC genes, the amount of the respective MHC in the heart and hence the intrinsic myosin ATPase activity and velocity of cardiac contraction, is at present perhaps the most well defined direct cardiac effect of thyroid hormone. These studies have provided substantial evidence for a direct effect of thyroid hormone on the heart, and have established a mechanism for the influence of thyroid disease on the rate of cardiac contractility.

Thyroid Hormone and Cardiac Sarcoplasmic Reticulum

Ventricular relaxation is consistently and predictably altered in thyroid disease. Alterations in the rates of ventricular contraction and relaxation occurring in hypo- and hyperthyroidism are associated with complementary alterations of calcium fluxes in the heart. It has been shown using the calcium sensitive dye aequorin, that calcium removal from cardiac myoplasm during relaxation is enhanced in hyperthyroid and impaired in hypothyroid ferret papillary muscle (22). Cardiac sarcoplasmic reticulum (SR) is considered to play a pivotal role in ventricular relaxation through active sequestration of myoplasmic calcium into its lumen, and the enzyme responsible for calcium transport is the calcium-ATPase (38). Therefore, calcium transport activity of the SR under different thyroid states has been extensively studied *in vitro*. SR calcium-transport activity and calcium-ATPase activity are increased in hyperthyroidism (39-42) and decreased in hypothyroidism (39,41). Data representative of the effect of hyperthyroidism on rat cardiac sarcoplasmic reticulum calcium transport activity is shown in *Figure 2*.

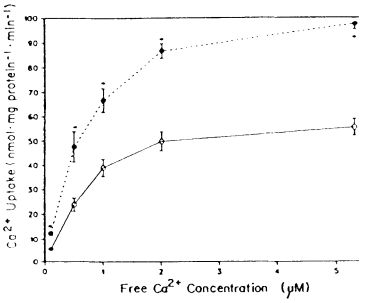

Figure 2. The effect of T_3 treatment on rat SR calcium transport activity determined as a function of free calcium concentration (0.1-5.3 uM). Euthyroid (o), hyperthyroid (•). *, significantly different from euthyroid, $p < 0.05$. Values are mean +/- SE, n=6. *From* Can. J. Physiol. Pharmacol. 66: 159-165, 1986. Used with authors' permission.

A number of mechanisms by which thyroid hormone may affect cardiac SR calcium-ATPase and calcium-transport activities have been proposed. Thyroid hormone increases the absolute amount of SR in rat heart after 24 days of thyroxine treatment (43), and there is an increased yield of SR membrane isolated from hyperthyroid rat heart (42). Indirect evidence that thyroid hormone may have a selective effect on cardiac SR protein synthesis is that altered calcium transport/ATPase activities are typically expressed on a per mg SR protein basis (39-42). Therefore the amount of calcium transported/ATP hydrolyzed is normalized to the total amount of SR protein present in the SR. Increased levels of the phosphoprotein intermediate of the SR calcium ATPase in SR isolated from hearts of hyperthyroid rat lend support to an inductive effect of thyroid hormone on the calcium pump (40,42). Further evidence of a thyroid hormone induced protein synthetic mechanism, is that increased cardiac SR calcium transport and increased calcium ATPase phosphoprotein intermediate of hyperthyroid rats is blocked by the administration of actinomycin D, cyclohexamide or puromycin (inhibitors of RNA and protein synthesis) concurrent with T_4 treatment (40). Thyroid hormone stimulated increases in SR calcium ATPase mRNA in hypothyroid rat heart is the most direct evidence to date that thyroid hormone may directly control SR calcium transport activity via induction of pump protein synthesis (44). In skeletal muscle SR, which responds to thyroid hormone analogously to the heart, it has been shown that developmental increases in SR calcium transport are directly related to the level of thyroid hormone (45). These studies also demonstrated that thyroid hormone effects on skeletal muscle SR were independent of growth hormone, an observation similar to the thyroid hormone mediated (growth hormone independent) expression and inhibition of cardiac alpha- and beta-MHC genes. Further research is required to determine if the effects of thyroid hormone on cardiac SR calcium ATPase are independent of growth hormone, and independent of cardiac adaptation to the peripheral effects of thyroid hormone.

In addition to a direct effect on the SR via an increase in the number of calcium pump sites, other mechanisms for the effects of thyroid hormone on cardiac SR have been proposed. Phospholamban, a 22,000 kDa regulatory protein of the SR membrane, the phosphorylation of which is correlated with enhanced calcium transport activity (38), is phosphorylated to a greater extent in the presence of cyclic 3',5'-adenosine monophosphate (cAMP) in cardiac SR from hyperthyroid rat

compared to euthyroid rat (46). However the significance of this is unclear since a separate study has shown cAMP-dependent protein kinase stimulation of hyperthyroid cardiac SR calcium transport is not enhanced (47). Enzymes involved in the desaturation of fatty acids, and hence the modelling of membrane phospholipid composition, are known to be affected by the thyroid state (49). SR membrane phospholipid alterations in hyperthyroidism include: increases in total phospholipids and phosphatidylcholine, C16:0, C18:0 and C22:5,n-6 and decreases in C18:2,n-6 and C20:4,n-6 (40). Since SR calcium pump activity is dependent upon the physicochemical nature of the lipid environment in which it is located (49), these lipid alterations may contribute to the altered SR calcium transport activity observed in different thyroid states. It has also been reported that the endogenous level of long chain acyl carnitines, known potent inhibitors of SR calcium transport and ATPase activities (50), are reduced in cardiac SR from hyperthyroid rat (42), suggesting the possibility that there may be reduced endogenous inhibition of the calcium pump in the hyperthyroid state.

The data discussed above largely considers the effects of thyroid hormone on the SR to be dependent on changes in protein synthesis and or degradation, either of the calcium pump itself or other metabolically relevant enzymes or regulatory proteins. There is also a body of evidence which suggests direct effects of thyroid hormone itself. Thyroxine and T_3 have been shown to directly stimulate, *in vitro*, erythrocyte membrane calcium ATPase (51), rabbit sarcolemmal calcium ATPase (52) and rat thymocyte plasma membrane calcium uptake (53) and calcium stimulated ATPase (54) activities at physiological concentrations (10^{-9}-10^{-10} M). Direct effects of thyroid hormone on enzymes mediating calcium flux across the cardiac cell membrane, and possibly the SR, represents another mechanism whereby thyroid hormone may control cardiac function.

It is evident that thyroid hormone is affecting SR function through a number of possible mechanisms. Thyroid hormone is considered to act via specific nuclear receptors to direct its effects on the cell. An increased number of SR calcium pumps in hyperthyroidism would conform to a nuclear site of action, and the increased SR mRNA following T_3 treatment (44) supports a nuclear site of action for the effect of thyroid hormone on the SR. The direct effects of thyroid hormone described above, however, indicate that the hormone's interaction with the SR may also occur

directly, independent of a nuclear interaction. The effects and mechanisms of action of thyroid hormone on cardiac subcellular calcium transport activity remains an interesting area of research.

Thyroid Hormone - Cardiac Adrenergic Receptor Interaction

The cardiac signs and symptoms of hyperthyroidism and hypothyroidism closely resemble those of catecholamine excess and deficiency, respectively. The clinical similarities between hyperthyroidism and catecholamine excess provoked consideration that cardiac adrenergic receptor alterations may be responsible for the cardiovascular manifestations of hyperthyroidism. Although the effects of thyroid disease resemble the effects of catecholamine excess or deficiency, paradoxically the plasma levels of noradrenaline (NA) are typically decreased or unchanged in hyperthyroidism and elevated in hypothyroidism, and plasma adrenaline (A) concentrations are generally not affected by the thyroid state (55) and the urinary excretion patterns of NA and it's metabolites reflect plasma levels (56). Since plasma catecholamines levels cannot explain the changes in cardiac function seen in the heart, research has focused on alterations in cardiac adrenergic receptor sensitivity or responsiveness to endogenous catecholamines as a means of explaining the cardiac alterations of thyroid disease. This aspect of thyroid hormone interaction with the heart has recently been reviewed in detail (57). Although there have been a number of reports in man and experimental animals (58-60) which indicate that adrenergic responsiveness is unaltered by the thyroid state, more recent *in vitro* and *in vivo* experiments in a number of animal species indicate otherwise. In hyperthyroid guinea pig right atria, left atria and papillary muscles the inotropic and chronotropic dose response curves to isoproterenol were shifted to the left (18). The shifts in the dose-response curves were reflected by 1.9, 5.5 and 2.6 fold increases in the pD_2 values for isoproterenol in the right and left atria and papillary muscle, respectively. Hyperthyroid rat right atria demonstrate a similar 1.6 fold increase in the ED_{50} value for the chronotropic response to isoproterenol (61). The maximum rate of force development in response to a near threshold concentration of isoproterenol (1 nM) in perfused interventricular septa from hyperthyroid rat was enhanced compared to euthyroid rat (47). Hypothyroid rat and guinea pig show changes in tissue sensitivity to isoproterenol opposite to those

seen in hyperthyroidism, with a decreased sensitivity to the inotropic and chronotropic effects of isoproterenol observed (18,62).

The question of whether the cardiac alterations of thyroid disease are mediated by alterations in cardiac adrenergic receptor sensitivity have also been investigated using *in vivo* models of thyroid disease. In the hyperthyroid dog with increased left ventricular systolic pressure, increased heart rate and cardiac output, propranolol reduced the contractile state of the heart to a greater degree than in the euthyroid dog (26). The greater sensitivity of the hyperthyroid dogs to the effects of propranolol remained when euthyroid dogs were paced at the hyperthyroid rate. Interestingly, and at variance with the current consensus, hyperthyroid dogs in this study did not demonstrate an increased cardiac sensitivity to exogenous noradrenaline or isoproterenol. In a longitudinal *in vivo* study using a porcine model, hyperthyroidism led to an increased resting and intrinsic heart rate (determined in the presence of atropine and propranolol) and increased systolic blood pressure (27). It has been shown that the concentration of isoproterenol required to increase the heart rate by 50% (ED_{50}) was reduced by 33% in hyperthyroid pigs. The slope of the line relating isoproterenol concentration to the change in heart rate was increased by 29% in the same animals. These increases in the *in vivo* chonotropic responsiveness (supersensitivity) to isoproterenol occurred coincident with an increase in the number

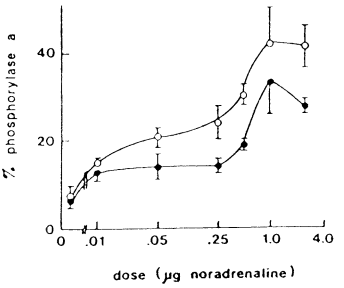

Figure 3. Plot of dose-response curve for noradrenaline-induced cardiac phosphorylase activation in isolated perfused hearts of euthyroid (o) and hyperthyroid (•) animals. Values are mean + / SE. *From* Can. J. Physiol. Pharmacol. 52: 373-383, 1974. Used with authors' permission.

of right atrial beta-receptors. An increase in the number of myocardial beta-receptors, and a decrease in alpha-receptors is a consistent finding in hyperthyroidism (57). Opposite changes in the number of myocardial alpha- and beta-receptors are observed in hypothyroidism (57). In addition to changes in tissue sensitivity, post-receptor responses to adrenergic agonists have also been shown to be altered by thyroid disease. Enhanced adrenergic receptor mediated phosphorylase *a* formation in hearts of hyperthyroid animals has been consistently shown (Figure 3) (19,63-65). The specific basal activity of phosphorylase kinase (the enzyme catalyzing phosphorylase *b* to *a* conversion) in hyperthyroid rat heart is twice that of the euthyroid animal (Figure 4) (65). The increased specific activity of phosphorylase kinase of hyperthyroid rat heart remained twice that of the euthyroid tissue when the enzyme was stimulated by either cAMP-dependent phosphorylation or

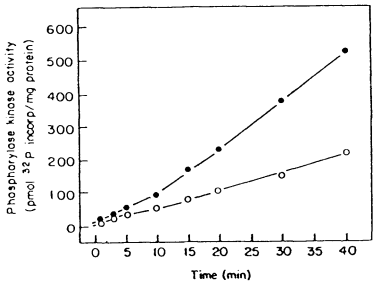

Figure 4. The effect of hyperthyroidism on basal phosphorylase kinase activity in ventricular tissue from euthyroid (o) and hyperthyroid (●) rats. *From* J. Mol. Cell. Cardiol. 15: 163-171, 1983. Used with authors' permission.

exogenous calmodulin. The effect of thyroid state on adrenergic receptor mediated increases in cAMP are variable, as both an increase (66) and no difference (64,65) in the accumulation of cAMP following adrenergic stimulation have been reported in the hyperthyroid heart. Myocardial adenylate cyclase (67,68) and phosphodiesterase (69) activities are not affected by the thyroid state. Therefore, the increased basal and stimulated intrinsic activity of cardiac phosphorylase kinase provides a rational explanation for the enhanced phosphorylase activation in the hyperthyroid state. A thyroid

hormone stimulated increase in phosphorylase kinase synthesis and/or the synthesis of a phosphorylase kinase isoenzyme with a higher intrinsic activity (analogous to the effect of thyroid hormone on myosin) are possible mechanisms for the increased acitivity of phosphorylase kinase in the hyperthyroid state.

It is apparent that the changes in cardiac function seen in thyroid disease are associated with changes in tissue sensitivity to adrenergic stimulation, the number of myocardial beta-receptors and changes in specific post-receptor events. These receptor related alterations contribute to the cardiovascular alterations of thyroid disease. However, in view of myosin and SR alterations, thyroid hormone induced adrenergic receptor alterations can not be considered to be solely responsible for the observed cardiac manifestations of thyroid disease.

Conclusions

The effect of thyroid hormone on the heart can be described as pleiotropic since intrinsic cardiac contraction and relaxation, and the regulation of these parameters by catecholamines are affected in thyroid disease. The molecular and subcellular components of the heart affected by thyroid hormone reviewed in this chapter allow for the realization that the physiological (pathological) effects of thyroid hormone are the result of multiple complex, integrated events. Although there has obviously been considerable progress in the understanding of thyroid hormone effects on the heart since Parry's (1) descriptions 200 years ago, more research is required to define all of the effects, as well as the relative importance of the various effects attributed to thyroid hormone. An unresolved question remaining is the time relationship of the different known effects of thyroid hormone on the heart. Knowledge of the interdependency of the various parameters affected by thyroid hormone will also enhance our understanding of the overall mechanism by which thyroid hormone alters cardiac function. Future molecular biological, biochemical and pharmacological research will yield the information required to understand the mechanisms responsible for thyroid hormone induced cardiac alterations.

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MEMBRANE ABNORMALITIES AND CHANGES IN CARDIAC CATIONS DUE TO ALTERATIONS IN THYROID STATUS

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INTRODUCTION

Thyroid hormones have been shown to induce marked changes in the cardiovascular system (1-4); however, the mechanism of cardiac alterations due to changes in thyroid status over a prolonged period are poorly understood. Since sarcolemmal Na^+-K^+ ATPase is known to serve as a pump for maintenance of the intracellular concentrations of Na^+ and K^+ in the myocardial cell (5-7), changes in the activity of this enzyme can be seen to alter the cation composition of the myocardium and thus may lead to changes in the contractile activity. Previous studies have revealed that changes in the plasma levels of thyroid hormones exert a dramatic effect on the myocardial Na^+-K^+ ATPase activity (8-11). While some investigators have attempted to correlate thyroid hormone induced changes in cardiac Na^+-K^+ ATPase with alterations in the Na^+ and K^+ contents (9,12,13), the results are conflicting. Furthermore, very little information concerning changes in myocardial Ca^{2+} and Mg^{2+} or plasma electrolytes due to alterations in thyroid status of the animal is available in the literature. This study was therefore undertaken to investigate serum and cardiac electrolytes in euthyroid, hyperthyroid and hypothyroid animals. Experiments were also

carried out to examine if the electrolyte changes due to hypothyroidism were reversible upon injecting the animals with thyroid hormone. The sarcolemmal Na^+-K^+ ATPase activity in euthyroid, hyperthyroid and hypothyroid hearts was monitored to seek relationship with changes in cardiac cation contents under these conditions. Furthermore, in view of varying degree of alterations in the Ca^{2+}-transport activities of the cardiac sarcoplasmic reticulum from hypothyroid and hyperthyroid animals (14-18), it was considered worthwhile to study changes in the sarcoplasmic reticular function by employing heart homogenates from the control and experimental animals. It should be pointed out that these experiments with heart homogenate will not only rule out the possibility of artifacts due to the procedure for isolation of the sarcoplasmic reticulum but will also help in identifying the specificity of membrane changes upon altering the thyroid status in animals.

METHODS

Sprague-Dawley rats were randomly distributed between euthyroid and hypothyroid groups; the hypothyroid groups received a 0.05% solution of propylthiouracil (P.T.U.) in their drinking water for 6-8 weeks while the euthyroid group received only water for the same period of time (19). In order to induce a hyperthyroid condition, the euthyroid animals were injected intraperitoneally with saline containing 25 ug thyroxine (T_4) per 100 g body weight. In some experiments, the hypothyroid animals received 50 ug triiodothyronine (T_3) per 100 g body weight per day. It is pointed out that hypothyroid animals receiving thyroid hormone were maintained on 0.05% P.T.U. during hormone administration. Serum and myocardial levels of sodium, potassium, calcium, and magnesium were determined by the method outlined elsewhere (12). The extracellular space was estimated by employing $^{35}SO_4$

as an extracellular marker (12).

For the determination of Ca^{2+} transport in heart homogenate, approximately 0.4 g of the apex region of the heart was removed and immersed in ice-cold 10 mM histidine, 0.25 M sucrose pH 7.2. The tissue was blotted, weighed, minced and homogenized in 16 volumes of the same buffer. Homogenization was performed 6 times for 15 s each with a Polytron PT 10-35 at a setting of 6. The final homogenate was filtered through Nylon gauze and kept in ice for 10 min. The Ca^{2+} accumulation into sarcoplasmic reticulum vesicles contained in the homogenate was determined at 37°C with constant stirring in 40 mM imidazol, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, 6 mM creatine phosphate, 10 mM K-oxalate, 10 mM MaN_3, 200 uM EGTA, 100 uM $^{45}CaCl_2$ (specific activity 2 uCi/ml) and 3 mg/ml wet heart tissue (20). The reaction mixture was preincubated for 5 min and Ca^{2+} transport was started by addition of 25 ul homogenate per 0.5 ml assay. At appropriate time points 0.1 ml of samples were taken and filtered through Millipore HA, 0.45 um filter by suction. Filters were washed twice with 2.5 ml ice-cold 40 mM imidazol, pH 7.0, 100 mM KCl and 2 mM EGTA. Radioactivity associated with the filter was determined by liquid scintillation counting. Linear rates of oxalate-supported Ca^{2+} uptake were calculated from data points at 0.5, 1, 2 and 3 min measured in duplicates and were expressed as umoles Ca^{2+} per g wet heart weight per min. The protein content as measured by the method of Lowry et al (21) in homogenates of euthyroid, hypothyroid and hyperthyroid myocardium were 213.7 ± 2.6, 200.0 ± 1.8 and 208.2 ± 3.3 mg protein per g wet heart weight, respectively.

The sarcolemmal fraction was isolated and purified according to the methods described earlier (22). For the estimation of Na^+-K^+ ATPase activity, membranes were incubated in a medium (0.225 ml) containing 50 mM

Tris-HCl, pH 7.4, 1 mM ETGA, 5 mM NaN_3, 6 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 2.5 mM phosphoenol pyruvate and 10 I.U./ml pyruvate kinase. The reaction was started by the addition of 0.025 ml 40 mM Tris-ATP and terminated 10 min later with 0.250 ml of 12% trichloracetic acid. The liberated phosphate was measured by the method of Taussky and Shorr (23). Values in the absence of Na^+ and K^+ were subtracted for calculating the Na^+-K^+ ATPase activity. The results were evaluated statistically by using the analysis of variance. Significant difference among different groups was determined by using the Duncan's New Multiple Range Post Hoc Test while significant difference between two groups was detected by the Student's "t" test.

RESULTS

Five groups of animals were used: a) Euthyroid, b) Hyperthyroid (7 days T_3 treatment), c) Hypothyroid (P.T.U. treated), d) Hypothyroid (P.T.U.) + 2 days T_3 treatment, and e) Hypothyroid (P.T.U.) + 7 days T_3 treatment. From the data in Table 1, it can be seen that the extracellular space (E.C.S.) was increased in hypothyroidism; this observation is similar to that reported after thyroparathyroidectomy (12). However, there was no reversal of this effect within the 7 days period of T_3 treatment. Furthermore, E.C.S. was unaltered in hyperthyroidism. Cardiac water contents with respect to dry weight [$(H_2O)/W_D$] are also given in Table 1. In general the tendency was for cardiac water to increase in hypothyroidism. On treatment with thyroid hormone, muscle water increased further after 2 days but at 7 days it had fallen below that in the hypothyroid animals. There was no difference between water content of hyperthyroid and euthyroid rat ventricles. An examination of extracellular and intracellular water with respect to dry weight revealed that changes in water content can be accounted

for by changes in extracellular water as would be expected by the change in E.C.S. noted earlier. Intracellular water content was significantly changed in hypothyroid animals after 7 days of treatment.

The effect of changes in thyroid status on plasma electrolyte concentrations is shown in Table 1. Plasma potassium concentration appeared to be directly related to thyroid status. Hyperthyroidism resulted in hyperkalemia while hypokalemia occurred in hypothyroidism; the latter was reversed by T_3 treatment. Plasma sodium was independent of thyroid status but hypocalcemia accompanied hypothyroidism and this was readily reversed by T_3 treatment. After only 2 days of T_3 treatment, calcium levels had risen above those in the euthyroid rats. While hyperthyroidism was accompanied by a tendency for calcium to increase, statistically significant difference was not achieved. Plasma magnesium levels were found to be related to thyroid state; these levels rose in hyperthyroid state and fell in hypothyroidism. The latter effect was reversed by T_3 administration so that after 2 days levels were close to those of euthyroid controls and after 7 days close to those of hyperthyroid rats. The data on plasma magnesium should be interpreted with some caution because the observed alteration may be related to the stress of surgery. In this regard it has been shown in humans that stress or pain may be associated with changes in serum magnesium levels (24).

Intracellular monovalent cation concentrations and their ratios as seen in euthyroid, hyperthyroid and hypothyroid hearts are given in Table 2. It must be pointed out that these values do not represent sarcoplasmic concentrations of free cations since the calculations do not take compartmentalization of cations into consideration. Intracellular potassium was not significantly altered in hypothyroidism or hyperthyroidism. However, treatment of hypothyroid animals with T_3 resulted in a significant increase

Table 1. Effect of altered levels of circulating thyroid hormone on rat ventricular extracellular space, tissue water content and plasma cations.

	Euthyroid (Control)	Hyperthyroid	Hypothyroid	Hypothyroid + 2 days T_3	Hypothyroid + 7 days T_3
A. Extracellular Space and Water Contents					
E.C.S.	0.153 ± 0.003	0.160 ± 0.012	0.194 ± 0.005*	0.204 ± 0.008*	0.212 ± 0.003*
$(H_2O)_m/W_D$	3.09 ± 0.02	3.12 ± 0.01	3.33 ± 0.05*	3.45 ± 0.05*	3.23 ± 0.04*
$(H_2O)_o/W_D$	0.627 ± 0.014	0.657 ± 0.049	0.836 ± 0.022*	0.901 ± 0.041*	0.897 ± 0.015*
$(H_2O)_i/W_D$	2.47 ± 0.02	2.46 ± 0.05	2.49 ± 0.05	2.54 ± 0.03	2.34 ± 0.04*
B. Plasma Cations					
$[K]_p$	2.59 ± 0.12	3.54 ± 0.23*	2.22 ± 0.10*	2.49 ± 0.13	2.70 ± 0.16
$[Na]_p$	143 ± 0.6	143 ± 1.8	141 ± 0.9	143 ± 2.2	146 ± 1.6
$[Ca]_p$	2.97 ± 0.05	3.24 ± 0.16	2.60 ± 0.06*	3.36 ± 0.10*	3.53 ± 0.12*
$[Mg]_p$	1.28 ± 0.09	1.64 ± 0.10*	0.84 ± 0.04*	1.19 ± 0.09	1.59 ± 0.07*

Values are means ± S.E. of 7-8 experiments. E.S.C. is expressed at gm extracellular water per gm wet weight. $(H_2O)_mW_D$ is gm muscle water per gm dry weight. $(H_2O)_oW_D$ is gm extracellular water per gm dry weight. $(H_2O)_iW_D$ is gm extracellular water per gm dry weight. Cation concentrations are given in mM.
* Statistically significant ($P < 0.05$) when compared to euthyroid values.

Table 2. Effect of thyroid status on intracellular monovalent and divalent cations.

	Euthyroid (Control)	Hyperthyroid	Hypothyroid	Hypothyroid + 2 days T_3	Hypothyroid + 7 days T_3
A. Monovalent Cations					
$[K]_i$ (mM)	163 ± 3.3	165 ± 3.9	168 ± 3.2	174 ± 2.8*	183 ± 2.8*
$[Na]_i$ (mM)	21.1 ± 1.7	26.3 ± 3.1	19.1 ± 1.0	20.6 ± 1.9	16.7 ± 1.1*
$[K]_i/[Na]_i$	7.73 ± 0.17	6.83 ± 0.88	9.00 ± 0.62	9.02 ± 0.62	11.32 ± 0.80*
B. Divalent Cations					
$[Ca]_i$ (mM)	0.969 ± 0.098	1.043 ± 1.147	0.743 ± 0.052	0.719 ± 0.083	0.972 ± 0.161
$[Mg]_i$ (mM)	14.1 ± 1.27	13.2 ± 0.94	17.8 ± 0.91	16.2 ± 1.77	16.4 ± 1.52
$[Mg]_i/[Ca]_i$	14.9 ± 1.94	15.3 ± 3.2	25.7 ± 2.78*	24.5 ± 3.04*	18.95 ± 1.66

Values are means ± S.E. of 7-8 experiments. * Statistically significant ($P < 0.05$) difference when compared to euthyroid values.

in potassium levels. Significant difference in intracellular sodium levels was only revealed when hyperthyroid and hypothyroid states were compared. Treatment of hypothyroid animals led to further decrease in intracellular sodium. The $[K]_i/[Na]_i$ was unaltered in the hypothyroid and hyperthyroid states but an appreciable change was seen after 7 days of thyroid hormone administration to hypothyroid rats when $[K]_i/[Na]_i$ increased significantly.

No significant change in calcium concentration was revealed (Table 2), although there did appear to be a tendency for the level to fall in hypothyroidism and to rise after thyroid hormone administration. Significant difference in intracellular magnesium could be seen only when hyperthyroid and hypothyroid conditions were compared; the increase in hyperthyroidism was partially reversed by T_3 treatments. $[Mg]_i$ depletion in hyperthyroidism and the reverse in hypothyroidism may reflect the failure of Mg^{2+} controlling mechanisms to keep pace with the rapidly expanding or constricting cell volume. $[Mg]_i/[Ca]_i$ ratio was increased in hypothyroidism; after 7 days, but not after 2 days, of T_3 administration this ratio was decreased so that it was no longer significantly different from control.

Fig. 1 shows that Na^+-K^+ ATPase activity in the sarcolemmal preparations in the absence of alamethicin from euthyroid and hyperthyroid hearts was not different from each other. However, the enzyme activity in the hypothyroid heart untreated membranes was about 20% higher than the control preparations. Treatment of membranes with alamethicin at a drug to protein ratio of 0.5 resulted in a significant increase in the Na^+-K^+ ATPase activity in hyperthyroid heart. Increasing the alamethicin to protein ratio to 1.0 showed an increase in the hyperthyroid sarcolemmal preparations and a decrease in the hypothyroid preparations. It should be mentioned that

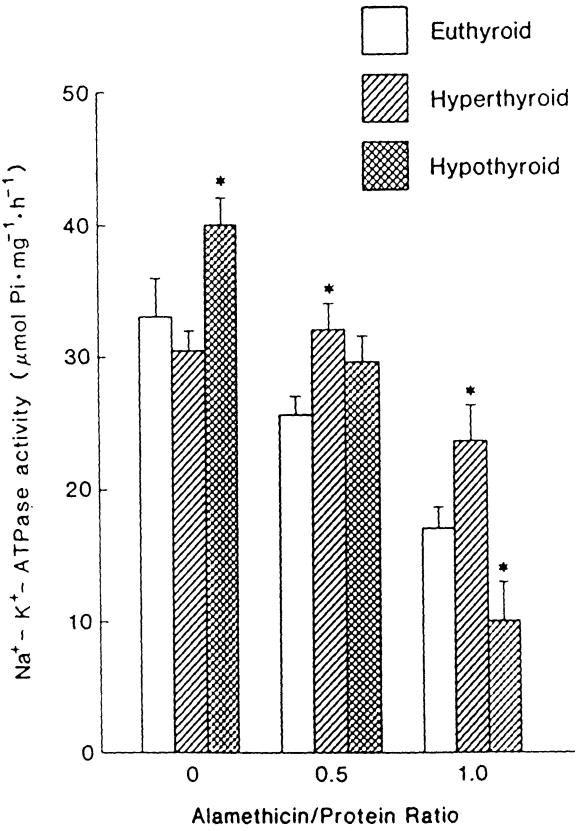

Figure 1. Na^+-K^+ ATPase activity in the absence or presence of alamethicin in sarcolemmal membranes isolated from euthyroid, hyperthyroid and hypothyroid hearts. Values are mean ± S.E. of 4 to 6 experiments. * - $P < 0.05$ when compared to the respective euthyroid value.

alamethicin is known to make pores in the membrane vesicles and thus all sites of the enzyme become available for expression. The results in Fig. 1 also indicate that alamethicin treatment depressed the sarcolemmal Na^+-K^+ ATPase activity. However, in comparison to the euthyroid preparations, the hypothyroid membrane showed greater sensitivity whereas the hyperthyroid preparations were less sensitive. Alamethicin treatment has been shown

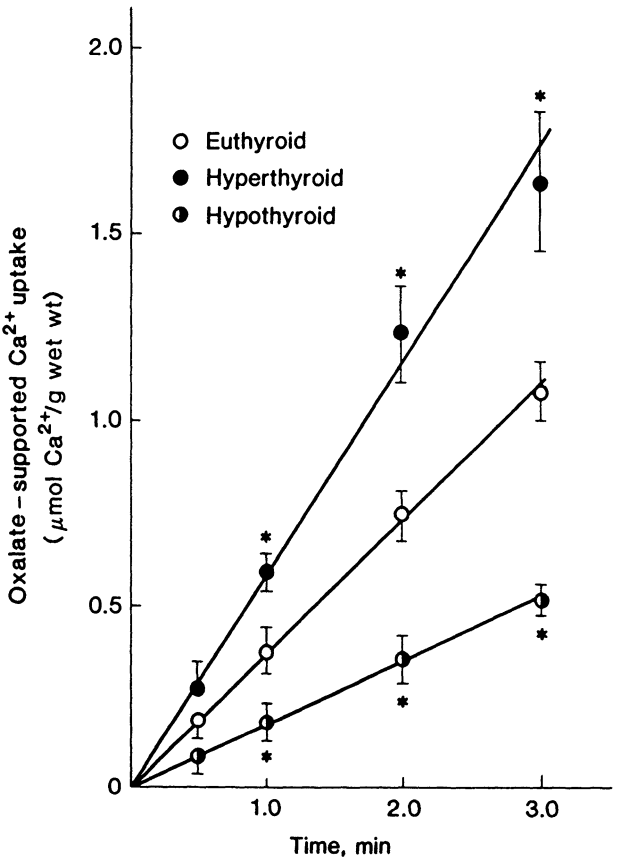

Figure 2. Time-course of oxalate-supported Ca^{2+} uptake in the ventricular homogenates from euthyroid, hyperthyroid and hypothyroid animals. Values are mean ± S.E. of 3 to 9 experiments. * - $P < 0.05$ when compared to the euthyroid value.

earlier to depress the sarcolemmal Na^+-K^+ ATPase activity in rat heart (25). Such effects of alamethicin on the sarcolemmal membranes may be attributed to its detergent like action where the enzyme-lipid complexes in the membrane are disturbed leading to an inhibition of the associated enzyme activity (26). The data in Fig. 2 show oxalate-supported Ca^{2+} uptake in

heart homogenates from euthyroid, hyperthyroid and hypothyroid rats. It can be seen that Ca^{2+} uptake occurred linearly in all homogenate preparations during 3 min. Hypothyroidism and hyperthyroidism were accompanied by decreased (v = 0.182 ± 0.016 umol Ca^{2+}/g wet wt/min) and increased (v = 0.5920.05 umol Ca^{2+}/g wet wt/min) rates of Ca^{2+} uptake when compared to euthyroid heart values (v = 0.352 ± 0.028 umol Ca^{2+}/g wet wt/min), respectively.

DISCUSSION

We have shown that the sarcolemmal Na^{+}-K^{+} ATPase activity was altered depending upon the status of thyroid hormones in the animals. Although the direction of changes in the enzyme activity was dependent upon the nature of alamethicin treatment, the results indicate a significant increase in Na^{+}-K^{+} ATPase in hypertrophied hearts and a decrease in hypothyroid preparations treated with alamethicin at a sarcolemmal protein ratio of 1.0. Nonetheless, the observed changes in the sarcolemmal membrane do not seem specific because the Ca^{2+}-transport function of the sarcoplasmic reticulum, as estimated by employing heart homogenate, was also altered in the hypothyroid and hyperthyroid hearts. It should be mentioned that the measurement of Ca^{2+} uptake in heart homogenate allows the estimation of sarcoplasmic reticular Ca^{2+}-transport rates per unit of muscle mass and avoids any error due to differences in the subpopulations of the sarcoplasmic reticular vesicles in purified preparations. The observed increase and decrease in the rates of oxalate supported Ca^{2+} uptake in heart homogenates from hyperthyroid and hypothyroid states, respectively, confirm earlier results with isolated sarcoplasmic reticular preparations (16,17).

In this study we did not observe any change in myocardial concentrations

of both Na^+ and K^+ in hypothyroidism. Such a finding is in contrast to what was expected on the basis of depressed sarcolemmal Na^+-K^+ ATPase activity in the hypothyroid rats (8,11). It is possible that the depression in sarcolemmal Na^+-K^+ ATPase activity in hypothyroid hearts may be an adaptive change to compensate for some other defect so that they exactly balance the requirement of the cell and no intracellular electrolyte changes are seen. This is analogous to metabolic changes which enable the cell to increase energy supply to match increased demand so that cytosolic high Energy compounds remain at or near normal levels (27). Thus it is not absolutely necessary that changes in cardiac Na^+-K^+ ATPase activity must be accompanied by changes in myocardial electrolyte contents. Although K^+ channels, Na^+-H^+ exchange and Na^+-Ca^{2+} exchange in sarcolemmal are also known to regulate the Na^+ and K^+ contents in the cell, no information regarding the effects of thyroid hormones is available at present. On the other hand, plasma electrolyte changes are believed to occur due to complex changes in absorption and excretion by the body. Expansion or reduction of cell volume and alteration in intracellular levels may also influence extracellular cation levels. In general the myocardium can be conceived to have little or no role in regulating plasma electrolyte levels; however, extracellular electrolytes may have profound effects on myocardial function. Whether changes in heart function in hypothyroid and hyperthyroid states (1,2) are due to alterations in extracellular electrolytes cannot be stated with certainty on the basis of this study.

As in other studies (9,28), the administration of thyroid hormone to hypothyroid rats for 48 hours or more caused an increase in intracellular potassium. This has been interpreted as reflecting enhancement of the Na^+-K^+ ATPase activity of hypothyroid hearts, which was depressed (11).

Nonetheless, these observations indicate that a simple relationship does not exist between intracellular potassium and Na^+-K^+ ATPase activity measured in vitro or the concomitant alterations in active monovalent ion uptake measured in isolated tissue (13,28). Similarly intracellular sodium did not vary as expected in euthyroid, hyperthyroid and hypothyroid states. Furthermore, intracellular sodium levels of hypothyroid rats were not different from those of euthyroid rats after T_3 administration. Although decreased intracellular sodium accompanied by increased potassium is expected when Na^+-K^+ ATPase activity increases, this type of relationship was only found after T_3 administration to hypothyroid rats. It should be noted that the hypothyroid state in this study was developed and maintained over a 6 to 8 week period and after this time intracellular levels were at or near normal. It may be that over a period, demands on transport mechanism and transport ability are modified so as to better match each other. In addition tachycardia, which is a characteristic of hyperthyroidism, increases the frequency of action potentials of the myocardium and thus the influx of Na^+ and efflux of K^+, will put greater demand on the Na^+-K^+ pump; the opposite will occur in hypothyroidism. In ventricular tissue of the frog it has been demonstrated that increasing $[K^+]_o$ increases the K^+ efflux from the cell (29). Since in this study, extracellular potassium was found to be related to thyroid state, the increased extracellular potassium in hyperthyroidism will increase K^+ efflux and the opposite will occur in the hypothyroidism.

Intracellular calcium content did not vary with thyroid state, although there appeared to be a tendency for it to decrease in hypothyroidism and for this to be reversed after thyroid hormone administration. However, the values of intracellular calcium represent total calcium content of the cell and not

cytosolic concentrations which may be influenced by a number of processes (30). The magnitude of Ca^{2+} influx has been shown in this study to be influenced by thyroid status and this may tend to increase intracellular calcium content. We have also demonstrated that in the presence of calmodulin, the Ca^{2+}-stimulated ATPase and ATP dependent calcium binding activities of cardiac sarcolemma were enhanced in the hypothyroid state (31). Therefore, extrusion of calcium from the cell will be increased in hypothyroidism. On the other hand, intracellular magnesium levels were not only altered when hypothyroid and hyperthyroid states were compared. Mechanisms which control intracellular magnesium levels are poorly understood. The Ca^{2+}/Mg^{2+} ATPase which has been suggested to be associated with a pumping mechanism for Mg^{2+} (6,32) was not altered by thyroid status. Increased or decreased $[Mg]_i$ may reflect the inability of cell to extrude or acquire magnesium at the same rate as the expansion or constriction of the cell volume.

It should be pointed out that $[Mg]_i/[Ca]_i$ ratio was found to be significantly increased in hypothyroidism. After 2 days but not 7 days of thyroid hormone treatment of hypothyroid animals, $[Mg]_i/[Ca]_i$ ratio was still above normal. Although it did not decrease in the hyperthyroid state, myocardial $[Mg]_i/[Ca]_i$ appeared to correlate well with thyroid status. Mg^{2+} has been demonstrated to influence calcium metabolism in muscle and in skinned skeletal fibers increasing free Mg^{2+} above 0.3 mM decreased submaximal tension development (33). Therefore the increased $[Mg]_i/[Ca]_i$ ratio of hypothyroid myocardial may play a role in the induction of depressed contractility in this state. However, in thyroidectomy no such change was seen (12) which would appear to rule out an obligatory role of altered $[Mg]_i/[Ca]_i$ in the depression of myocardial contractility.

SUMMARY

Hypothyroidism in rats was induced by propylthiouracil treatment for 6 to 8 weeks whereas hyperthyroidism was induced by injecting thyroxine for 7 days. Treatment of the sarcolemmal preparations with alamethicin at a drug to protein ratio of 1.0 showed an increase in the Na^+-K^+ ATPase activity in hypertrophied hearts but a decrease in the enzyme activity was apparent in hypothyroid hearts. The sarcolemmal preparations from hypothyroid animals were more sensitive whereas those from hyperthyroid rats were less sensitive to alamethicin in comparison to the euthyroid preparations. The observed changes in the sarcolemmal membranes were not specific because the Ca^{2+}-transport function of the sarcoplasmic reticulum, as measured by the oxalate-supported Ca^{2+} uptake in heart homogenate, was increased in hyperthyroid state and decreased in hypothyroid animals. Myocardial intracellular sodium and potassium did not change in hypothyroid and hyperthyroid rats; however intracellular sodium and potassium increased after thyroid hormone administration to hypothyroid animals. Intracellular calcium did not change significantly with changes in thyroid status, whereas intracellular magnesium was significantly altered only when hypothyroid and hyperthyroid states were compared. Myocardial $[Mg]_i/[Ca]_i$ ratio was significantly increased in the hypothyroid state but not after triiodonthyronine treatment. These results suggest that a defect in the sarcolemmal Na^+-K^+ ATPase in hypothyroidism may not be associated with apparent changes in myocardial Na^+ and K^+ contents whereas an increase in $[Mg]_i/[Ca]_i$ may play a role in the manifestation of depressed contractile state in hypothyroidism.

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12

INHIBITORY EFFECTS OF CAPTOPRIL ON THE ONSET OF CARDIOMYOPATHY IN CARDIOMYOPATHIC HAMSTERS

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INTRODUCTION

Attempts at treating idiopathic cardiomyopathy have been made both clinically and especially using cardiomyopathic hamsters. Cardioprotective effects of drugs such as Ca antagonists(1), β-blockers, as well as α_1-blockers, *l*-carnitine, ubiquinone, α-tocopherol, etc. have been tried on cardiomyopathic hamsters. Although these drugs have shown some degree of effect both histopathologically and biochemically, no effect in inhibiting the progress of degeneration has been observed. At this point, we administered angiotensin-converting-enzyme inhibitor (ACE inhibitor), captopril to cardiomyopathic hamsters. And compared protective effects against its myocardial damage by examining the ECG, biochemically by using serum malondialdehyde (MDA), serum CPK activity, serum aldolase and ventricular myosin isoenzyme as indices.

METHODS

Cardiomyopathic hamsters (J-2-N) We bred a male cardiomyopathic hamster (Bio. 14.6) with a female Golden hamster and the F_1 female obtained was bred with a Bio. 14.6. The F_2 female obtained was bred with the same Bio 14.6 male parent and the F_3 hamster thus obtained was a cardiomyopathic hamster with a higher reproduction rate than the Bio 14.6 hamster. This hamster was named J-1-N (Figure l) and J-2-N hamsters were obtained by interbred some generations between J-1-N female and Bio 14.6 male hamster.

Thus obtained J-2-N hamsters were divided into cardiomyopathic and normal groups based on ECG measurements (2).

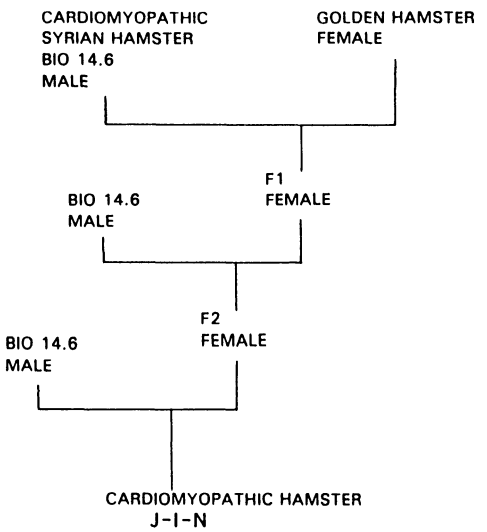

Fig. 1 Cross-breedings of cardiomyopathic hamster

EVALUATION OF ECG

	score
LAD or RAD	1
ST depression	1
QS (Ⅱ,Ⅲ,aV$_F$)	1-2
QS (V$_{1-2}$)	1
QS (V$_{3-5}$)	1-2
low voltage	1
pulmonaly P	1
arrythmia	1

Fig. 2 ECG score in our laboratory

Figure 2 shows ECG scores obtained in our laboratory; animals with a score of 4 or more had clearly developed cardiomyopathy and were designated the cardiomyopathic group (CH group). We used animals with normal ECGs as the control group (C group) and also used Golden hamsters for reference (G group) at the same time.

Serum CPK, serum aldolase, serum LDH, and serum MDA in these 3 groups was determined.

Malondialdehyde determination (3, 4) Serum was heated at 90°C for 30 minutes in a mixture composed HCl and thiobarbituric acid. The resultant color was extracted with 15% methanol containing n-butanol and read at 535 nm. Results were calculated using standard solutions of 1,1,3,3-tetraethoxy propane (5).

Captopril administration At age 5 weeks male cardiomyopathic hamsters J-2-N were divided at random into two groups; the captopril treated group received 15 mg/kg of captopril orally for 10 weeks by dissolving the drug in drinking water. The other group was called the non-treated group. Body weights were periodically determined from beginning to end of the experimental period. ECGs were taken using both limb and chest leads from around the beginning of the experimental period. At the end of the experimental period, blood was drawn under pentobarbital anesthesia from abdominal aorta and serum CPK, serum aldolase, serum LDH, serum MDA were determined. Extirpated heart was studied both histopathologically and biochemically. For histopathological examination, extirpated heart was embeded, sliced and dyed with HE and Azan Stain. The fibrotic ratio was then determined by the point count method. Biochemically, myocardial ventricular myosin isoenzyme patterns were compared by the following method.

Myosin isoenzymes Native myosin was extracted from left and right ventricles in accordance with the method of Martin et al. (6), myosin isoenzymes were separated by pyrophosphate gel electrophoresis (T=4%, C=3%, acrylamide) by the method of Hoh et al. (7). 10 µl samples of myosin solution (1 µg/µl) were placed on the gel and electrophoresis was carried out for 27 hours at 2°C. Gels were stained by the method of Blakesley et al (8).

RESULTS

The left figure of Figure 3 shows the ECG of normal hamsters which were except for left axis deviation. The right shows the ECG of the

cardiomyopathic group which exhibit a QS pattern at III, and aV_F. We compared serum CPK and aldolase in both groups, as shown in Figure 4. In the normal ECG group serum CPK did not rise and showed the same level as the Golden hamsters, but in the CH group there was a marked rise in serum CPK, showing good correlation with ECG score (9).

During captopril administration, 3 animals in the non-treated group died after the 8th week (13 weeks of age). Due to this a decrease in mean body weight was observed in the non-treated group at the 8th week. But at the end of the administration period in the 10th week, no significant difference was observed between groups in regard to body weight, heart weight, and heart/body weight ratio.

Figure 5 shows serum CPK and serum aldolase activity. In the non-treated group serum CPK rose to 2231 ± 409 ($n = 10$), but in the captopril group it only rose to 685 ± 143 ($n = 14$), a marked inhibition. Aldolase showed almost same tendendency. Elevated level of serum MDA was observed in the non-treated group but these rises were also markedly inhibited in the captopril group. LDH elevation of non-treated group was not significant (Figure 6).

Figure 7 shows the myosin isoenzyme in left and right ventricles of the non-treated and captopril groups. In the non-treated group a reduction in myosin V_1 and an increase in myosin V_3 were observed, but the captopril group displayed almost identical ventricular myosin isoenzyme patterns as the normal ECG J-2-N and Golden hamsters. Figure 8 shows relative ratios of V_1, V_2, and V_3 in each group.

In this way we confirmed that myosin isoenzyme patterns in the captopril group showed almost no differences with those of normal hamsters. That is

to say, captopril administration provided good prophylaxis against myocardial damage in cardiomyopathic hamsters from the aspect of constituent protein.

ECGs of cardiomyopathic hamsters J-2-N used in this experiment, revealed severe myocardial damage, as shown in Figure 9. Hamsters administered captopril however showed no such findings, but rather displayed ECGs similar to those of normal hamster in Figure 10. In support of these ECG findings, myocardial tissue damage in the non-treated group was observed to be severe, in the captopril group only minimal changes were observed; fibrotic ratio in the non-treated group was 8.0% (mean) but was only 4.8% (mean) in the captopril group. Fibrotic ratio in the Golden hamsters was 3.5% (mean).

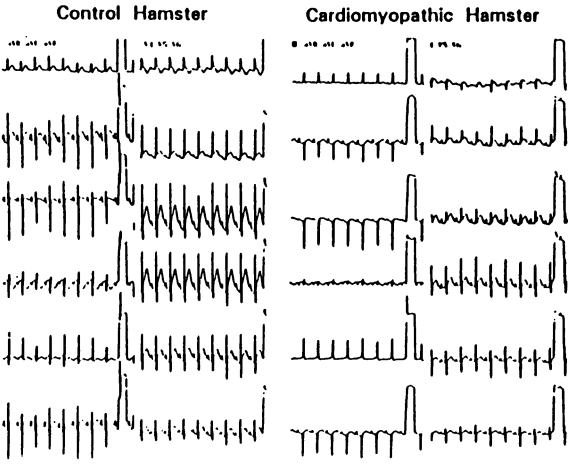

Fig. 3 Electrocardiogram of cardiomyopathic hamster J-2-N
The left figure is the electrocardiogram of the normal ECG hamster, the right shows the ECG of the cardiomyopathic group exhibiting a QS pattern at III and aV_F.

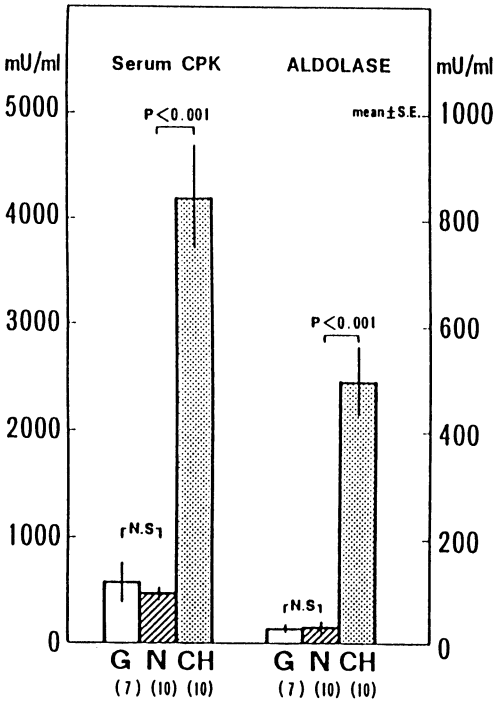

Fig. 4 Serum CPK and aldolase activities of Cardiomyopathic hamster

Muscle tissue cell membranes throughout the body were markedly impaired in the cardiomyopathic group. G: Golden hamster, N: ECG normal hamster, CH: Cardiomyopathic hamsters J-2-N with ECG score of 4 or more. Values represent mean ±S.E.

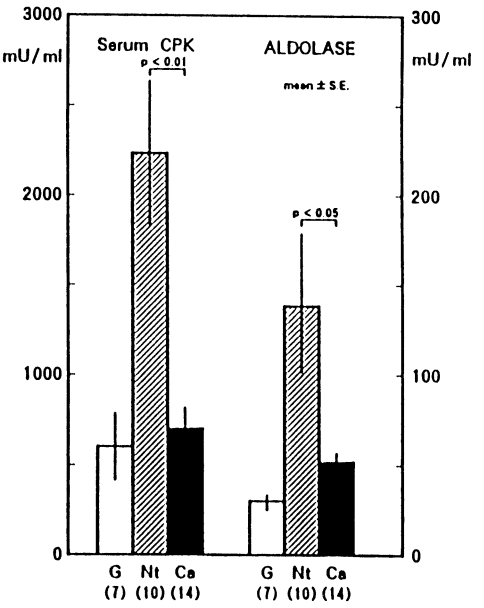

Fig. 5 Serum CPK and aldolase activities of captopril group

This figure shows serum CPK and aldolase activities. In addition to progressive cardiomyopathy in the non-treated group, serum CPK rose significantly, but this rise was markedly inhibited in the captopril group. G: Golden hamster. Nt: Non-treated group. Ca: Captopril group. Values represent mean ±S.E.

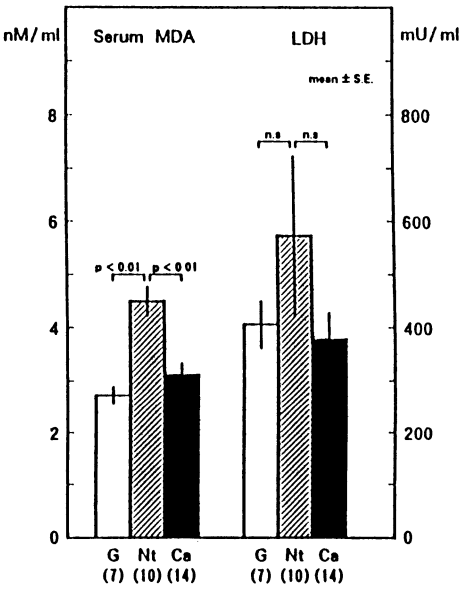

Fig 6 Serum MDA and LDH activities of Cardiomyopathic hamster

Elevated level of serum MDA was observed in the non-treated group but these rises were also markedly inhibited in the captopril group. G: Golden hamster. Nt: Non-treated group. Ca: Captopril group.

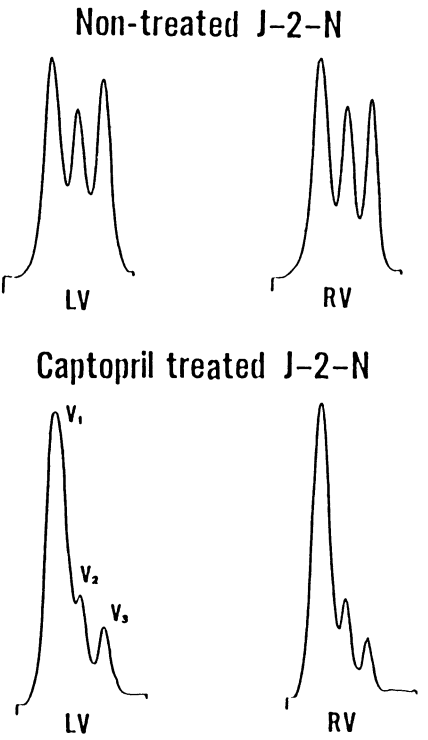

Fig. 7 Ventricular myosin isoenzyme typical patterns

This figure shows myosin isoenzyme in the left and right ventricles of the non-treated group and captopril group. The captopril group ventricular myocardial myosin isoenzyme showed the same pattern as the ECG normal J-2-N hamsters and Golden hamsters.

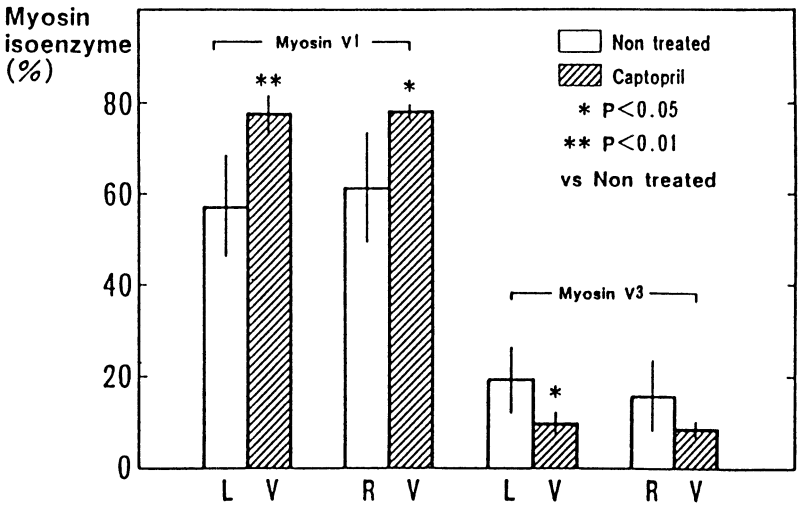

Fig. 8 Relative ratios of myosin isoenzyme V_1, V_2 and V_3 in the non-treated and captopril groups

Percentages of right and left ventricular myosin isoenzyme V_1 of the captopril group were significantly higher than the non-treated group. Values are mean ±S.D.

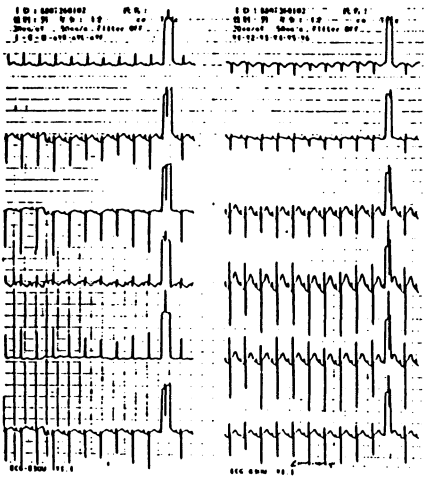

Fig. 9 Electrocardiogram of non-treated hamster

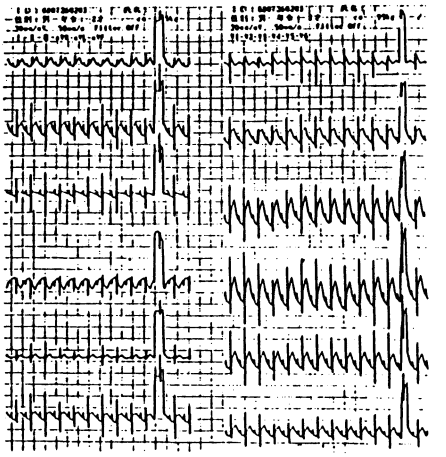

Fig. 10 Electrocardiogram of captopril treated hamster

DISCUSSION

Attempts at treating cardiomyopathic hamsters have been conducted before; looking at recent reports, Factor (10) and Tapp (11) reported that the Ca antagonist Verapamil, alleviated myocardial cellular damage. They stated that this inhibition of damage was due to the prevention small blood vessel contraction which leads to advancement of myocardial cellular necrosis during the initial stages of cardiomyopathy. Kobayashi et al. (12)

showed that the calcium channel in the myocyte increases at a relatively early period. Finkel et al. (13) in an experiment [^{3}H] nitrendipine, which binds to the calcium channel, found that at around the age of 4 months calcium channels increase and thereafter relatively decrease. β-blockers have mainly been clinically applied. In 1975 Waagstein et al. reported that by using metoprolol, which has no intrinsic sympathomimetic activity (ISA) on patients with dilatory type cardiomyopathy they obtained improvement in both subjective and objective symptoms as well as a reduction in mortality. Thereafter, successive reports appeared indicating its clinical efficacy. However, evaluation of its therapeutic effects are still not definite. Selection of cases and careful administration for cardiomyopathy are presently being carried out. Results based on experimental use of β-blockers are relatively few, but recently Sole et al. (14) stated that although no improvements were observed using the β-blocker propranolol, the α_1-blocker prazosin was effective. Further clinical and experimental studies are needed.

A series of studies have been carried out in regard to *l*-carnitine, all agreeing that in cardiomyopathic hamsters Bio 14.6, Bio 53.58, the concentration of carnitine in the myocardium is reduced, and Yamashita et al. (15) administered *l*-carnitine to Biol4.6 hamsters and reported that myocardial necrosis, fibrosis and calcification all decreased.

Other drugs tested on cardiomyopathic hamsters include α-tocopherol, α_1-blocker (16), digoxin (17), etc. At our laboratory we also administered verapamil and noted a significant improvement, but using myosin isoenzyme as an index the effect proved to be somewhat limited.

In this study we administered captopril and obtained wide-ranging improvements in the above-mentioned indices. These results exceeded our expectations. The mechanisms of action of captopril's effects include: 1) inhibition of conversion of angiotensin I into angiotensin II in the renin-angiotensin system, 2) increases in vasodilative prostaglandin, 3) increase in tissue bradykinin and 4) possible scavenger effects of the sulphydril component.

It is thought that of drugs tested up to the present, captopril is the most effective (18), but at the present time it is not known if captpril's effects are due to suppression of cardiomegaly via the ACE-inhibtor action, or whether the increase in vasodilatory prostaglandin and increase in bradykinin are important (19), or to what degree the sulphydril component is involved. At present we are trying to clarify the mechanism of action of captopril by conducting two or three more experiments including administration of Enalapril, which, despite being an ACE inhibitor, has almost no bradykinin stimulating or PGE_2 (prostaglandin E_2) formation promoting action.

SUMMARY

Various drugs have been tested on cardiomyopathic hamsters, but effects inhibiting the progress of damage have not been observed. In this study, 15 mg/kg of captopril was administered to J-2-N hamsters at 5 weeks of age for 10 weeks; age matched J-2-N hamsters were used as non-treated hamsters. At the end of captopril administration, blood was collected from abdominal aorta, and serum malondialdehyde, serum CPK, aldolase, and LDH were determined and myosin isoenzyme patterns of extirpated myocardium were

compared. Additionally, ECGs were compared and the fibrotic ratio was determined. Serum MDA, CPK, aldolase increased significantly in the cardiomyopathic group, whereas these indices were significantly inhibited in the captopril group. High ECG scores and V_3 predominant myosin isoenzyme patterns were also much improved in the captopril group. These results suggest that captopril administration is beneficial in preventing myocardial damage development in cardiomyopathic hamsters. We are now attempting to clarify whether these effects are due to 1) its action as an ACE inhibitor, 2) increase in vasodilatory prostaglandin, 3) action to increase bradykinin in the tissue, or 4) whether action as a scavenger due to sulphydril component is involved.

ACKNOWLEDGEMENTS

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Altered myocardial contractility and energetics in renovascular hypertensive rats.

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INTRODUCTION

Cardiac hypertrophy has been regarded as an important adaptive change in response to cardiac overload, in order to maintain adequate pump function, and is all the more important as a stage in the development of contractile failure. In the present study, cardiac hypertrophy in rats, induced by sustained pressure-overload in experimental renovascular hypertension, was examined from the view point of myocardial contractility and myocardial energetics as represented by ventricular myosin isoenzyme patterns.

METHODS

Hypertension was induced in 8-week-old male Wistar rats by constriction of the right renal artery with a silver clip (Goldblatt rats). Age-matched sham-operated male Wistar rats served as controls. Systolic blood pressure and heart rate were measured by a rat tail manometer-tachometer system (Natsume KN-210-1, Tokyo). Mechanical studies were performed using isolated left ventricular papillary muscles excised 16-17 weeks after the operation. Left ventricular free walls were used for the determination of myosin isoenzyme patterns.

Papillary muscles were stimulated at 32°C with a frequency of 0.2 Hz and a voltage 30% above threshold and were perfused with Tyrode solution containing 1.1M Ca^{2+}. After the

steady state was attained at Lmax, developed and resting tension, dT/dt, time to peak tension, time to half relaxation, and total contraction time were recorded. The responses of mechanical parameters to isoproterenol (10^{-7} M) were also estimated at Lmax, following the interposition of Tyrode solution for 25-30 min, and 10^{-5} M dibutyryl cyclic AMP (DBcAMP) was administered. The response of each parameter was obtained by comparing two paired values. One value was measured in the steady state prior to isoproterenol or DBcAMP administration and the other was the maximum value measured after isoproterenol or DBcAMP administration.

Polyacrylamide gel electrophoresis in the presence of pyrophosphate was performed as described elsewhere (1-3). The gel contained 3.8% acrylamide and 0.12% N,N'-methylene-bisacrylamide. The electrophoresis buffer was 20 mM $Na_4P_2O_7$ (pH 8.8) containing 10% glycerol. Native myosin from the left ventricle was extracted with a solution consisting of 100 mM $Na_4P_2O_7$ (pH 8.8), 5 mM 1,4-dithiothreitol, 5 mM EGTA and 5 μg/ml leupeptin. Electrophoresis was carried out for 30h at 2°C and a voltage gradient of 13.3 V/cm.

Student's *t-test* was used for statistical comparisons.

RESULTS

Ventricular weights in hypertensive rats were about 40% heavier than those in the controls (Table 1). There were no significant differences in either active tension (AT) or resting tension (RT) between hypertensive and control rats (Fig. 1). As for time rate parameters, +dT/dtmax decreased significantly and -dT/dtmax tended to decrease in hypertrophied myocardium from Goldblatt rats as compared with myocardium from control rats (Fig. 2). Myocardial mechnical responses to isoproterenol were significantly depressed in Goldblatt rats (Fig. 3) and responses to DBcAMP tended to decrease in Goldblatt rats

Table 1 Blood pressure, pulse rate, body weight, ventricular weight and papillary muscle size

	B P (mmHg)	P R (beats/min)	B W (g)	V W (mg)	Papillary muscle size	
					L (mm)	CSA (mm^2)
Control (n=5)	148 ± 8	390 ±19	561 ±58	1218 ±162	5.6 ±0.5	0.94 ±0.13
Goldblatt (n=5)	225 ±27	414 ±17	536 ±85	1692 ±394	5.5 ±0.4	1.03 ±0.15
	P<0.01	ns	ns	P<0.05	ns	ns

BP : blood pressure, PR : pulse rate, BW : body weight, VW : ventricular weight, L : length, CSA : cross sectional area, Values are means±SD, ns : not significant

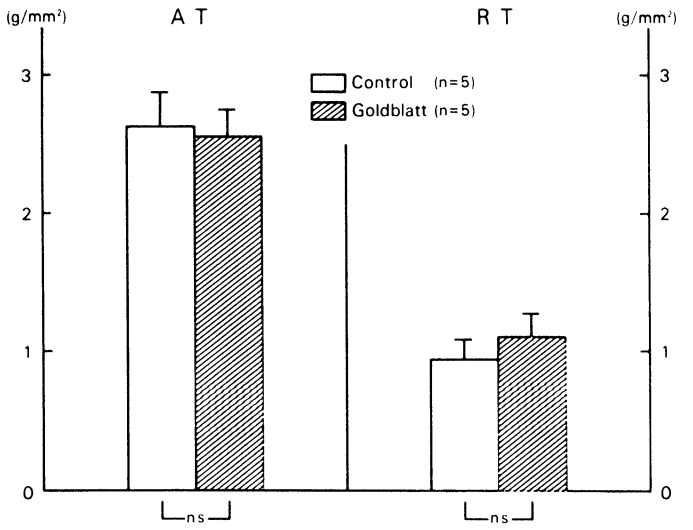


Fig. 1 Comparisons of mechanics (I)

AT : active tension, RT : resting tension
Vertical lines indicate SD ns : not significant

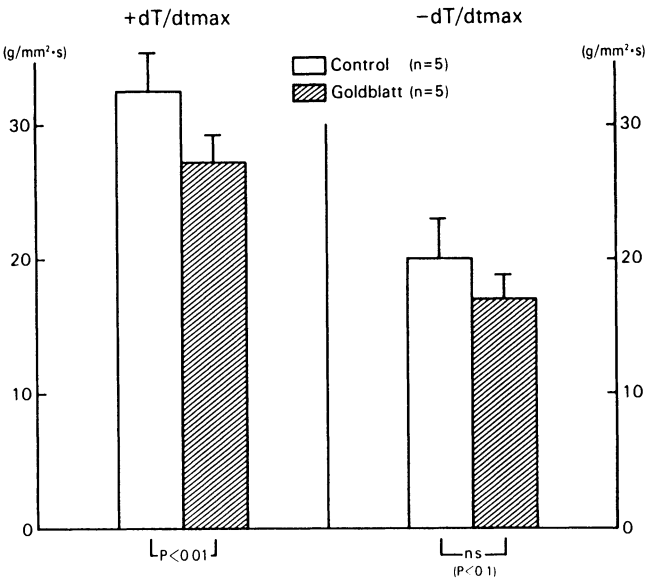


Fig. 2 Comparisons of mechanics (II)

Vertical lines indicate SD. ns : not significant

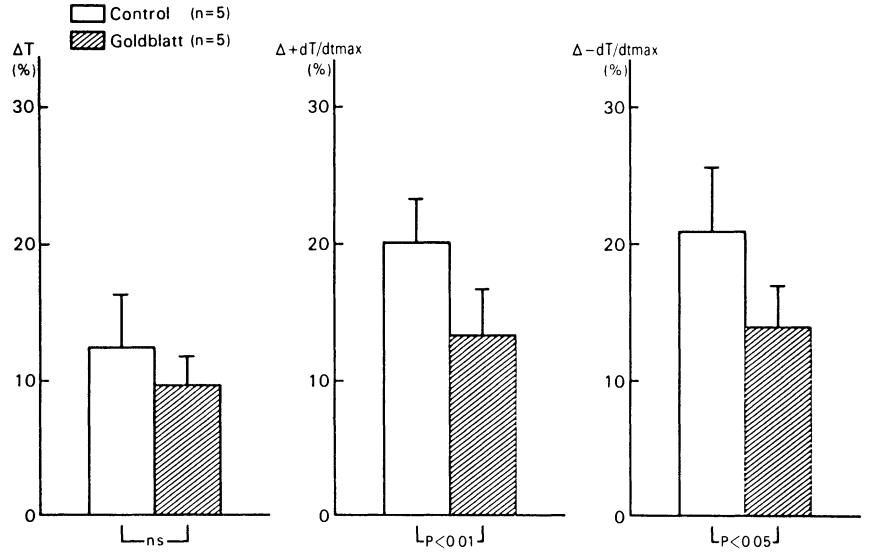


Fig. 3 Myocardial mechanical responses to isoproterenol

Vertical lines indicate SD. ns : not significant

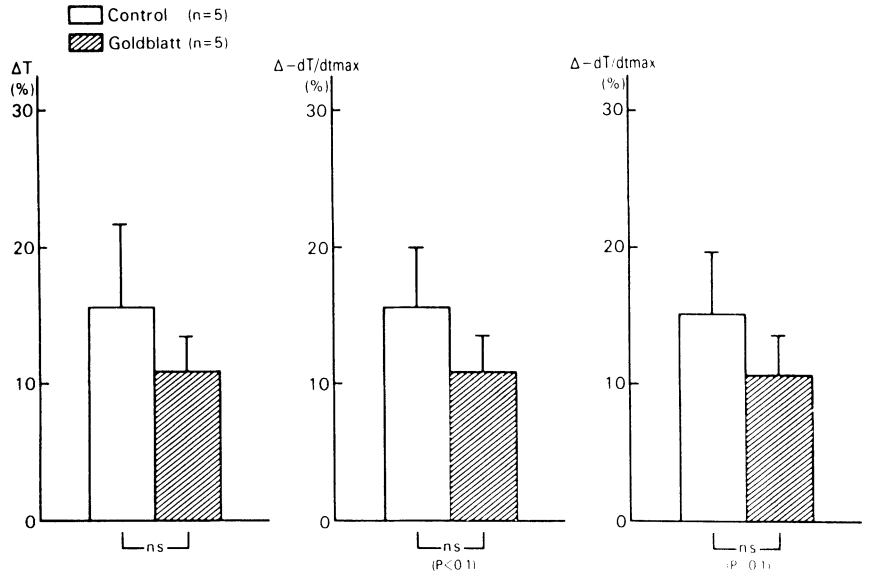
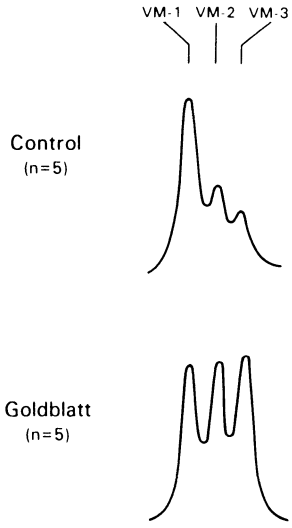


Fig. 4 Myocardial mechanical responses to DBcAMP

Vertical lines indicate SD. ns: not significant



	VM-1	VM-2	VM-3
		(%)	
Control (n=5)	55.9 ±4.2	27.2 ±2.5	16.9 ±2.2
Goldblatt (n=5)	32.2 ±4.1	33.3 ±1.4	34.4 ±5.4
	P<0.001	P<0.01	P<0.001

Fig. 5 Left ventricular myosin isoenzyme pattern

Values are means±SD

(Fig. 4). Left ventricular myosin isoenzyme patterns in Goldblatt rats were significantly shifted toward VM-3, which has the lowest ATPase activity (Fig. 5).

DISCUSSION

Isometric developed tension (T) of isolated left ventricular papillary muscles from hypertrophied myocardium remained unchanged in the present study. This is consistent with a report to the effect that isometric tension in Goldblatt hypertension was significantly increased in the early stage and then decreased continually to a value slightly below the control level in the late stage (4). Time rate parameters, i.e., +dT/dtmax decreased or displayed decreasing tendencies in Goldblatt rats. The subcellular causes of these mechanical changes are sarcolemmal or sarcoplasmic reticular dysfunction in hypertrophied myocardium (5-8). In addition, collagen remodelling in pressure-overloaded hypertrophied myocardium has been reported (9). Hydroxyproline concentration in the left ventricular tissue of Goldblatt rats was unchanged in the early stage and was increased in the later stage (10). The shift of myosin isoenzyme patterns toward VM-3 in Goldblatt rats may be considered as an adaptation for economical contraction (11-13). Our data are consistent wih the idea that the time rate parameters are more affected by myocardial transformation than developed tension and working capacity (14,15). Myocardial mechanical responses to isoproterenol decreased significantly in hypertensive rats. This can be explained in tearms of decreased myocardial β-receptors in pressure-overloaded myocardium (16). Factors related to post-receptors may also play a role in depressed myocardial catecholamine responsiveness in hypertensive rats. In the present study, the observed myocardial response to DBcAMP, which passes the myocardial surface membrane and exerts positive inotropic effects without stimulating β-receptors, was generally similar to that described in our previous reports (17,18), although the depressed response noted in

the present study was not statistically significant. Factors relating to post-receptors include sarcolemmal or sarcoplasmic reticular function. Alteration in myosin isoenzyme patterns could also be of significance if the degree of increase in contractility resulting from activation of the cAMP-regulated system varies with the relative concentration of VM-1 (19). Furthermore, the adrenaline responses of papillary muscles containing VM-1 and VM-3 differ in the rate of cross-bridge cycling (20).

SUMMARY

Alterations in myocardial contractility and energetics were investigated in rats with experimentally induced renovascular hypertension (Goldblatt rats). Hypertension was induced in 8-week-old male Wistar rats by constricting the right renal artery with a silver clip. Mechanical studies were performed with isolated left ventricular papillary muscles excised 16-17 weeks after the operation. Left ventricular myosin isoenzyme patterns, which are closely related to myocardial energetics, were obtained by pyrophosphate gel electrophoresis. There was no significant difference in isometric developed tension (T) between control and hypertensive rats, but the maximum rate of increase in tension (dT/dtmax) was lower in the latter group. Myocardial mechanical response to isoproterenol was depressed and the response to DBcAMP tended to decrease in hypertensive rats. Left ventricular myosin isoenzyme patterns in hypertensive rats shifted toward VM-3, which has the lowest ATPase activity.

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CARDIAC PHOSPHATIDYLETHANOLAMINE N-METHYLATION IN NORMAL AND DIABETIC RATS TREATED WITH L-PROPIONYLCARNITINE

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INTRODUCTION

The mitochondrial oxidation of long-chain fatty acids is a major pathway for energy production in the heart. L-carnitine, a naturally occurring highly polar compound, is essential for transporting long-chain fatty acids across the inner mitochondrial membrane to the site of oxidation, as well as for the export of intramitochondrially produced short-chain acyl esters and for the disposal of unphysiological acyl metabolites (1,2). In fact, myocardial carnitine deficiency has been documented in several heart diseases, both in humans (3,4) and in experimental animal models (5,6). Accordingly, carnitine deficiency can be seen to be associated with an energy deficit arising from the unavailability of fatty acids within the mitochondria. Moreover, the accumulation of long-chain fatty acids and their derivatives, fatty acyl CoA-thio esters and fatty acylcarnitine esters due to carnitine deficiency could produce deleterious effects on cardiac structure and function. These metabolites are active detergents and bind to the cell membranes (7), and there is evidence (7,8) that these lipid intermediates alter the functional properties of the myocardial membranes. It has also been suggested that the long-chain acyl derivatives may contribute to the decline of myocardial contractility and may cause intracellular Ca^{2+} overload (8).

Phospholipid N-methylation reaction consists of the sequential addition of three methyl groups from the physiological donor S-adenosyl-L-methionine (AdoMet) to the amino moiety of an intramembranal phosphatidylethanolamine (PE) molecule (9,10). The final synthesis of phosphatidylcholine (PC) is preceded by the formation of intermediates, monomethyl and dimethyl derivatives, in the presence of the membrane-bound methyltransferase system. Accordingly, the methylation of PE results in the formation of phosphatidyl-N-monomethylethanolamine (PMME), which is then converted into PC via phosphatidyl-N, N-dimethylethanolamine (PDME). Previous studies in our laboratory using rat heart subcellular membranes have shown the existence of three methyltransferase catalytic sites (I, II and III) for PE N-methylation, each exhibiting different kinetic parameters, pH profile, sensitivity to divalent cations, and pathobiological behavior (10-13). These three sites can be identified at 0.055 uM (site I), 10 uM (site II), and 150 uM (site III) concentrations of AdoMet (10-12). Under optimal conditions, predominant synthesis of specific N-methylated phospholipids, namely PMME, PDME and PC was found to occur in cardiac membranes at sites I, II and III, respectively (11,12). It should be pointed out that phospholipid N-methylation has been suggested to be an important mechanism for changing several membrane associated functions (9) including the control of Ca^{2+} fluxes in the myocardium (14-16), and it has been shown to be defective in several heart diseases (12,13,17).

Although the sarcolemmal (SL) membrane from chronic diabetic rat hearts has been reported to exhibit a reduced methylation rate (18), the exact mechanism responsible for this altered membrane function is unknown at present and this defect is likely to be one of the many metabolic derangements in diabetes (19). In this regard, carnitine deficiency has been shown to occur

in diabetic hearts (20) and the associated elevation of myocardial free fatty acids (21) has been suggested to alter the membrane protein function (7). In fact, the depressed Na^+,K^+-ATPase and Na^+-Ca^{2+} exchange activities of the diabetic heart SL were partially normalized by treating the diabetic animals with L-propionylcarnitine (22), one of the most potent carnitine analogues (23). Recent studies (24) also suggest that L-propionylcarnitine may specifically reduce the membrane damage due to oxygen derived free radicals and their by-products; these toxic chemical species have been indicated to contribute to the diabetic heart dysfunction (19) and have been shown to inhibit the cardiac methylation process (25). Such findings indicate that the correction of carnitine-dependent metabolic defects in the diabetic heart by L-propionylcarnitine supplementation may be beneficial to the membrane PE N-methyltransferase system. The present study was therefore undertaken to examine the PE N-methylation activities in cardiac subcellular membranes obtained from normal and chronically diabetic rats with or without L-propionylcarnitine treatment.

METHODS

Male Sprague-Dawley rats (150-175 g) of the same age were used in this study. Diabetes was induced under ether anesthesia by a single intravenous (tail vein) injection of streptozotocin (65 mg/kg body weight) dissolved in a citrate-buffered vehicle (pH 4.5); control rats were injected with citrate buffer alone. All rats had unrestricted access to food and water throughout the experimental period until they were sacrificed. The control and experimental diabetic animals were subdivided randomly for the administration of L-propionylcarnitine which was injected intraperitoneally at a dosage of 250 mg/kg body weight (22); the drug treatment started 2 days after the

streptozotocin injection and continued daily for the duration of the experimental period. All animals were sacrificed by decapitation 8 weeks post-streptozotocin injection. Hearts were removed, atria and large vessels were carefully trimmed, and the ventricular tissue was processed for the isolation of subcellular membranes. Blood samples were taken at the time of sacrifice and analyzed for plasma insulin concentration by a standard radioimmunoassay technique (Amersham) and for glucose levels by using the Sigma glucose reagent kit. The above experimental protocol is similar to that employed elsewhere for inducing the diabetic cardiomyopathy (18, 26, 27). Purified heavy or light SL preparations were isolated from pools of two or three hearts according to the methods described by Dhalla et al (28) and Pitts (29), respectively. The fragments of sarcoplasmic reticular membranes (microsomes) were obtained according to the procedure outlined by Sulakhe and Dhalla (30). All these procedures were carried out at 0-5 ^{o}C, and the membrane activities were assayed immediately after completing the isolation protocol. In agreement with previous observations (18,26,27), the marker enzyme (ouabain-sensitive $Na^{+}K^{+}$-ATPase for SL; cytochrome c oxidase for mitochondria; rotenone-insensitive cytochrome c reductase for SR; K^{+}-EDTA ATPase for myofibrils) examination of the SL and SR preparations from control and experimental groups revealed a minimal but an equal extent of cross contamination by other subcellular organelles in both control and experimental heart preparations.

Phospholipid methyltransferase activity was assayed by measuring the incorporation of [^{3}H] methyl groups into membrane phospholipids in the presence of S-adenosyl-L-([^{3}H]methyl) methionine ([^{3}H]-AdoMet) as described earlier (10,11). Assays were performed with 0.5 mg membrane protein in 0.5 ml reaction medium under optimal conditions for the three catalytic

sites involved in the methyltransferase reactions, as indicated previously (10,11). Unless otherwise mentioned, incubation for catalytic site I was carried out in the presence of 1 mM $MgCl_2$, 0.055 uM [^{3}H]-AdoMet (75.2 Ci/mmol) at pH 8.0 (50 mM Tris-glycylglycine buffer). For the catalytic sites II and III, incubation was performed without $MgCl_2$ using 10 uM [^{3}H]-AdoMet (200 uCi/mmol), pH 7.0 (50 mM imidazole buffer) and 150 uM [^{3}H]-AdoMet (200 uCi/mmol), pH 10.0 (50 mM sodium hydroxide-glycine buffer), respectively. After a preincubation period of 10 min at 37^{o}C, the reaction was initiated by adding [^{3}H]-AdoMet and was terminated 30 min later with the addition of 3 ml of chloroform:methanol:2 N HCl (6:3:1, by volume), followed by three times wash with 2 ml of 0.1 M KCl in 50% methanol. The N-methylated phospholipids (PMME, PDME and PC) were separated by thin layer chromatography and the methyl group incorporation in these lipid products was determined (10,11). Protein concentration was measured by the method of Lowry et al (31) with bovine serum albumin (fraction V) as a standard.

Results are presented as a mean ± SE. The statistical differences between mean values for two groups were evaluated by the Student's t test. P value less than 0.05 was considered statistically significant.

RESULTS

Eight weeks after the injection of streptozotocin, the experimental animals showed elevated levels of plasma glucose and decreased levels of plasma insulin. These diabetic animals exhibited depressed body weight and heart weight in relation to control animals but showed a higher heart weight/body weight ratio (data not shown). Such characteristics of the diabetic animals are similar to those reported earlier (18,23,26,27) and were not modified by L-propionylcarnitine treatment (23). The untreated control

and L-propionylcarnitine-treated control animals also did not show differences in any of the above parameters.

The data in Table 1 indicate that all three catalytic sites of the PE N-methyltransferase system were significantly depressed in the heavy SL fraction isolated from diabetic hearts, and this confirms our previous findings (18). Treatment of the diabetic animals with L-propionylcarnitine did not show any modification of changes in the methylation activities. It may however be noted that the L-propionylcarnitine supplementation resulted in a 35% reduction of the methylation rate at site II in control hearts but did not further aggravate the already depressed site II activity in the diabetic hearts (Table 1).

Table 1. Activities of PE N-methyltransferase catalytic sites I, II and III in the heavy sarcolemmal fraction isolated by the hypotonic shock-LiBr treatment method from control, propionylcarnitine-treated control, diabetic and propionylcarnitine-treated diabetic rat hearts.

Experimental Groups	Catalytic site		
	I	II	III
Control	0.72 ± 0.03	8.7 ± 0.4	127 ± 9
Propionylcarnitine-treated control	0.68 ± 0.05	5.7 ± 0.5*	121 ± 8
Diabetic	0.53 ± 0.02*	4.4 ± 0.4*	94 ± 7*
Propionylcarnitine-treated diabetic	0.58 ± 0.02*	4.5 ± 0.6*	92 ± 6*

Values are means ± S.E. of four experiments. Membranes were analyzed for the intrinsic PE N-methyltransferase I, II and III activities upon incubation at 37°C for 30 min in the presence of 0.055, 10 and 150 uM [^{3}H] AdoMet, respectively. The results are expressed as pmol [^{3}H] methyl groups incorporated into N-methylated phospholipids (PMME + PDME + PC)/mg/30 min.
* Significantly ($P < 0.05$) different from control.

Table 2. PE N-methyltransferase activities in the light sarcolemmal fraction isolated by sucrose-density gradient method from control, propionylcarnitine-treated control, diabetic and propionylcarnitine-treated diabetic rat hearts.

Experimental Groups	Catalytic site		
	I	II	III
Control	0.67 ± 0.11	9.4 ± 0.7	123 ± 7
Propionylcarnitine-treated control	0.73 ± 0.13	6.2 ± 0.5*	139 ± 7
Diabetic	0.42 ± 0.02*	5.6 ± 0.3*	76 ± 4*
Propionylcarnitine-treated diabetic	0.46 ± 0.10	6.0 ± 0.2*	81 ± 3*

Values are means ± S.E. of four experiments. The results are expressed as pmol [^{3}H] methyl groups incorporated into N-methylated phospholipids/mg/30 min. Other details are same as described in the legend of Table 1.
* Significantly ($P < 0.05$) different from control.

To exclude the possibility that the observed changes in sarcolemma were due to any artifact associated with one particular membrane preparation from the diabetic heart, PE N-methylation was carried out in the light SL prepared by the sucrose-density gradient method (29). The results obtained with this second SL fraction (Table 2) were similar to those presented in Table 1, which supports the view that low N-methylation rate at the cardiac SL level is a distinct feature of the 8 week stage of experimental diabetes. In fact, subcutaneous injections of 3U protamine zinc insulin/day for the last two weeks to the 6 week diabetic animals were found to normalize the defective SL methylation (data not shown). On the other hand, L-propionylcarnitine treatment failed to modify the altered SL methylation in diabetes, while it depressed site II activity in non-diabetic animals. It should be noted that

in statistically comparing the site I values for propionylcarnitine-treated diabetics vs controls, the P value was more than 0.05 due to the high standard errors. In order to examine if L-propionylcarnitine exerted any direct effect on the enzyme, PE N-methyltransferase activities of the heavy SL fraction purified from normal rat hearts were assayed in the absence and presence of different concentrations of the drug. The incorporation of radiolabeled methyl groups at site II was markedly depressed at 10^{-8} - 10^{-3} M L-propionylcarnitine, whereas no effect was detected at sites I and III (Figure 1).

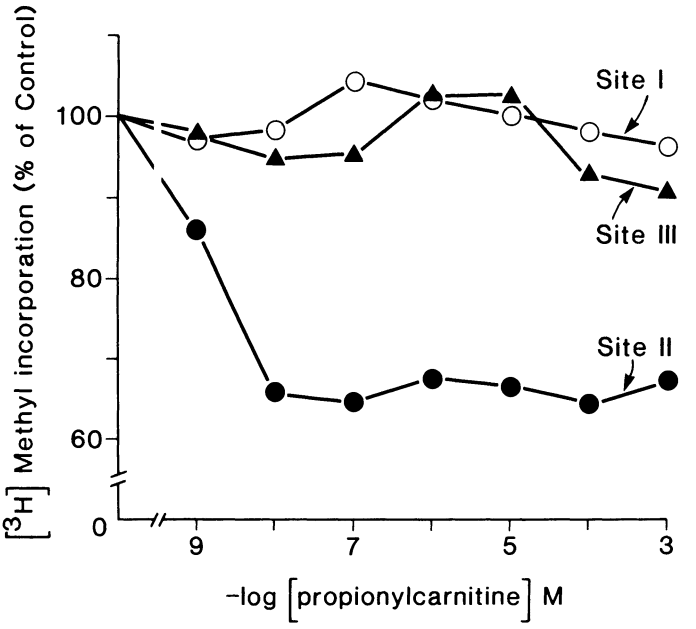

Figure 1. In vitro effect of L-propionylcarnitine on the catlytic sites involved in the PE N-methyltransferase activity in cardiac heavy sarcolemmal fraction. Each point represents an average of four experiments. Control values in the absence of the drug for sites I, II and III were 0.71 ± 0.1, 7.3 ± 1.0 and 110 ± 12 pmol [^{3}H] methyl groups incorporated into total N-methylated phospholipids/mg/30 min, respectively.

Studies in this laboratory have shown that PE N-methylation activity is not only present in the sarcoplasmic reticular membrane (11) but it also stimulates the SR Ca^{2+} pump (15). Earlier experiments have shown that the site I in the SR methylation process was activated in 8 week-diabetic hearts, and this change was normalized by the insulin treatment (32). Results in Table 3 confirm that the site I methylation activity, unlike sites II and III, was stimulated in the diabetic SR. L-propionylcarnitine treatment did not modify the N-methyltransferase activity of both control and diabetic SR preparations (Table 3).

Table 3. PE N-methyltransferase activities in the sarcoplasmic reticular fraction isolated from control, propionylcarnitine-treated control, diabetic and propionylcarnitine-treated diabetic rat hearts.

Experimental Groups	Catalytic site		
	I	II	III
Control	1.47 ± 0.08	3.9 ± 0.2	103 ± 5
Propionylcarnitine-treated control	1.37 ± 0.10	3.8 ± 0.3	111 ± 10
Diabetic	2.17 ± 0.09*	4.2 ± 0.5	112 ± 25
Propionylcarnitine-treated diabetic	2.01 ± 0.08*	3.8 ± 0.3	101 ± 7

Values are means ± S.E. of five experiments and are expressed as pmol [^{3}H] methyl groups incorporated into N-methylated phospholipids/mg/30 min.
* Significantly ($P < 0.05$) different from control.

DISCUSSION

The present study has demonstrated that all three catalytic sites of the SL methyltransferase system were depressed in diabetic myocardium while an increased activity was evident in SR at site I, which is the rate limiting

step in the process of N-methylation (9). It is unlikely that these abnormalities in PE N-methylation were artifacts because two different types of SL preparations revealed similar results and the SR site I stimulation was specific in nature since sites II and III in SR were unaltered. Furthermore, these changes, which confirm previous findings (18,32), appear to be secondary to diabetes per se because treatment of the diabetic animals with insulin for 2 weeks reversed the values towards the control levels (18,32). Therefore, it is evident that at the same stage of diabetes, the methylation system in SR differs from that in SL both in terms of the number of catalytic sites affected in these preparations and the type of alterations at site I. Similar differences were observed also in experimental cardiac hypertrophy (12). Thus, the differential behavior of SL and SR PE N-methylation activities in different disease states of the heart indicates that these activities may have a distinct role in the myocardial cell function. In fact, we have provided evidence that perfusion of the isolated heart with methionine, a precursor for the methyl donor AdoMet, induces a powerful and long lasting positive inotropic effect and that PE N-methylation, probably at the level of SR Ca^{2+} pump, plays an important role in this phenomenon (33). The positive inotropic effect is preceded by a transient mild negative inotropic action, probably related to the intrasarcolemmal accumulation of N-methylated phospholipids and subsequent changes in SL Ca^{2+} transport activities (33), which confirms the involvement of multiple methylation sites in heart function. At this point we may speculate that a decreased methylation at SL level and a simultaneous increased methylation at SR might constitute an integrated compensatory mechanism to sustain the contractile force at 8 week stage of the diabetic cardiomyopathy.

Although the function of L-carnitine as a endogenous cofactor of fatty acid metabolism is established, its role as an exogenously administered

therapeutic agent is less defined. Contrary to our expectations, treatment of the diabetic animals with one of its more potent analogues, L-propionylcarnitine, did not modify the diabetic alterations of SL and SR methyltransferase activities. However, in normal heart, both in vivo and in vitro experiments showed a clear inhibition of the sole SL methyltransferase site II by L-propionylcarnitine. This highly selective effect further confirms that the three SL sites possess distinct characteristics (10-13) and suggests that the intramembranal organization for site II may be different in SL and SR. It is interesting to note that maximal methylation-dependent changes of the SL Ca^{2+} pump and Na^{+}-Ca^{2+} exchange activities were associated with predominant synthesis and intramembranal accumulation of PDME catalyzed by site II (14,16). Phospholipid methylation-dependent alterations in Ca^{2+} pump and Na^{+}-Ca^{2+} exchange seem to cause the initial decrease in contractile force development upon methionine perfusion of a normal heart (33). Thus, it is possible that the inotropic potential attributed to L-propionylcarnitine (23) may be due, at least partly, to the drug inhibition of SL site II and subsequent attenuation of the negative inotropic component related to the SL phospholipid methylation. This would be compatible with the proposal that the multifactorial effects by which carnitine treatment benefit the cardiac function cannot be explained only on the basis of an improved oxidative metabolism (34).

SUMMARY

Phosphatidylethanolamine (PE) N-methylation was examined in cardiac sarcolemmal and sarcoplasmic reticular membranes after inducing chronic (8 weeks) experimental diabetes in rats by an intravenous injection of 65 mg streptozotocin/kg. The N-methylation activities in diabetic sarcolemma were significantly depressed at all three catalytic sites (I, II and III) of the

methyltransferase system. On the other hand, an increase in radiolabeled methyl group incorporation from S-adenosyl-L-methionine was evident at site I without any change at sites II and III in the diabetic sarcoplasmic reticulum. L-propionylcarnitine treatment did not modify the changes in cardiac PE N-methyltransferase in diabetic animals but selectively inhibited the normal sarcolemmal site II methylation activity both under in vivo and in vitro conditions. These results confirm an abnormal PE N-methylation in subcellular membranes from diabetic hearts but these changes were not prevented by L-propionylcarnitine. However, L-propionylcarnitine-induced depression of sarcolemmal site II in normal hearts may be of some significance in terms of the inotropic potential of this drug.

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III

ISCHEMIC HEART DISEASE AND CARDIAC FAILURE

15

DIETARY N-3 POLYUNSATURATED FATTY ACIDS AND ISCHEMIC HEART DISEASE

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INTRODUCTION

Epidemiological studies have indicated that diets containing fish, fish products, or products from other marine animals are beneficial in the prevention of cardiovascular disease (1-5). Modification of several risk factors for the development of atherosclerosis (high levels of plasma triglyceride and total cholesterol, low levels of plasma HDL cholesterol, high aggregability of platelets, leukocyte and monocyte reactivity, high viscosity of blood and, possibly, hypertension) by the n-3 polyunsaturated fatty acids (PUFA) eicosapentanoic (EPA) and docosahexanoic acid (DHA), abundantly present in marine oils, most likely contribute to this prevention (4,6-10). The mechanisms by which EPA and DHA modify these factors are not well understood. Several of the effects may be mediated through changes in prostanoid formation, e.g. by a competitive inhibition of production of arachidonic acid-derived thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) or by an increase of production of EPA-derived prostanoids (TXA_3 and PGI_3).

Dietary fish oil not only affects the rate of development of atherosclerosis but there is also some evidence for its anti-ischemic and anti-arrhythmic action. Several investigators reported a reduction in infarct size, less tissue leakage of creatine kinase and a lower incidence of arrhythmias after *in situ* coronary ligation in fish oil dogs and rats (11,12). Gudbjarnason *et al* have reported that the mortality of rats during chronic norepinephrine treatment was increased by feeding with cod liver oil (13). However, we could not confirm this observation in pigs and rats as (see below). Moreover, at present

it is not clear how n-3 PUFA could influence the outcome of ischemic insult and sensitivity to adrenergic stress.

In patients undergoing percutaneous transluminal coronary angioplasty (PTCA) the incidence of restenosis was lower when fish oil supplements were taken before and after the clinical intervention (14), but the results are not equivocal (15). Dietary fish oil likely exerts this beneficial action by reducing thromboembolism and inflammatory reactions. In analogy, improved graft patency associated with altered platelet function was induced by marine fatty acids in dogs (16).

The potential benefit of dietary intervention with n-3 PUFA may be offset by free radical-induced lipidperoxidation ,which is believed to play a role in the development of ischemia-reperfusion damage in myocardium (17-19), and by oxidative modification of low density lipoprotein (LDL) which could enhance its atherogenicity (20). We now report the effect of dietary fish oil on the rate of lipidperoxidation of the normoxic and ischemic pig myocardium. Harmful effects were not seen which may be related to adaptation of free-radical scavenger systems such as gluthathion reductase (21).

Several studies indicate an increased need for antioxidants like vitamin E to prevent side effects caused by marine oils (22,23). Reduction in platelet aggregation and prolongation of bleeding time, associated with bleeding diathesis (8) and impaired leukocyte function, may lead to infections, delayed healing after tissue injury and carcinogenicity. Most of the marine oils are hydrogenated to yield raw materials for margarine and other edible fats. Hydrogenation destroys the n-3 fatty acids and some new monounsaturated docosanoic acid isomers are formed (9,24). Excess amounts of monounsaturated fatty acids with 22 carbons cause a transient triacylglycerol lipidosis in the heart and other tissues but not in the liver (25). Daily intake of 30 - 40 ml cod liver oil for a long period of time also increases the risk of vitamin A and D toxicity (26).

The currently available concentrated preparations of EPA and DHA are supplemented with vitamin E and are low in cholesterol, monoene fatty acids, vitamins

A and D. These purified extracts facilitate further clinical investigations and experimental trials (7). In our studies a purified mackerel oil preparation, having these special properties, was used.

FISH OIL AND PROGRESSION AND REGRESSION OF ATHEROSCLEROSIS

Critical interactions between 4 types of cells - monocytes, platelets, endothelial cells and smooth muscle cells - are believed to be involved in atherogenesis (27). The initial lesion in cholesterol-fed monkeys is deposition of clusters of leukocytes, primarily monocytes, on arterial endothelium (8). These monocytes migrate to the subendothelium and assume the shape of foam cells in fatty streaks. The overlying endothelium retracts and platelet adherence and aggregation leading to mural thrombosis occurs. Smooth muscle proliferation and migration from media to intima and accumulation of lipids within the smooth muscle are prominent features of fibrous plaque formation. Prostanoid metabolism probably is involved in the molecular basis for many of these events. In order to understand the mechanism leading to attenuation of the platelet-vessel wall interaction, knowledge of the basic prostaglandin metabolism is mandatory (28,29). Monocytes / macrophages produce the potent chemoattractant leukotriene B_4, a product formed from arachidonic acid by the lipooxygenase pathway (30). Clusters of leukocytes also produce other leukotrienes (such as C_4, D_4 and E_4), which promote platelet aggregation and cause vascular vasoconstriction and may be instrumental in reducing blood flow in atherosclerotic hearts (31). Elevated levels of plasma lipoproteins, especially the LDL and very low density (VLDL) fractions, are often associated with an increased risk of atherosclerosis. Hyperlipidemia probably participates in atherogenesis by adding injury to endothelium damaged by increased membrane viscosity (32). Hyperlipidemic endothelium may also permit monocyte adhesion and may promote growth factor formation in the endothelial cells (6,8).

The effect of fish oil on experimental atherosclerosis (including regression) has not been extensively studied. Positive studies have been reported in rats (33), swine (34-

36) and rhesus monkeys (37). In rabbits (38,39) and in another study in rats (40) the results were negative. The negative results in one of the studies on rabbits could be explained by an improper control of the diet composition (41). The study of Weiner *et al* (34) showed that cod liver oil reduced the severity of atherosclerotic lesions in hypercholesterolemic swine without affecting serum lipid levels. They therefore attributed the fish oil effect to the reduction of serum TXA_2 levels. It appears that when serumcholesterol is increased by exogenous cholesterol and bile acids, the marine oils are not capable to reduce plasma cholesterol. We found indeed a marked reduction of LDL and HDL cholesterol during fish oil feeding to normolipidemic pigs (42). On the other hand, dietary fish oil not only reduced the number of lesions in aorta and carotid artery but also lowered serum cholesterol in hypercholesterolemic rhesus monkeys (37). The effects on the levels of serum, LDL and HDL cholesterol may ,however, be less important than the effects on the chemical composition of the lipoproteins. Information in the literature on the influence of fish oil on cholesterol over phospholipid ratio and the PUFA composition of cholesterolesters and phospholipids in LDL and HDL is still scarce. Parks *et al* showed that the reactivity of lecithin:cholesterol acyltransferase (LCAT) in the LDL fraction is reduced in fish oil fed monkeys (43). Thus the cholesterol over phospholipid ratio of lipoproteins can be affected by changes in LCAT activity. The capacity of HDL to carry out reversed cholesterol transport could be influenced by changes in its surface properties which can be caused by alterations in e.g. the cholesterol over phospholipid ratio.

Suppression of atherosclerosis is important but regression of lesions by dietary intervention may be clinically more relevant. Recently we observed the addition of n-3 fatty acids (0.3 g EPA/kg body weight) to a low-cholesterol diet resulted in regression of atherosclerosis in the aorta but not in the coronary arteries (36,44,45). During the regressionperiod the dietary n-3 fatty acids both lowered platelet aggregation and serum cholesterol. How these findings relate to the mechanism by which fish oil induced regression can not be assessed. Superoxide production by polymorphonuclear cells

isolated from the blood of hypercholesteremic pigs was measured. Eight months fish oil feeding reduced the zymosan-stimulated O_2 production, which indicates a possible involvement of the leukocyte functions in the fish oil effects on progression and regression of atherosclerosis. Because atherosclerosis is a complex process involving important perturbations in "cell-cell interaction", cell proliferation, lipoprotein metabolism and arachidonic metabolism it is also premature to extrapolate the observed effects of fish oil to the clinical situation. Therefore, investigations on the effect of dietary fish oil on single factors contributing to the development of atherosclerotic lesion are needed. Some of these factors include the mechanically or chemically-induced damage of the endothelial surface, platelet adherence and aggregation at the impaired sites, smooth muscle cell proliferation, lipid infiltration into the arterial wall and formation of the fibrous plaque (44).

We investigated the effect of fish oil on platelet-vessel wall interaction in pigs with a chronically stenosed coronary artery (46,47). For this purpose we fed the animals either fish oil (0.3 g EPA/kg body weight per day) or lard fat. After 8 weeks a non-resting flow-limiting stenosis was applied by placing a 2 mm plastic constrictor around the left anterior descending coronary artery. Eight weeks later the animals were again anesthetized and regional perfusion (by radioactive microspheres) and systolic segment shortening (by echocardiography) were measured in order to determine whether proliferation of the endothelial layer close to or at the site of constriction affected myocardial regional bloodflow and function. When the hearts were stressed by raising the heart rate to 160 beats/min the myocardium, especially the subendocardium, of the lard fat fed animals nourished by the partially constricted coronary artery was hypoperfused and did not function normally (47). Histological examination revealed that the fish oil fed animals had significantly less intima and media proliferation at the site of the stenosis. Dietary fish oil also markedly reduced serum triglyceride (50%) and cholesterol (20%) levels. However, the lipid levels of the lard fat fed (2.5 mM for cholesterol and 0.6 mM for triglycerides) do not lead to the development of

atherosclerotic lesions in normal coronary arteries during that period (46,48). We also showed that serum concentrations of TXA_2 and PGI_2 were markedly reduced by fish oil (47,49). Aggregation of platelets in whole blood was reduced in the mackerel oil fed animals. The blood viscosity tended to decrease with fish oil feeding. These effects of fish oil on blood viscosity could favorably influence abnormally high mechanical cell to surface actions and shear forces disrupting the barrier function of the endothelium around the site of the constrictor. It is feasible that in the above described study (47) the triggering of intimal proliferation response was prevented by fish oil through inhibition of platelet adherence to the vessel wall, platelet aggregation and production of platelet derived growth factor (46). It is unlikely that factors such as endothelium-dependent relaxations (8,50), increased neutrophil aggregation, accumulation of mast cells in the coronary arteries and release of leukotrienes (51) are involved, because the constrictor produced a fixed stenosis.

As mentioned before, a prominent effect of EPA on the metabolism of prostaglandins and leukotrienes can not be excluded. Such an effect could alter constrictory, thrombotic and inflammatory responses that are critical to plaque formation following endothelial injury (30,52).The fatty acid 20:5n-3 inhibits the formation and antagonizes actions of 20:4n-6 derived prostaglandins. EPA is a competitive inhibitor and a poor substrate for cyclo-oxygenase. Small quantities of TXA_3 and PGI_3 are also formed of which only PGI_3 is known to have weak agonistic properties. This explains why the ratio TXA over PGI decreases after fish oil ingestion. PGI and TXA play opposite roles in the regulation of platelet aggregability, vasoconstriction. Hence the ratio of their concentrations will determine the reduction in platelet aggregation. For the same reasons that as with cyclo-oxygenase, the lipooxygenase will be affected by dietary fish oil. EPA behaves as a poor substrate and so competes with 20:4n-6 for lipo-oxygenase. For example, less LTB_4 but more LTB_5 molecules are formed and this may affect the function of leukocytes and vascular endothelium.

In human volunteers the synthesis of interleukin-1 (IL-1) and tumor necrosis factor

(TNF) by mononuclear cells is suppressed by supplementation with n-3 PUFA (53). These cytokines induce local inflammatory changes and mediate several systemic acute-phase responses. Moreover, IL-1 and TNF often act synergistically on vascular endothelium and the synthesis of arachidonic acid. The effects of fish oil on platelet aggregation and mononuclear cell synthesis of cytokines may both contribute to a lower rate of restenosis after PTCA (15).

The beneficial effects of EPA and DHA on the process of atherosclerosis have been attributed frequently to the diet-induced changes in serum lipid levels (9,40,48,49,54,55). Fish oil definitely reduces circulatory triglyceride but the reports on the responses of total cholesterol, LDL and HDL levels are equivocal. HDL levels are not affected or decrease depending on the daily intake of EPA and DHA (40,54,42,56). There is abundant evidence that fish oil primarily inhibits the production of VLDL by the liver (6,9,42,57,58). LDL is a metabolic product of VLDL catabolism and surface fragments of VLDL are used to assemble plasma HDL. Therefore, secondary effects of dietary fish oil on serum LDL and HDL levels may be expected. Fish oil also increases peroxisomal beta-oxidation activity and suppresses membrane-bound diacylglycerol acyltransferase (58,59) by modification of the membrane phospholipid fatty acid composition. These effects contribute to the hypotriglyceridemic response. The lipoprotein catabolizing enzymes, lipoprotein lipase and hepatic endothelial lipase are not involved in the lowering of the triglyceride levels (54,60). We observed indeed a decrease in lipoprotein lipase activity in fish oil fed pigs (42)

FISH OIL, MYOCARDIAL MEMBRANE FUNCTION AND SENSITIVITY TO ADRENERGIC AND ISCHEMIC STRESS

Myocardial membrane function

Marine oils produce, in addition to the antiplatelet, vasodilatory, antiinflammatory and hypolipidemic effects, marked changes in fatty acid profiles of myocardial

phospholipids (61-63). The primary effects include a reduction in the content of n-6 fatty acids such as linoleic and arachidonic acid and a concomitant increase in n-3 fatty acids, such as EPA and DHA. Alteration of the fatty acid composition of membranes may affect numerous cellular functions such as the production of eicosanoids, membrane receptor characteristics, membrane enzyme activities and permeability to various ions. Clearcut evidence for those effects of fish oil in myocardium is lacking (52,64). Since fish oil causes replacement of the n-6 by n-3 fatty acids the double bond index (DBI) of cardiac membranes increases markedly (52,54,55). The DBI is one of the main determinants of the fluidity of the membrane core. Several other factors must be considered before alterations in fluidity can be directly attributed to changes in fatty acid unsaturation. Changes in membrane composition (the neutral triglyceride content, the phospholipid/protein ratio, the phospholipid class distribution and cholesterol/phospholipid ratio) must also be known (52,64). For example, an increase in the cholesterol/phospholipid ratio tends to increase the order of lipid motion in bilayer (65). We found in sarcolemma of hearts of mackerel oil fed pigs a higher DBI (1.85 *vs* 1.38) and a higher cholesterol/phospholipid ratio (0.64 *vs* 0.38) than in those of lard fat fed pigs (52). These observations confirmed results of earlier studies performed in hearts from cod liver oil fed rats (61). The 24:1 content of sarcolemmal sphingomyelin fraction increases after mackerel oil nutrition which represents another adaptive response to a high DBI-induced membrane fluidity change. The sarcolemmal sphingomyelin fraction contains mostly saturated and monoene fatty acids and therefore determines membrane fluidity in the same negative manner as the cholesterol fraction (52). The increase of sphingomyelin's relative content of long-chain monoene fatty acids reduces membrane fluidity, making it even more difficult to predict the direction of changes in membrane fluidity by dietary intake of PUFA.

In our studies on porcine myocardium the phosphatidyl inositol (PI) fraction of the sarcolemmal phospholipids showed an unexpected low content of 20:4 n-6, although replacement of linoleic and arachidonic acid by EPA and DHA due to fish oil nutrition

was again present (52). A receptor-mediated phospholipase A_2 (PLase $A_{2)}$ probably catalyzes hydrolysis of 20:4n-6 from the PI lipids which subsequently acts as second messenger. However, studies on the role of PLase A_2 in signal transduction in myocardium are lacking. In general, a small fraction of the membrane phospholipids phosphatidylethanolamine (PE) and PI can be converted rapidly into phosphatidylcholine (PC) by PE-N-methyltransferases and into phosphatidyl inositol-4.5-bisphosphate (PIP_2) by the hormone-receptor stimulated PIP_2-specific phospholipase C (PLase C), respectively. In myocardium, respectively ß- and α_1-adrenergic receptor stimulation initiate those phospholipid responses, but the conversion rates of PE and PI phospholipids are relatively low (52,66-70). It is feasible that fish-oil induced modification of PUFA composition of PE and PI in the membrane affects those signal transduction processes. Furthermore, the activation of protein kinase C by the formed diacylglycerol (DG) during the increased rate of PI turnover depends on the molecular species of the DG which are derived from PIP_2 molecules (69). The role of protein kinase C induced protein phosphorylation is not completely understood but effects on downregulation of adrenoceptors and activation of Ca^{2+} channels have been described (69). In fact, PUFA are involved in several steps in the α_1-adrenoceptor mediated phosphatidylinositol turnover : 1) in the phospholipidbilayer in which the adrenoceptor and PLase C are embedded ; 2) in the substrate PIP_2 for the PLase C ; 3) in DG, the natural activator of protein kinase C ; 4) as free EPA and 20:4n-6 which are both substrates for cyclo-oxygenase. Indeed, some recent reports indicated that an EPA and DHA containing diets produced changes in the response of the heart to catecholamines, particularly in the α_1 type (13,61,71)

We have also investigated the functional properties of myocardial sarcolemma isolated from mackerel oil fed pigs (52). No changes were found in the Ca^{2+} permeability and Na^+/Ca^{2+}-exchange activity of the isolated membrane fragments. Heart sarcolemma exhibited an increased 5'-nucleotidase and Ca^{2+} pumping ATPase activity. Adenylate cyclase activity was unchanged although its sensitivity to isoproterenol

stimulation was higher. It is not clear whether the enzymatic changes are due to changes in the membrane DBI or cholesterol content.

Myocardial adrenergic and ischemic stress

The relevance of all findings on phospholipid fatty acid composition and enzyme activities in isolated membranes for the *in vivo* function of plasma membranes of normal and pathological myocardium is yet unknown. Dietary induced changes in the composition of the phospholipids in the heart alter coronary flow rate and contractile function (52,63,72) and the response to (ischemic) stress (12,52,62,73).However, it remains difficult to discriminate between a role for membrane phospholipids and eicosanoid synthesis in producing these effects. The observed increase in sensitivity of adenylate cyclase to isoproterenol may be related to the increased mortality due to norepinephrine stress in cod liver oil fed rats (74). Gudbjarnason *et al* (74) and we (61) showed that chronic administration of norepinephrine to rats produced marked changes in fatty acid distribution in the major phospholipids of the myocardium. A substantial replacement of n-6 fatty acids by n-3 fatty acids took place. The question arises whether the alteration in fatty acid composition here was a result from α_1 or ß adrenergic receptor stimulation. If the answer would be α_1, it means that a high PI turnover leads to preferential incorporation of DHA above 18:2n-6 in the major phospholipids of myocardium

Mortality of cod liver oil fed rats increased after chronic isoproterenol treatment (6), but decreased when arachidonic acid-rich diets were used (52). We could not confirm the increased mortality during catecholamine stress of fish oil fed rats, although the extent of replacement of n-6 by n-3 PUFA was quite similar (6,61). The discrepancy between the mortality data and the lack of knowledge about the cause of death of the norepinephrine treated rats does not permit to draw a conclusion concerning the role of dietary n-3 fatty acids on the sensitivity of the heart to stress. Moreover, in another comparative study we found no major differences in cardiovascular performance of

mackerel oil and lard fat fed pigs (myocardial contractility, pre- and afterload, cardiac output and myocardial work), when at baseline conditions and after the heart was stressed by atrial pacing (heart rate 160 beats/min).

Dietary supplementation with fish oil has even been shown to reduce infarct size and the incidence of arrhythmias after coronary artery ligation in dogs (11).However, the effect of fish oil on the incidence of arrhythmias is controversial as in addition to antiarrhythmic (11,73) also arrhythmogenic (62) actions have been reported. We did not observe any differences in the incidence of ventricular arrhythmias and recovery of regional myocardial function during and after multiple coronary artery occlusions between anaesthetized pigs fed with mackerel oil (0.3 - 0.6 g EPA/kg body weight per day) and those fed with lard fat for 8 to 16 weeks (49,55). It is possible that the heart should be subjected to longer periods of ischemia to demonstrate dietary fish oil-induced changes in recovery of function and arrhythmias. Much lower baseline and peak levels of TXA_2 and PGI_2 were found in coronary venous blood of the mackerel oil fed animals which indicated that also in this study EPA efficiently competed with 20:4n-6 for the cyclo-oxygenase. The more vigorous hyperemic response found in the mackerel oil fed animals during the last reperfusion period most likely was caused by a reduction of the TXA_2/PGI_2 ratio (49,55).

The loss of creatine kinase of hearts of cod liver oil fed rats has been examined during *in situ* coronary artery ligation (12) and low flow ischemia using the Langendorff procedure (75). Only in the in situ model the creatine kinase release was 36% reduced by dietary fish oil which was measured after 6 hours of ischemia. However, the authors did not relate the observed difference in ischemic damage to the extent of recovery of contractile function. The mechanism by which n-3 fatty acids exert their protection in the latter studies is not known although alterations in the membrane phospholipid fatty acid composition and eicosanoid synthesis could again have played a role (11,12,52). The pharmacological effects of the cyclo-oxygenase products of arachidonic acid or EPA on the heart can be both beneficial and deleterious. Their precise role in modulating the

response of the heart to ischemic challenges is therefore difficult to assess, irrespective of the changes in the prostanoid concentrations that occur after fish oil consumption (76). Moreover, structural and functional changes of myocardial cell membranes due to the alteration of fatty acid composition may be equally important (52).

FISH OIL AND LIPIDPEROXIDATION

Several reports have shown elevated endogenous generation of free radicals and their products, as malondialdehyde(MDA) and those detectable by electron spin resonance spectrometry in lyophilized tissue. The direct and indirect role of oxygen radicals in the development of myocardial necrosis due to ischemia and reperfusion has been well documented (17-19,77-79). A large body of evidence has been accumulated demonstrating that treatment with free radical scavengers (e.g. superoxide dismutase, catalase and mannitol) reduced the extent of myocardial injury.

Membrane PUFA are very susceptible to oxygen free radicals ($O_2^{\cdot -}$ and $OH^{\cdot}$) and so are the organic free radicals are formed from their reactions with membrane PUFA.The divinyl methane structure within the PUFA chain is particularly prone to abstraction of the allylic hydrogen, resulting in formation of fairly stable lipid-free alkylradicals (77) and rearrangement of the double bonds into the diene configuration. In the presence of O_2^-, these lipid-free radicals initiate a chain of autoxidation reactions starting with the formation of peroxyradicals (initial phase). The peroxyradical can abstract hydrogens from surrounding molecules (PUFA, alkyls or proteins) and those can be converted into lipidperoxides. These lipidperoxides can spontaneously or metalcatalyzed (Fe) break up into alkoxyradicals and hydroxylipid (catalysis and propagation phase). The alkoxyradicals can be oxidized (termination phase) to fatty aldehydes, volatile hydrocarbons (pentane for n-6 PUFA family and ethane for the n-3 PUFA family). The endproduct MDA is only formed when the original PUFA has 3 or more double bonds.

Cells contain a broad spectrum of antioxidants and free radical-controlling enzymes

such as superoxide dismutase, catalase and selenium-dependent gluthathion peroxidase. The antioxidant vitamin E is largely found in association with membrane lipids and this localization probably serves as a local defense mechanism against PUFA oxidation because $O_2^{\cdot -}$ is far more soluble within the non-polar lipid phase of the membrane (77). Since free radicals play a pivotal role in the extension of myocardial damage during reperfusion following a period of ischemia (17-19,77-79), it is quite feasible that fish oil-induced alterations in PUFA composition of cardiac membrane has consequences for the vitamin E content and rate of lipid peroxidation of myocardium before and during ischemia-reperfusion. Larger amounts of MDA have been found in heart tissue from rats fed with cod liver oil for 14 to 21 days (80). The earliest symptoms of vitamin E deficiency are a disorder of fat depots, in adipose tissue, liver cell degeneration, inflammation, fibrosis and accumulation of the lipofuscin pigment (19). Pigs are particularly sensitive to yellow fat disease. However, we observed none of these symptoms in pigs in which the fish oil-rich diet was supplemented with vitamin E and selenium (48,54). During the whole dietary period plasma levels of MDA and water-soluble fluorescent substances were even lower than those in the lard fat pigs (81).

We also showed that hearts of mackerel oil but not those of lard fat fed pigs produced significant amounts of MDA under normoxic conditions (52, 81). The MDA production of the mackerel oil fed pigs, however, was not affected by a repeated sequences of 5 min periods of ischemia interrupted by 10 min of reperfusion. The free radical induced cyclization reaction in PUFA is , in fact, a chemical equivalent to the enzymatically reactions catalyzed by cyclo-oxygenase (75,81). Decomposition of cyclic peroxides formed by cyclo-oxygenase also yields MDA. However, the contribution of prostanoid synthesis to the MDA formation is likely of minor importance because mackerel oil feeding strongly reduces plasma levels of TXA_2 and PGI_2. The fish oil-induced increase in MDA formation is also not derived from altered activity of circulating polymorphonuclear cells as the function of these cells is usually depressed by dietary fish oil. In the occlusion-reperfusion experiments the recovery of regional heart

function and incidence of ventricular arrhythmias were similar for the mackerel oil and lard fat fed pigs. These results suggest that the increased susceptibility of heart membranes to free radical generated peroxidation in mackerel oil fed animals does not affect the extent of ischemic damage. Ischemia-reperfusion did also not alter sarcolemmal PUFA composition which finding indicates that reacylation processes are sufficiently active during myocardial ischemia.

Sarcolemmal membranes isolated from myocardium of mackerel oil fed pigs exposed *in vitro* to a free radical generating system (Fe^{2+} ions complexed with ADP plus dihydrofumarate) showed a much higher MDA production than those from myocardium of lard fat fed pigs (81). This finding indicates that EPA and DHA, incorporated into sarcolemmal phospholipids, are indeed more susceptible to attack of oxygen free radicals (52,81). It should be noted that MDA production in these experiments started after a lag phase of approximately 20 min. The lag phase is probably caused by the endogenous vitamin E exogenously added to both the fish oil and lard fat containing diets. Hammer and Wills (82) demonstrated that supplementation with vitamin E markedly reduced MDA formation in rat liver microsomes (measured in the presence of ascorbate) after both lard fat and herring oil diets. In that study we can not evaluate the effect of dietary vitamin E supplementation on the duration of the lag phase because information on the time course of MDA production is lacking. In conclusion, the results indicate that the observed PUFA-induced enhancement of membrane lipid peroxidation is not critical for the incidence of ventricular arrhythmias and the development of contractile failure during coronary occlusion-reperfusion.

SUMMARY

Epidemiological studies indicate that marine diets are beneficial for the prevention of cardiovascular disease. Subsequent work on experimentally-induced atherosclerosis in several species has indicated that the n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), present in marine oils, contribute to this prevention by

modifying plasma levels of cholesterol and triglyceride, platelet aggregation, monocyte and leukocyte reactivity, blood viscosity and perhaps also arterial blood pressure. The mechanism by which EPA and DHA induce these effects are not completely understood but changes in the eicosanoid metabolism may be involved. In our experimental studies in rats and pigs we could not confirm earlier observations that dietary fish oil increases the sensitivity of the myocardium to adrenergic and ischemic stress. Substantial incorporation of EPA and DHA at the expense of linoleic and arachidonic acid, which occurs in membrane phospholipids of myocardium in pigs fed mackerel oil, did not alter the susceptibility to ischemic damage and arrhythmias. Supplementation of the fish oil containing diets with vitamin E did not prevent an increase in susceptibility of myocardial tissue phospholipids to peroxidation *in situ* as well as *in vitro* sarcolemmal membranes but the free radical-induced process did not jeopardize the myocardium.

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ROLE OF FREE RADICALS IN THE DEVELOPMENT OF ISCHEMIC HEART DISEASE

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INTRODUCTION

Over the past 5 - 6 years there has been a growing interest in the concept that oxygen radicals play a role in the pathogenesis of myocardial ischemia and infarction (1-6). Up to 6 years ago it was generally believed that the degree of ischemic damage following a coronary occlusion was simply determined by the ratio of oxygen supply to the oxygen demand of the heart. Consequently, therapeutic interventions have been employed to reduce the demand or to increase the supply of oxygen to the myocardium. Only recently has the concept emerged that oxygen can be toxic and that oxygen free radicals could be the determinant of the degree of ischemic damage in the heart. Much of the work in the field has dealt with coronary reperfusion models as coronary reperfusion following myocardial ischemia is characterized by reintroduction of oxygen into the system.

Often the evidence is based on experimental data indicating i) increased rates of lipid peroxidation in diseased tissue, or ii) the ameliorating effect of antioxidants, or both. Such studies often use experimental end points of cell and tissue damage that may not accurately reflect the human disease process, and these should be interpreted cautiously. Whereas end products of lipid peroxidation certainly may be harmful to the cell, lipid peroxidation may occur as a consequence of tissue damage (7) and it is not necessarily related to the primary mechanism of tissue injury.

Thus, oxygen radicals can be implicated in almost all disease processes in which cell injury occurs.

It should be pointed out that the enzymatic production of lipid peroxides, such as in the arachidonic acid cascade, are not necessarily harmful. It follows that the finding of lipid peroxidation end products may be significant, but it does not necessarily convey information about the primary process of cell injury. For these reasons we have determined the occurrence of oxidative stress as an index of oxygen free radical damage. Oxidative stress is a condition in which the redox state of the cell is shifted towards oxidation because of an increased production of radicals or altered metabolism. Oxidative stress is reflected by oxidation of labile thiols of proteins and small molecules and is a prerequisite for major cellular alterations; it can be accurately detected by simultaneously measuring tissue formation and rate of release of oxidized glutathione.

The objective of this paper is to summarize the evidence in favour of a role for oxygen free radicals in the pathogenesis of myocardial reperfusion injury which we have accumulated in our laboratories. Several topics are considered in detail: the role of oxygen free radicals in the ischemic and reperfused myocardium; the effects of ischemia on the defence mechanisms against oxygen free radicals; the occurrence of oxidative stress during reperfusion in isolated heart preparations and during surgically induced cardiac arrest in a clinical setting. Some of the data presented here have already been published (8-16).

OXYGEN FREE RADICALS AS MEDIATORS OF PATHOPHYSIOLOGIC PROCESSES

The inherent nature of the oxygen molecule makes it susceptible to univalent reduction reactions to form superoxide anions ($O_2^{\bullet -}$), a highly

reactive free radical (17). A free radical is any substance which has one or more unpaired electrons. Although oxygen itself is actually a biradical, it is not very reactive, even though it has two unpaired electrons. This is because both of the electrons in oxygen spin in the same direction. Other reactive products of oxygen metabolism can be formed from subsequent intracellular reduction of O_2, including hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH). The half-life of the hydroxyl radical is about a billionth of a second. It is the most dangerous radical in biological systems and is the most potent oxidizing agent known thus far.

In a cell the ·OH radical becomes free to attack unsaturated fatty-acid side chains by assimilating a hydrogen atom and leaving a carbon radical. The lipid radical subsequently rearranges to produce a conjugated diene and, in the presence of oxygen, results in formation of organic oxygen radicals (18) which removes hydrogen from additional fatty-acid side chains resulting in an oxidative stress via a chain reaction which manifests the production of lipid peroxides. The rate of lipid peroxidation is increased in the presence of iron or copper salts and results in an increased fluidity, increased permeability and loss of membrane integrity.

In vivo studies suggest that two important pathophysiologic processes, central nervous system injury and the acute inflammatory reaction, involve the generation of free radicals with subsequent tissue destruction. Demopoulos et al (19) reviewed the role of oxygen free radicals as mediator of damage in the central nervous system of animals subjected to ischemia; head trauma or hypertension can be prevented or reduced by treatment with oxygen free radical scavengers, suggesting that oxygen free radicals are partly responsible for the endothelial lesions seen in ischemic, traumatic or hypertensive injury. The most likely source of oxygen free radicals in the brain is via the

metabolism of arachidonate (20).

Oxygen free radicals are also thought to play a role in the acute inflammatory response. Free radical scavengers have protective effects in different models of inflammation, including the reverse passive Arthus reaction, carrageenin-induced foot edema, and passive immune-complex glomerulonephritis (21). The source of oxygen free radicals in the acute inflammatory response are neutrophils which are known to generate superoxide anions and hydrogen peroxide during phagocytosis (22).

Ischemic and reperfusion-induced myocardial injury is another pathological condition in which oxygen free radicals may play a role. The following discussion provides an overview of evidence supporting their involvement in pathogenesis of ischemic and reperfusion damage.

SOURCES OF FREE RADICALS IN THE ISCHEMIC AND REPERFUSED MYOCARDIUM

Free radicals in the myocardium may arise from a number of sources and cellular mechanisms; their production may occur under normal and pathological conditions. Mitochondria are one of the most important sources of oxygen-free radicals in the myocardium, as production of $O_2^{\cdot -}$ and H_2O_2 is enhanced under ischemia and reperfusion in this organelle while the electron transport chain remains in the reduced state (23,24).

The xanthine oxidase pathway is also an important site of free radical production, particularly during ischemia when cytosolic calcium increases and ATP is broken down to AMP, the molecule which is ultimately metabolized to hypoxanthine. The concomitant elevation of cytosolic calcium concentration enhances the conversion of xanthine dehydrogenase, an enzyme localized in the endothelium of the capillaries of coronary arteries, to xanthine oxidase (25,26). According to this concept, when molecular oxygen is reintroduced to

cells containing high concentrations of hypoxanthine, this enzyme causes the release of $O_2^{\bullet -}$ and H_2O_2. However studies on the distribution of xanthine oxidase among various tissues and species provides evidence that the enzyme activity varies widely and certain organs in certain species show essentially no activity. The rabbit, pig and human hearts have little or no xanthine oxidase activity (27-30). Endoperoxide intermediates which arise from the conversion of arachidonic acid also lead to the production of oxygen-free radicals by leukocytes and other cell types in the heart (31).

Another possible mechanism of free radical generation are the neutrophils. Neutrophils accumulate in the vascular space of reperfused ischemic myocardium where they may adhere to the endothelium and release oxygen-free radicals (32). Although there are multiple stimuli for neutrophil migration, one chemoattractant may arise from the interaction of plasma lipids with oxygen-free radicals derived from the activity of cyclooxygenase, or xanthine oxidase on their respective substrates. Engler et al (33) have proposed that capillary plugging by leukocytes contributes to the occurrence of "no reflow" after reperfusion of ischemic myocardial tissue. Finally, the autoxidation of catecholamines could provide another source of free radicals under ischemic conditions (34,35).

DEFENCE MECHANISMS AGAINST OXYGEN-FREE RADICALS

In the heart there are defence mechanisms which are able to protect the cell against the cytotoxic oxygen metabolites. These include the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase plus other endogenous antioxidants such as vitamin E, ascorbic acid, and cysteine (36).

The primary mechanism for clearance of superoxide anions is the superoxide dismutase enzyme. This enzyme isolated in 1969 by McCord and

Fridovich, catalyzes the dismutation of superoxide anions to H_2O_2 and O_2 (37). The reaction can proceed also spontaneously, but superoxide dismutase is able to increase the rate of intracellular dismutation by a factor 10^9. At least three separate forms of SOD have been characterized. One contains copper and zinc and it is present in the cytosol. Another contains manganese and it is present in the mitochondria. The third one contains iron and it is associated with the cytoplasm of Escherichia Coli.

Two enzyme systems are important in the metabolism of H_2O_2 produced by the univalent reduction of superoxide anion (38,39). The first is catalase, an enzyme found mainly in the cytosol, which catalyzes the reduction of H_2O_2 to water. Catalase is present only at very low concentrations in the myocardium; on the other hand, glutathione peroxidase is present at significant concentrations in the cytosol of the heart (40). The hexose monophosphate shunt produces the reducing equivalents (NADPH) for the action of glutathione reductase through glucose-6-phosphate oxidation. Reduced glutathione (GSH) is then utilized by GSH peroxidase to form oxidized glutathione (GSSG) which is in a dynamic equilibrium with all cellular sulphydryl groups. Glutathione disulfides mixed with proteins constitute an important part of total cellular glutathione pool and the entire equilibrium is regulated by thiol transferases.

It follows that GSH, as a co-substrate of glutathione peroxidase, plays an essential protective role against oxygen-free radicals and prevents the peroxidation of membrane lipids; the activity of superoxide dismutase in the heart being nearly four times less than in liver, and catalase activity being extremely low (12). This protective mechanism results in an increased rate of the intracellular oxidized glutathione (GSSG) formation.

It follows that the changes of glutathione status provide important

information on the cellular oxidative events and tissue accumulation and/or release of GSSG in the coronary effluent is a sensitive and accurate index of oxidative stress (9-12,15,16).

EFFECTS OF ISCHAEMIA AND REPERFUSION ON THE DEFENCE MECHANISMS AGAINST OXYGEN-FREE RADICALS

The basic premise for the involvement of oxygen in reperfusion damage is that ischemia and reperfusion have altered the defence mechanisms against oxygen toxicity. To investigate the possible role of oxygen in reperfusion injury, we have determined the effects of ischemia in isolated and perfused rabbit hearts. The activity of mitochondrial and cytosolic SOD which represent the first line of defence against oxygen toxicity and of glutathione peroxidase (GPD) and glutathione reductase (GRD) which are the second line of defence against oxygen-free radical production. Our data (12) indicate that severe ischemia specifically reduces the activity of mitochondrial SOD but does not affect the activity of cytosolic SOD.

A prolonged ischemia induces a significant and specific alteration of the mitochondrial SOD. This is remarkable when considering that mitochondria are one of the most important sites of production of oxygen-free radicals in conditions of ischemia and reperfusion. Under these conditions, GPD is the second line of defence against oxygen toxicity, and should be highly stimulated, causing an imbalance of the cellular glutathione status. Reduction of coronary flow did not affect the activity of GPD or GRD, but ischemia induced a reduction in myocardial GSH/GSSG ratio. This was due to a significant reduction of tissue content of GSH while GSSG remained unchanged. Reperfusion did not modify GPD or GRD activity but further reduced the tissue content of GSH, which was concomitant with an important increase of tissue

GSSG, resulting in a further decline of GSH/GSSG ratio. On reperfusion there was a sustained release of GSH and GSSG into the coronary effluent. This finding is of importance because during reperfusion there was a massive production and release of GSSG indicating that the cellular content of this compound was increased at least five-fold (10-13,16). These results suggest that ischemia induces metabolic alterations capable of reducing the defence mechanism against oxygen toxicity. The prime alteration seems to be at the level of mitochondrial SOD, as its activity was reduced 50% after severe ischemia.

Under these conditions the readmission of molecular oxygen is likely to stimulate the production of $O_2^{\cdot -}$ radicals above the neutralizing capacity of mitochondrial SOD; consequently GPD is likely to be highly stimulated. We found a severe alteration of the glutathione status, indicating that myocardial oxidative damage had occurred, and that it probably had been counteracted at this level. The changes of glutathione status occurring during ischaemia and reperfusion which we found, provide important information regarding the cellular oxidative events; the tissue accumulation and release of GSSG in the coronary effluent is a sensitive and accurate index of oxidative stress (10).

It is pointed out that in animal studies the evidence of oxidative stress on reperfusion is correlated with the duration of the ischemic period (15). Reperfusion after a short period of ischemia (30-60 min) does not result in oxidative stress, probably because the defence mechanisms are still able to protect the myocardial cells against the burst of oxygen-free radicals generated by readmission of oxygen with coronary flow. Reperfusion after more prolonged periods of ischemia, when the defence mechanisms are likely to be reduced, results in further damage with no recovery in function.

OCCURRENCE OF OXIDATIVE STRESS IN HUMANS

Clinical evidence for the occurrence of oxidative stress in man is still very poor. This is partly due to the inadequacy of reliable indices able to detect the occurrence of oxidative damage in man, and is partly due to the difficulty of standardizing the ischemic and reperfusion period. We have attempted to resolve this problem by measuring the arterial and coronary sinus difference of GSH and GSSG of coronary artery disease (CAD) patients subjected to different periods of global ischemia followed by reperfusion during coronary artery by-pass grafting (9,15). Because of the high rate of glutathione autooxidation and disappearance in the blood, we have determined plasma levels of GSH and GSSG with a method modified by us (41), treating the blood immediately after collection with thiol reagents, di-thio-nitrobenzoic acid (DTNB) for GSH and N-ethylmalmeimide (NEM) for GSSG determination, respectively.

The patients were divided in two groups according to the ischemic period: i) shorter or ii) longer than 30 minutes. Before clamping, in all patients there was a small positive arteriovenous (A-V) difference for GSH and GSSG. During the following 30 or 60 min of global ischemia, the A-V difference for GSH remained constant except in one patient. In this case, 15 min after clamping, the concentration of GSH in the coronary sinus rose above the arterial levels which did not change. During global ischemia there were no major changes in the A-V difference of GSSG except in two patients showing a small release of GSSG in the coronary sinus, one after 30 and one after 60 min of clamping. During reperfusion after 30 min of clamping, there was a transient release of GSH and GSSG into the coronary sinus reaching a peak 5 min after the onset of reperfusion. During the following 15 min the GSH and the GSSG concentrations in the coronary sinus declined and fell below the

arterial values reflected by the positive myocardial A-V difference. However, reperfusion after 60 min of clamping resulted in a pronounced and sustained release of GSH and GSSG from the myocardium and at the end of the procedure the concentration of GSH and GSSG in the coronary sinus greatly exceeded the arterial levels leading to a negative A-V difference.

Reperfusion reinstated after 60 min of ischemia led to a significant release of GSH and GSSG, which continued at the end of the procedure. This is similar to the effects of reperfusion after prolonged ischemia in the isolated heart and presumably implies oxidative stress. We suggest that these cases illustrate some aspects of pathophysiology of reperfusion of the human heart. They indicate that reperfusion may induce oxidative damage after a prolonged period of ischemia and suggest that oxygen-free radicals may be involved in reperfusion damage. These findings are of importance as they constitute the rationale for therapeutic interventions designed to improve the efficacy of myocardial reperfusion.

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17

CARDIOVASCULAR MEMBRANES AS MODELS FOR THE STUDY OF FREE RADICAL INJURY

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INTRODUCTION

Following ischemia - a condition characterized by oxygen depletion, acidosis, and retention of tissue metabolites (1) - severe oxidative damage to cardiovascular tissues occurs upon reperfusion. However, early reperfusion remains the major clinical method to salvage ischemic myocardium and further elucidation of the multiple injury mechanisms operative during ischemia/reperfusion is necessary to solve this paradoxical dilemma. In the process of ischemia/reperfusion, mounting evidence has confirmed that the generation of oxygen-derived free radicals participates in the injury process (2-4). At the whole organ level, the reported protection of cardiovascular tissues by antioxidant-type enzymes (eg. superoxide dismutase and catalase), antioxidant drugs (eg. deferoxamine) and vitamins (eg. vitamin E) suggest a role for free radicals in the reperfusion injury. In recent years studies employing electron spin resonance spectroscopy and spin-trapping agents have shown that increased production of free radicals occurs during ischemia and myocardial reperfusion (4-11). The specific cellular sites of generation of these toxic free radicals

are not known. Certainly white cells migrating into ischemic/reperfused areas of myocardium are capable of generating significant amounts of superoxide anions (2,12-14); endothelial cells (15-17) and cardiomyocytes (18) have been reported to generate oxygen-derived free radicals as well. Nevertheless, the relative contribution of each of the various cell types to the overall process of injury of cardiovascular tissues remains an area of intense investigation. One significant challenge for this area of investigation is whether or not sufficient radicals accumulate to overwhelm the intrinsic cellular protective mechanisms.

In addition to determining the site of free radical production, it is also essential to identify the concomitant metabolic perturbations occurring during ischemia that may alter the threshold of injury to oxygen-derived free radicals. It has been reported that acidosis may induce impairment of calcium-transport by sarcoplasmic reticulum and that this loss may be inhibited by scavengers of free radicals (19). Perhaps, an even more important membrane site of oxidative injury is the plasma membrane. It has been stated that "The plasma membrane is a critical site of free radical reactions" for several reasons. Extracellularly generated free radicals must cross the plasma membrane before reacting with other cell components and they initiate toxic reactions with the membrane. The unsaturated fatty acids present in membranes [phospholipids, glycolipids, glycerides, and sterols] and transmembrane proteins containing oxidizable amino acids, are susceptible to free radical damage" (20). Our laboratory has focused upon the plasmalemma of cardiomyocytes, the sarcolemma,

both to determine the consequences of free radical-induced injury (21,22) and as a model to determine the effects of ischemic-like perturbations (23,24) and pharmacological interventions (25,26).

DIRECT FREE RADICAL MODIFICATION OF MEMBRANES

Since the participation of oxygen-derived free radicals in the ischemia/reperfusion model of injury and other pathological conditions remains difficult to quantify in situ, our present understanding of free radical involvement in tissue/membrane injury must rely heavily on in vitro model systems. Exposure of biological membranes to oxygen free radicals results in profound changes. We have shown that free radical injury of sarcolemma results in the rapid production of lipid peroxidation products and the loss of membrane-bound enzyme activity (21). The oxidation of membrane lipids and proteins subsequent to the rapid generation of oxygen derived radicals may result in alterations in membrane structural and functional properties. In our laboratory, we found that the physical properties of isolated myocytic sarcolemma and sarcoplasmic reticulum-enriched membranes were altered by exposure to free radicals in vitro. We performed these studies utilizing the technique of steady state fluorescence polarization and used dihydroxyfumerate and Fe^{3+}-ADP to generate hydroxyl free radicals. Figures 1 and 2 show the marked "stiffening" of membranes that appears to correlate with alterations in phospholipid composition (Table 1). Concurrently,

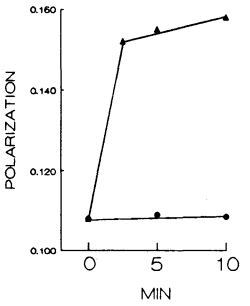

Figure 1. Effect of hydroxyl free radicals on diphenyl-hexatriene fluorescence polarization in sacrolemmal membranes (lower line, Control; upper line, +hydroxyl radicals)

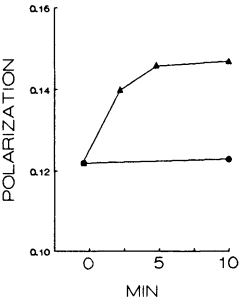

Figure 2. Effect of hydroxyl free radicals on diphenyl-hexatriene fluorescence polarization in sarcoplasmic reticulum (lower line, Control; upper line, +hydroxyl radicals)

the loss of membrane-bound enzyme activities (Na,K-ATPase and rotenone-insensitive NADH cytochrome c reductase) has been shown to occur within minutes of free radical exposure (21), indicating that enzymatic activity was altered. Our findings suggest that the cascade of lipid degradation events initiated by free radicals may have significant secondary effects on lipid dependent enzyme conformation and function.

Table I. Loss of canine sarcolemmal and sarcoplasmic reticulum phospholipids following exposure to the hydroxyl radical generating system. Values are expressed as percent loss of control phospholipids for each component

Phospholipid	Sarcolemma	Microsomes
Phosphatidylcholine	- 32	- 47
Phosphatidylethanolamine	- 85	- 67
Phosphatidylinositol + Phosphatidylserine	- 46	- 60
Sphingomyelin	+ 3	- 2

SECONDARY INJURY FOLLOWING FREE RADICAL EXPOSURE

In addition to direct perturbations by free radicals, the products of lipid peroxidation are toxic: lipid peroxidation "scission" products have been demonstrated to cause cellular toxicity in the rat lens grown in organ culture (27), and in mixed primary cultures of myocytes, endothelial cells, and fibroblasts (28). Highly reactive species, like malondialdehyde, which cross-link proteins in biological membranes as well as other macromolecules, may injure the cells by altering these macromolecules (29-31). Lipid hydroperoxides and short chain scission products may also act as promoters of additional oxidative damage through the well known autocatalyzed chain of lipid peroxidation (32). The alteration of physical properties of biological membranes, by the oxidative loss of unsaturated fatty acids from membrane phospholipids and, by cross-linking of membrane proteins, may ultimately lead to loss of cellular integrity and cell death. In addition to the above physical perturbations, enzymatic activities of catabolic enzymes, such as phospholipases and proteases, may be enhanced by the alterations of their potential substrates. Van Deenen (33) originally proposed that phospholipids containing peroxidized fatty acids may serve as better substrates for phospholipases. Servanian (34) reported in an in vitro study that phospholipid epoxides were two-fold more susceptible to hydrolysis by phospholipase A2 than the corresponding non-epoxide-containing phospholipids. Others have shown that rat liver mitochondria (35) and peritoneal fluid are more susceptible to exogenous phospholipase A2 following exposure to exogenous free radical injury

(35,36). It has also been suggested (37) that phospholipase A2 catalyzed removal of fatty acyl hydroperoxides from peroxidized lipids is a necessary first step in the glutathione and glutathione transfer/detoxification of these lipids.

ISCHEMIA-INDUCED INCREASES IN SENSITIVITY TO FREE RADICAL INJURY

Lysophosphatides and long chain acyl carnitines accumulate in ischemic tissues (1); in this regard, our laboratory has been investigating the role of lipid amphiphiles in free radical-mediated membrane injury (23). We studied the effects of different species of acyl CoA (18:0, 18:1), acyl carnitine (16:0), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and free fatty acids (16:0, 18:1) on free radical-induced lipid peroxidation. LPC was shown to accelerate lipid peroxidation in sarcolemma during exposure to a superoxide/hydroxyl radical generating system (23): 50 IM LPC increased peroxidative injury by 200% over amphiphile untreated controls.

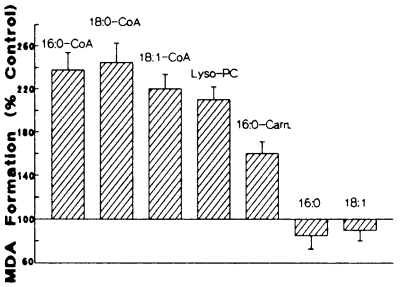

Figure 3. Effect of various lipid amphiphiles on membrane lipid peroxidation in response to the hydroxyl radical system. (Modified from Mak, Kramer and Weglicki; J. Biol. Chem. 261:1153,1986; with permission)

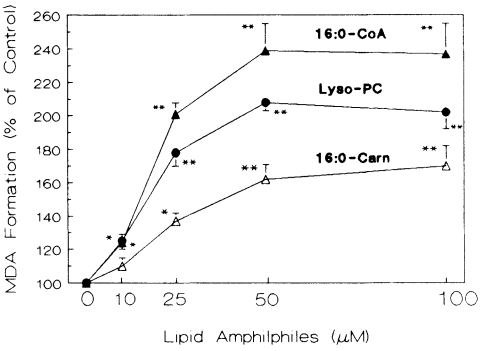

Figure 4. Stimulating effect of lipid amphiphiles on membrane lipid peroxidation. (Modified from Mak, Kramer and Weglicki; J. Biol. Chem. 261:1153,1986; with permission.)

Similarly, palmitoyl carnitine and other amphiphiles were observed to promote sarcolemmal lipid peroxidation (Figures 3 & 4) and increase the susceptibility of the lipid-dependent Na,K-ATPase (23) to free radical injury. Significant promotion of peroxidation by acyl carnitine occurred at concentrations less than 25 IM, which may be lower than its critical micellar concentrations. The interaction of these amphiphiles might involve binding of their monomeric form into the lipid bilayer, resulting in altered membrane physical properties and enhanced susceptibility of peroxidation-sensitive sites within the membrane bilayer to oxygen-derived radicals. Such interactions may also stimulate autocatalyzed chain-propagation mechanisms. Thus, the ischemia-related impairment of lipid metabolism and subsequent accumulation of

lipid amphiphiles within the cardiovascular membranes may "prime" cardiac tissue for oxygen-derived free radical injury during reperfusion.

SUMMARY

Present methodology does not permit in situ studies of accumulation of tissue amphiphiles and free radicals by means other than invasive tissue disrupting techniques. In the future, noninvasive methodologies may emerge which will allow us to determine whether such changes observed in vitro are similar to those occurring in situ, and to better evaluate therapeutic interventions against free radical-induced tissue damage.

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18

OXIDATION OF MYOFIBRILLAR THIOLS: A MECHANISM OF CONTRACTILE DYSFUNCTION REVERSIBLE BY DITHIOTHREITOL

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INTRODUCTION:

In recent years, experimental and clinical evidence has determined that the reperfusion of previously ischemic myocardium may lead to accelerated cellular damage. In the past this has been attributed to the disruption of cellular membranes (1, 2) following the endogenous generation of oxygen-free radicals (OFR), but more recently there is increasing evidence that damage to cellular proteins is also involved (3, 4). Attempts to quench the endogenous generation of OFR's with superoxide dismutase and catalase has met with some success, but the long term results have not shown a major reduction in tissue loss (5, 6). OFR's, such as superoxide anion or the hydroxyl radical, are highly reactive compounds, but have extremely short half-lives (ie 10^{-9} seconds). However, much of the observed damage to the myocardium occurs several hours after reperfusion (7). This suggests a role for exogenously generated OFR's or related oxidizing agents in the late development of myocardial reperfusion injury .

Reperfusion of a previously ischemic region of the heart often leads to the accumulation of polymorphonuclear leukocytes (PMN's) within the tissue (8, 9). Within 15 minutes of reperfusion, the number of PMN's in the injured region can increase by up to 25% (10) often leading to capillary plugging followed by the 'no-reflow' phenomena (11). Once in the injured area, the PMN's are known to marginate and diapedis through the capillary wall , entering into the underlying already damaged tissue. Within 12 hours

there is evidence of extravascular PMN accumulation (10). Once in the extravascular compartment, the PMN's attach onto damaged cells and undergoe a respiratory burst, releasing large concentrations of proteases, OFR's and related oxidant species directly onto the surface membrane of these cells (12). Unfortunately, PMN's will also attach to and secrete OFR's onto surrounding cells which where not previously damaged, thereby leading to the destruction of otherwise healthy tissue. Several studies (13, 14, 15) have shown that depleting circulating PMN's in dogs results in reduced infarct size and diminished cellular damage in the reperfused myocardium. In many tissues, this respiratory burst by activated PMN's may aid in the healing process by removing these damaged cells so that new tissue can be produced. However, in organs such as the heart, where loss of any myocyte is permanent, this may be life threatening. It is therefore of interest to understand what agents are being produced by PMN's, and how they are involved in the development of myocardial damage.

PMN's generate and release many OFR's, including superoxide ($\cdot O_2^-$), hydroxyl radical ($\cdot OH^-$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (12). HOCl is of particular interest because it is probably the most reactive of the oxidant species produced within the body (16). Figure 1 is a schematic representation of the events leading up to the production and release of HOCl by activated neutrophils following attachment to a target cell.. It has been shown (17) that PMN's release their proteolytic and oxidizing agents directly at the site of attachment to the cell, and not randomly into the systemic circulation. Therefore, while the concentration of HOCl may be in the nanomolar range in the surrounding extracellular fluid, the concentration directly at the site of release onto the cell surface may be much higher. The effects that potentially micromolar concentrations of this agent have on the cellular mechanisms in the myocardium are still virtually unknown.

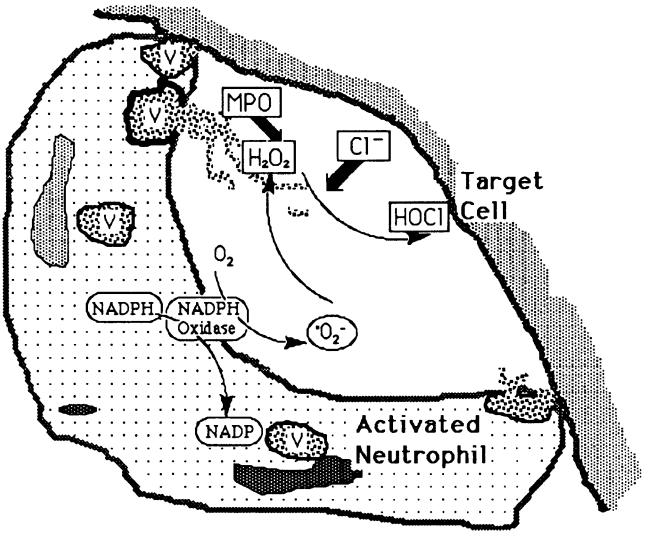

Figure 1: A possible mechanism of oxygen-metabolite production in an activated PMN. Superoxide radical, produced by the NADPH - NADPH Oxidase system is converted into H_2O_2 by Catalase. The release of myeloperoxidase (MPO) from cellular vesicles (V) into the cleft between the PMN and the target cell will catalyse the conversion of H_2O_2 plus chloride anion (Cl^-) into HOCl. At physiological pH, about 50% of the HOCl (pKa 7.2) will be in the non-dissociated form and hence can cross cellular membranes to effect intracellular sites.

The decline in developed force and the ensuing contracture as observed in isolated rat papillary muscles exposed to hypochlorous acid (HOCl) has been reported to correlate with a decrease in the level of reduced protein thiols (18). This decline in cellular thiols following oxidative stress has been reported in several models, including isolated hepatocytes (19), skeletal muscle (20) and cardiac muscle (21). We reported (18) the recovery of both P-SH and contractile function in papillary muscles isolated from rat myocardium by subsequent treatment with the disulfide reducing agent, dithiothreitol (DTT). However, no mechanisms to account for this recovery of contractile function has

been proposed. Given that a highly significant amount (>40%) of the total cellular protein thiols are oxidized, we may assume that the major proteins of the tissue (ie actin and myosin) are involved. The fact that DTT is able to partially restore function of these papillary muscles suggests that thiol oxidation may be instrumental in the HOCl-induced contracture, and that this oxidation is reversible. The specific location of these thiols is still unclear.

In order to elucidate the mechanisms involved in oxidant-induced contractile dysfunction in the heart, we studied the effects of HOCl perfusion on the contractile function of isolated coronary perfused, isovolumically contracting rat heart. We relate the observed changes in the contractile performance to changes in the thiol redox status of the contractile proteins (P-SH), and examine the ability of DTT to restore both the contractile performance and the P-SH levels in these proteins. We conclude that DTT may be useful for restoring myocardial contractile function following oxidative stress.

METHODS:

Hearts were excised from male Sprague-Dawley rats (275 - 300 grams) following cervical dislocation, and mounted on a modified Langendorff perfusion apparatus. The hearts were perfused at constant flow with oxygenated (95% O_2 / 5% CO_2) Krebs Ringer buffer (in mM: NaCl 120; KCl 4.8; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_2$ 25.3, EDTA 0.5) pH 7.40, 37°C via the coronary arteries at an initial mean aortic root pressure (ARP) of 50 mm Hg. The atria were removed and the hearts were paced at 270 / minute via an electrode inserted through the free wall of the right ventricle using a Grass S88 stimulator. An latex balloon attached to a Statham P23 pressure transducer was inserted through the mitral valve into the left ventricle and gradually distended with water to attain maximal developed pressure. Hearts were considered acceptable if their left ventricular end

diastolic pressure (LVEDP) was between 0 and 10 mm Hg, and end systolic pressures (LVESP) between 90 and 120 mm Hg prior to the initiation of the experiments. Left ventricular pressure measurements as well as the aortic root pressure (ARP), which represents the perfusion pressure for the coronary vasculature, were recorded using a Grass 4 channel polygraph.

Seperate reservoirs containing either a) normal KR buffer; b) KR with HOCl (100 mM); or c) KR with DTT (500 μM) maintained the solutions at 37°C under constant oxygenation. Following a 15 minute stabilizing period, the hearts were perfused as follows: 1) normal KR buffer for 80 minutes (control); 2) normal KR for 80 minutes, with DTT added for the final 60 minutes; 3) KR with HOCl for 80 minutes; 4) KR with HOCl for 20 minutes followed by 60 minutes of washout with normal KR; or 5) KR with HOCl for 20 minutes followed by 60 minutes of KR with DTT. Following each experimental period, the hearts were perfused with normal KR buffer for 2 minutes to remove any residual solutions, then rapidly chilled by dropping into iced saline. The right ventricular free walls were cut away, and samples of the left ventricular free wall were removed for total tissue SH determination. The remaining tissue was homogenized immediately for isolation of actin and myosin as described below.

Total tissue protein sulfhydryl (P-SH) and total non-protein sulfhydryl (NP-SH; mainly glutathione) determinations were made as described previously (18). Briefly, from each heart, three small (20 mg) samples of the left ventricular free wall were homogenized seperately for 1 minute in 200 μl of iced 5% Trichloroacetic acid (TCA) containing 5 mM ethylenediaminitetraacetic acid (EDTA), using a 0.5 ml glass / glass microhomogenizer, then transferred to a chilled microcentrifuge tube and kept on ice for 30 minutes. The homogenate was centrifuged (13,000 x G) for 2 minutes to sediment the proteins, the supernatant was collected, and brought to pH 7.6 with NaOH for NP-SH determination. The protein pellet was resuspended and washed 3 times with 200 μl of the iced TCA

solution, then resuspended in 200 µl of buffer containing TRIS, 0.1 M; SDS, 0.5%; pH 7.6 and brought to pH 7.6 with NaOH to dissolve the proteins. Aliquots of this suspension and of the supernatant were assayed for P-SH and NP-SH respectively using the DTNB (5,5'-dithiobis(2-nitrobenzoic acid) assay (22). Protein concentration was determined in triplicate using the Bradford (BIORAD) method.

The myofibrillar proteins were isolated using a low salt/high salt technique. The septum and the remaining left ventricular free wall of the hearts (app 200 mg wet weight) were homogenized in 500 µl of Low Salt Buffer (LSB in mM: KCl, 20; K_2HPO_4, 2; EGTA, 1) with 25 µl of phenylmethylsulfonyl fluoride (PMSF) using a glass/Teflon homogenizer for 2 minutes on ice, then transfered into chilled 1.5 ml microcentrifuge tubes. The homogenizer was washed with a further 500 µl of LSB. The tubes were centrifuged (0°) for 45 seconds (8800 X G). The pellet was resuspended in 500 µl of High Salt Buffer (HSB in mM: $Na_4P_2O_7$ 40; MgCl, 1; EGTA, 1), allowed to stand on ice for 15 minutes at pH 9.5 (NaOH), then centrifuged (0°) for 10 minutes (8800 X G). The supernatent, containing the myofibril complex with a protein yield of 25 - 50 µg / mg of tissue homogenized, was collected and the protein concentration was adjusted to 1 mg / ml with HSB.

Iodoacetamide (IAA) is a compound which is known to bind selectively to reduced thiol groups without significantly altering the structure or charge of the protein involved (23). To label the free thiol residues in the contractile proteins, an aliquot of the homogenate, containing approximately 500 µg protein was incubated at 37°C in the dark in the presence of approximately 1 µCi of ^{14}C-IAA. The reaction was terminated by the addition of excess (10 µM) DTT. The proteins were seperated using a 7.5% SDS-Polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Blue stain (Figure 4a). A standard curve (1,5, and 25 µg each of purified rabbit skeletal actin and myosin) was run on each gel for estimation of protein concentration using laser densitometry (LKB

2222-020). Following densitometric scanning , the gels were dried and exposed against X-Ray film for 2 days at -80°C for autoradiography (Figure 4b). To quantitate the level of ^{14}C-IAA binding to each protein, the bands were cut from the gel, digested in 30% H_2O_2 for 3 hours at 60°C, then counted using liquid scintillation. The results are expressed in Table 1 in ^{14}C counts per minute / μg protein

Values shown are means ± SE, with n = 8 for each treatment group. Differences in mechanical response between the treatment groups were determined using a one-way Anova technique and subsequent Tukeys test ($p<0.05$). Differences between P-SH, NP-SH, and ^{14}C-IAA binding were determined using one-way Anova and subsequent Bonfaroni's test ($p<0.05$).

RESULTS:

Figure 2 summarizes the contractile response of isolated rat hearts under control conditions or during perfusion with 100 μM HOCl for 80 minutes. Control hearts functioned well, showing only slight decrease in pulse pressure (PP = LVESP - LVEDP) from 100 ± 6.9 to 88 ± 6.4 mm Hg by 80 minutes and an elevation of ARP from 51 ± 0.5 to 65 ± 2.8 mm Hg. Perfusion with DTT alone for the final 60 minutes induced a slight elevation in LVEDP that, with the exception of the 80 minute value, was not significantly different from control. There were no differences in ARP or PP values between control and DTT perfused hearts at 80 minutes.

The presence of HOCl in the perfusate produced a gradual decline in the contractile performance of the hearts. In four of the 24 hearts initially perfused with HOCl, the PP increased transiently by 5 - 10 mm Hg (data not shown) within the first two minutes then returned to initial values by 10 minutes, while the remaining 20 hearts remained stable for the first 10 minutes. Continued perfusion with HOCl induced a rise in LVEDP

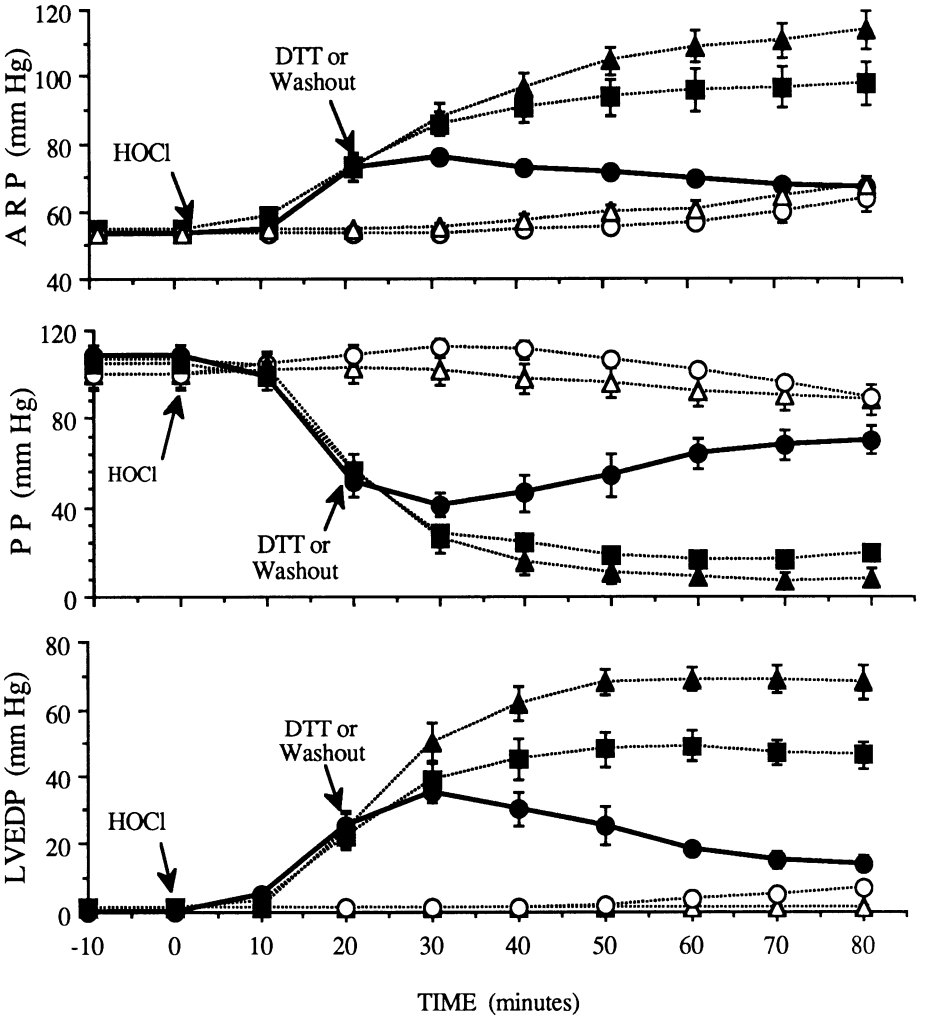

Figure 2: The effects of 80 minutes of perfusion with: Δ = CONTROL; ▲ = HOCl ONLY; O = DTT ONLY; ● = HOCl / DTT; ■ = HOCl / WASHOUT; on aortic root pressure (ARP), pulse pressure (PP), and left ventricular end-diastolic pressure (LVESP). HOCl was added at Time = 0 minutes, while DTT was added (or WASHOUT) following 20 minutes of HOCl perfusion. DTT was added at 20 minutes to non-HOCl perfused hearts as well (DTT ONLY). + dDP/dt values (not shown) followed the PP values. Values shown here are means ± SE, with n = 8 for each group.

concommitant with a decrease in LVESP, depicted in Figure 2b) as a decrease in PP, by 15 minutes. ARP rose steadily over this period, depicting a rise in coronary vascular resistance.

At 20 minutes of HOCl perfusion there were no differences between the ARP, LVEDP, or PP values for the three experimental groups described in Figure 2. Continued perfusion (80 minutes) with HOCl lead to the development of contractile failure, defined here as the failure of the left ventricle to develop >50% of its initial PP, and contracture, or inability of the left ventricle to relax during diastole. ARP rose steadily, possibly due to increased vascular tone, constriction of the vasculature due to the contracture, or edema. By 70 minutes of HOCl perfusion, many of the hearts developed dysrythmias or the contractions became independent of the electrical stimulations. By 80 minutes, five of the eight HOCl-perfused hearts failed to contract at all in response to electrical stimulation.

Removal of HOCl from the perfusate (Washout) at 20 minutes attenuated but did not prevent the development of contracture. ARP and LVEDP values were significantly lower in the Washout hearts as compared to the HOCl-perfused hearts at 80 minutes. PP was also significantly greater in Washout hearts, although three of the eight hearts were still nonfunctional at 80 minutes. Conversely, when the perfusate was changed from KR with HOCl to KR with DTT at 20 minutes, contractile performance continued to decline (ie. from 20 - 30 min), but then 'recovered' significantly over the remainder of the protocol. By 10 minutes of DTT perfusion, both ARP and LVEDP begin to decrease, often approaching control levels by the end of the experiments. At 80 minutes, ARP was not significantly different from the control values. PP recovers back to 70 ± 6.5 mm Hg, or greater than 78% of control values. Longer perfusion (up to 120 minutes) or a higher concentration of DTT (up to 1 mM) did not have any greater ameliorative effect. Any

dysrythmias or evidence of electrical uncoupling that developed during HOCl perfusion were alleviated within 60 minutes of DTT perfusion.

Figure 3 expresses the level of total tissue protein (P-SH) and total non-protein (NP-SH) sulfhydryls in the above hearts at the end of their perfusion protocol. HOCl perfusion for 80 minutes induced a significant depression in the level of both the NP-SH (8% of control) and P-SH (59% of control) pools. Washout did not prevent this decline in P-SH, although the NP-SH values were significantly greater than those recorded for the HOCl-perfused hearts. The levels of P-SH isolated from those hearts perfused with HOCl-DTT for the final 60 minutes showed a significantly higher level of thiols as compared those isolated from HOCl-perfused or HOCl-Washout perfused hearts, although it is still significantly diffferent from the control values. Because the NP-SH assay is not

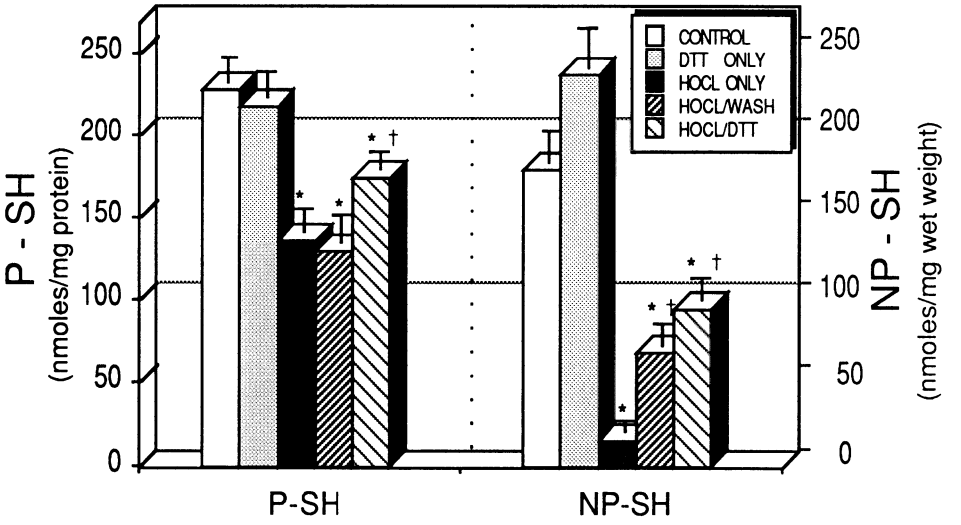

Figure 3: Protein (P-SH) and Non-protein (NP-SH) sulfhydryl residues extracted from rat myocardium following 80 minutes of perfusion as described in Methods. Values are means ± SE of n=8 experiments. * = different ($p<0.05$) from "CONTROL", † = different from "HOCl ONLY" values.

specific for intracellular glutathione, and will also include residual DTT, the elevation of NP-SH that is observed following DTT perfusion can not be necessarily considered as an increase in cellular glutathione levels.

Figure 4 depicts a Coomasie-blue stained gel (a) and its corresponding autoradiogram (b) depicting actin and myosin seperated from the above described hearts. Figure 4a) shows a fairly equal concentration of both actin and myosin (heavy chain), as well as several other less prominent proteins, in each of the lanes. The autoradiogram in b) shows that the level of ^{14}C-IAA bound to the proteins was not equal. Lane 3 contains the proteins isolated from HOCl-ONLY perfused hearts, and shows substantially less

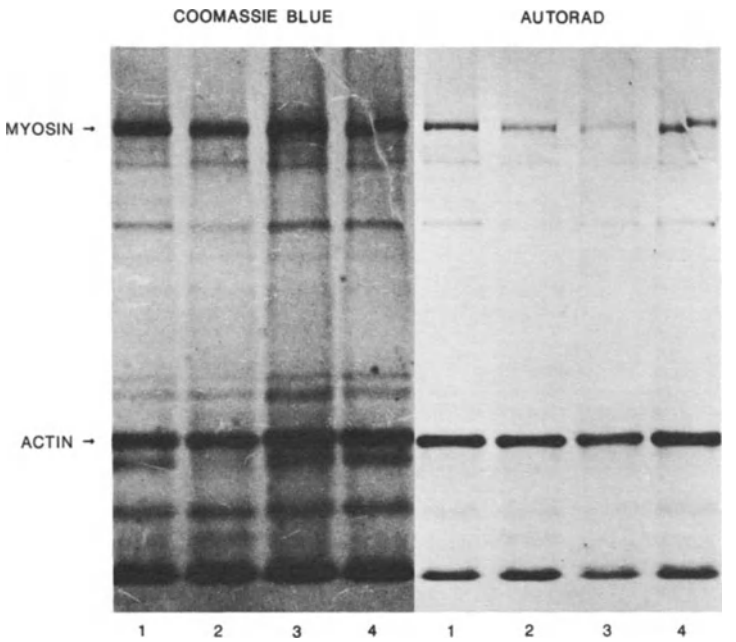

Figure 4 Coomasie Blue stained gel (a) and its corresponding autoradiogram (b) showing actin and myosin (MHC) extracted from rat hearts perfused as follows: Lane 1 - HOCl / DTT; Lane 2 - HOCl / WASHOUT; Lane 3 - HOCl ONLY; Lane 4 - CONTROL. Protein content per band was determined using densitometric scanning against a standard curve of purified actin and myosin.

radiolabel in all the visible proteins, especially in the myosin band, as compared to Lane 4 (CONTROL) or Lane 1 (HOCl / DTT). This shows that the availability of free thiols for binding to the IAA is substantially reduced following HOCl perfusion. The proteins in Lane 1 show a partial restoration of thiols available for IAA binding in both the actin and myosin molecules. Lane 2 (HOCl - WASHOUT) shows no recovery of thiols following the removal of the HOCl from the perfusate.

Table 1 summarizes the level of ^{14}C-bound to each of the major protein bands as determined by scintillation counting. Following HOCl perfusion, it appears that as few as 25% of the sulfhydryl residues in actin, and 18% in myosin were available for binding to ^{14}C-IAA as compared to control (100%). Some preservation of thiols was observed in the contractile proteins isolated from HOCl - WASHOUT perfused hearts, while the proteins isolated from the HOCl-DTT perfused hearts showed significantly higher levels of available thiols in most of the proteins shown here, including both the actin and myosin molecules (65% and 66% respectively).

	CPM (PER µG PROTEIN)		PERCENT OF CONTROL	
	ACTIN	MYOSIN	ACTIN	MYOSIN
CONTROL	199.9 ± 18.3	94.2 ± 11.4	100%	100%
HOCl ONLY	48.7 ± 7.2	11.1 ± 3.6	24%	18%
HOCL / WASHOUT	50.3 ± 7.6	24.8 ± 5.9	25%	26%
HOCl / DTT	130.5 ± 11.0	61.5 ± 13.5	65%	66%

Table 1: Levels of ^{14}C - IAA (CPM·µg protein^{-1}) bound to actin and myosin as an indication of free thiols.

DISCUSSION:

Pressure development due to the contraction of cardiac muscle is only the final expression of a very complex sequence of biochemical events occuring in the myocytes. The initial depolarization of the sarcolemmal membrane and the subsequent transarcolemmal Ca^{++} influx, followed by the release of sarcoplasmic reticulum (SR) Ca^{++} and its binding with Troponin C, lead to the activation of the myofibril ATPase, and the force-generating myosin/actin cross-bridge formation. Many of the proteins involved in this sequence of events contain reduces thiol groups which are critical to their biochemical function. The inactivation of any one of these proteins by thiol oxidation could lead to the impairment of the contractile process. For example, oxidation of as few as one cysteine residue will lead to the inactivation of the sarcolemmal and the SR Ca^{++} ATPase's (24, 25), the sarcolemmal Na^{+}/K^{+}-ATPase (26), or the myosin ATPase (27). However, most of the above data was derived by direct modification of very specific thiol groups, using DTNB, N-ethylmaleimide, or other thiol-specific agents, in purified enzyme preparations. There is little direct evidence available to address whether the exogeneous application of non-specific oxidant species will impair the normal functioning of the contractile apparatus through the alteration of the myofbril protein thiol redox status.

The development of contracture as observed in both the isolated rat papillary muscles (18) and in the coronary perfused hearts may be due in part to lipid peroxidation and subsequent membrane disruption leading to a cytoslic calcium overload. DTT would not be expected to facilitate the repair of damaged membranes, therefore DTT's ability to restore contractile function in these two models strongly suggests that the oxidation of protein sulfhydryl residues is involved in this damage. In this study we demonstrate that the decreased mechanical performance resulting from the exposure of perfused rat hearts to HOCl correlates with thiol oxidation within the contractile proteins. We show that the

contractile proteins themselves are highly susceptible to HOCl-induced thiol oxidation, and that these thiol levels can be restored with DTT.

In order to understand how thiol oxidation within the contractile proteins could alter the contractile performance of the myocardium, we must first consider the structure of the proteins involved. In a recent review paper, Audemand et al (28) describe the structure of the myosin molecule as discerned using various enzymatic treatments. Cleavage of the myosin molecule with chymoptrypsin allows for the dissociation into a 200 kD myosin heavy chain (MHC) portion and two different light chains, commonly referred to as the alkali and the regulatory light chains (see Figure 5). The MHC can be further cleaved to produce two 95kD S1 segments (the globular heads of the myosin molecule) which have been shown to retain all the ATPase activities of the myosin molecule (29). This S1 region has been shown to contain the actin binding domain (30) as well as the nucleotide binding site (31), which are spatially separate but highly interdependent.

Rabbit cardiac myosin contains between 32 - 36 cysteine residues (32), of which six are located on the light chains, and a total of eight are distributed throughout each of the S1 heavy chain subfragments (28) as depicted in Figure 5. Pfister et al (33) showed that four reduced SH residues, located adjacent to the nucleotide binding site on the 20 kD S1 subfragment, are essential for maintaining the activity of the K^+-stimulated myfibril ATPase as well as the Ca^{++} stimulated myofibril ATPase. These thiols include the two highly reactive thiols known as SH1 and SH2 (34) which are known to be critical for maintenance of the ATPase activity. The binding of ATP to the nucleotide binding site will induce a configurational change in the 20 kD subfragment, thereby exposing these thiols to oxidation. Wells and Yount show that by crosslinking the SH1 and SH2 residues following binding of the nucleotide, they could 'trap' the nucleotide within the binding site thereby inactivating the enzyme (Figure 5 D). Of great interest is a study by Chaussepied et al (27) which shows that the modification of SH1 with DTNB leads to a loss of moysin

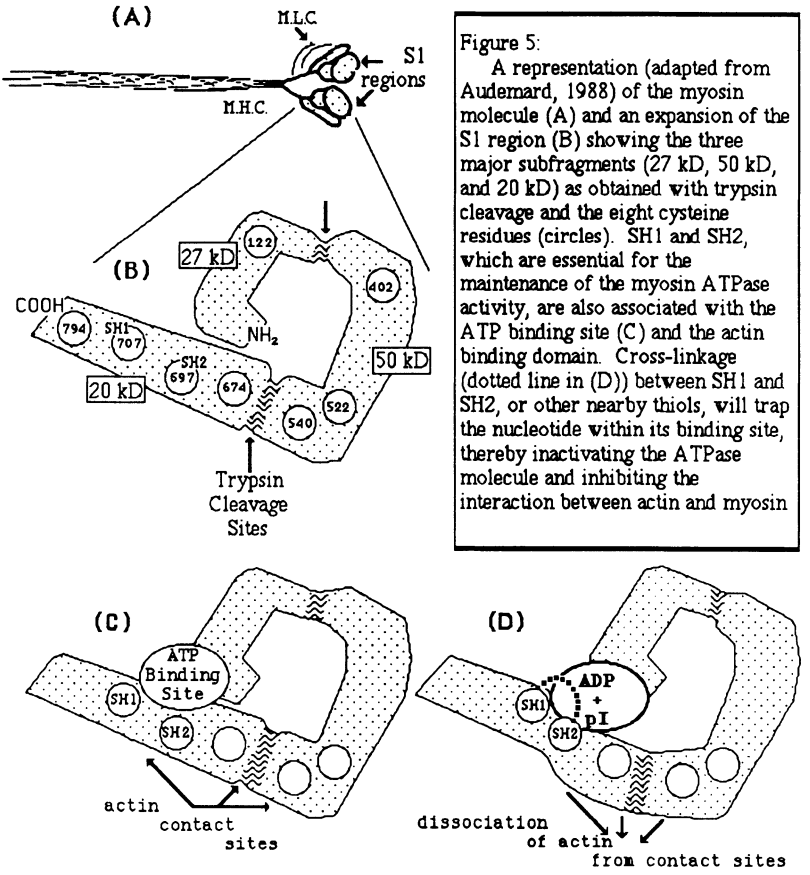

Figure 5:
A representation (adapted from Audemard, 1988) of the myosin molecule (A) and an expansion of the S1 region (B) showing the three major subfragments (27 kD, 50 kD, and 20 kD) as obtained with trypsin cleavage and the eight cysteine residues (circles). SH1 and SH2, which are essential for the maintenance of the myosin ATPase activity, are also associated with the ATP binding site (C) and the actin binding domain. Cross-linkage (dotted line in (D)) between SH1 and SH2, or other nearby thiols, will trap the nucleotide within its binding site, thereby inactivating the ATPase molecule and inhibiting the interaction between actin and myosin

ATPase activity, but subsequent treatment with dithioerythreitol (DTE), a thiol reducing agent similar to DTT, restores this ATPase activity. This suggests that the loss of ATPase activity following thiol oxidation may be a reversible reaction as well.

In another study, Kasprzak et al (30) located the contact site between actin and myosin (actin binding domain) close to the nucleotide binding site (Figure 5 C). They showed that the binding of the ATP to the nucleotide binding site caused a significant decrease in the distance between the SH1 and SH2 thiols, and a concommitant increase in the distance between SH2 and the nearby actin binding domain. This conformational change was shown to disrupt the electrostatic and hydrophobic actin contacts (27), producing a dissociation between actin and myosin (Figure 5 D). These data suggest that crosslinking between SH1 and SH2, or between SH1 and the other thiols within the myosin molecule will alter the conformation of the actin binding domain and inhibit the binding of actin to the myosin S1 region. In the intact muscle, such a reduction in cross-bridged available for cycling would lead to a decrease in force development during a contraction. Conversely, if these thiols are crosslinked before complete hydrolysis of the ATP molecule has occurred, then the cross-bridges may remain in the pre-power configuration. This could impair the release of the cross-bridge formed between actin and myosin, resulting in the formation of rigor bonds (35).

Actin (rabbit skeletal) has been sequenced (36) and shown to contain 5 free cysteine residues, with no disulfides under normal condition. The availability of these thiol residues to bind with Iodoacetamide (IAA) changes during the contraction cycle (36), either due to changes in conformation of the protein during the interaction, or due to the involvement of these thiols in the actual binding of actin to myosin, This suggests that thiol residues may be actively involved in the contractile process, perhaps by maintaining the integrity of the binding site for myosin. In a study using isolated hepatocytes(19), the depletion of free thiols within the actin molecule of the cytoskeleton following exposure to an oxygen-free radical generating system resulted in the cross-linking between several actin molecules. This led to the formation of large molecular weight aggregates which severely compromised the viability of those cells. Prior exposure to DTT offered protection against

this actin crosslinking, while the subsequent application of DTT reduced the number of aggregates formed and partially reversed the cytoskeletal contractile dysfunctions observed following OFR generation. This suggests that actin in also very sensitive to thiol redox status, and that the presence of reduced thiols in the actin myosin molecule may be essential for its functionality.

In view of the above, it appears that thiol modification within the contractile proteins of the heart may significantly alter their functional characteristics. While the results obtained in this study cannot directly link the decline in contractile performance to a decline in the activity of the myosin ATPase, it is interesting to speculate about this possibility. We do present evidence that protein thiols within the actin and myosin molecules are depleted following exposure to extracellular HOCl, and that this can be reversed by the subsequent exposure to DTT. This repletion of protein thiols with DTT correlates with a significant restoration of contractile function in the isolated heart model. The fact that DTT does not induce a full recovery of thiols or contractile function suggests that some of the proetein thiols may have been irreversibly oxidized, or were in a configuration not affected by DTT. The fact that a greater level of thiol oxidation was evident in the isolated myosin (Table 1) vs the total P-SH (Figure 3) suggests that not all myocardial proteins thiols are equally susceptible to the HOCl perfusion. Given that the cysteine residues in actin and myosin become more accessible for binding during the conformational changes involved in each contractile cycle, it may be that these thiols are more susceptible to oxidation than those associated with other cellular proteins.

In conclusion, we have shown that the contractile dysfunction that is observed in the coronary perfused rat heart following exposure to HOCl correlates with a decrease in protein thiols within the contractile proteins. This contractile failure is at least partially reversible by the subsequent perfusion with DTT, coinciding with a restoration of protein thiols. Further work needs to be done describing the redox status of the myofibril proteins

in vivo in response to neutrophil infiltration, including the measurement of the activity of the myofibril ATPase. The use of reducing agents such as DTT to counter the oxidation of reactive protein thiols may prove to be useful for the in vivo restoration of contractile performance to the damaged myocardium.

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CARDIAC CONTRACTILE FAILURE DUE TO OXYGEN RADICALS IN AN EX VIVO SYSTEM

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INTRODUCTION:

Molecular oxygen is a life supporting agent but because of the two unpaired electrons in it's outer valence orbit, oxygen remains potentially toxic to all aerobic life. Under normal conditions more than 95% of oxygen, taken up by the cells undergoes tetravalent reduction to water via the cytochrome oxidase system in the mitochondria. However, 3-5% of oxygen is known to be consumed in a mono and/or divalent reduction pathway with the generation of different reactive oxygen intermediates including active oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical. Collectively, these reactive intermediates are termed here as partially reduced forms of oxygen (PRFO). PRFO are ubiquitous in nature and are present in a variety of biological and non-biological systems. Because of their unique electronic configuration, most of PRFO are highly reactive and have a very short half life.

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Because PRFO are continually being generated by constitutive metabolic processes, many aerobic cells possess antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, which protect the cell and neutralize the damaging effects of PRFO. Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen. Catalase and glutathione peroxidase both catalyze the reduction of hydrogen peroxide to water and oxygen. In addition, a number of non-enzymatic antioxidants exist within the cell to prevent free radical generation and cell damage. However, in disease states or conditions of imposed oxidative stress, PRFO generated from intra and extracellular sources may exceed the protective capacity of the cellular antioxidants and cause cell damage.

Since the discovery of the enzyme superoxide dismutase (1), many investigators have undertaken to elucidate the involvement of oxygen radicals in both health and disease. Over the past two decades several reports have provided evidence suggesting a role of PRFO in a variety of pathological conditions including inflammation (2), irradiation injury (3), ischemia-reperfusion injury (4-11), drug-induced toxicity (12-13), catecholamine-induced cardiomyopathy (14) as well as degenerative diseases such as aging, arthritis and immunedeficiences (15).

Over the recent years a variety of in vivo and ex vivo model systems have been used to study the effects of oxygen radicals on myocardial structure and function. Significant contributions have been made by these studies in establishing a role for PRFO as mediators of myocardial cell damage. Although the oxidant species generated and their relative concentrations in these experimental systems are not well defined, these approaches have been helpful in understading the PRFO-mediated damage in many pathological conditions. However, conclusions based on these studies should be interpreted with caution as results obtained with one model system can easily differ from those obtained with an alternative

experimental model. In view of the increasing awareness for the role of oxygen radicals in heart disease and use of different PRFO sources to understand cardiac injury, we describe the effects of PRFO on rat hearts in an ex vivo perfusion system and contrast these data with that obtained with different model systems. In the end PRFO-induced myocardial cell damage and heart failure is explained according to the hypothesis proposed earlier (19).

SOURCES OF PRFO AND CONTRACTILE CHANGES IN DIFFERENT MODEL SYSTEMS:

Both a monovalent and/or divalent reduction of oxygen can be brought about by the enzyme xanthine oxidase which catalyzes the oxidation of purine to hypoxanthine, of hypoxanthine to xanthine, and of xanthine to uric acid (16). In the past, purine (17), hypoxanthine (18), and xanthine (19-21) have been used as substrates to generate PRFO. When isolated rat hearts were perfused with xanthine-xanthine oxidase (x-xo) as the source of PRFO, a significant decline in contractile function was seen within 5 min of perfusion which became more severe in 10 min (Fig 1). Complete contractile failure with a large increase in resting tension occured at about 20 min of perfusion (Figs 1 and 2). There was no further change in resting tension at 40 min of perfusion with x-xo. Some studies have reported a transient increase in developed force in response to PRFO (22). We have also seen this effect, but not consistantly and therefore it remains to be established whether this effect is real or it is an artifact of the perfusion system. Addition of free radical scavengers such as superoxide dismutase (SOD), catalase and mannitol shows protection against the decline in mechanical function as well as rise in resting tension indicating that the negative inotropic effect is indeed mediated by PRFO (20,23).

Using hypoxanthine as substrate, Yetrehus and associates (18)

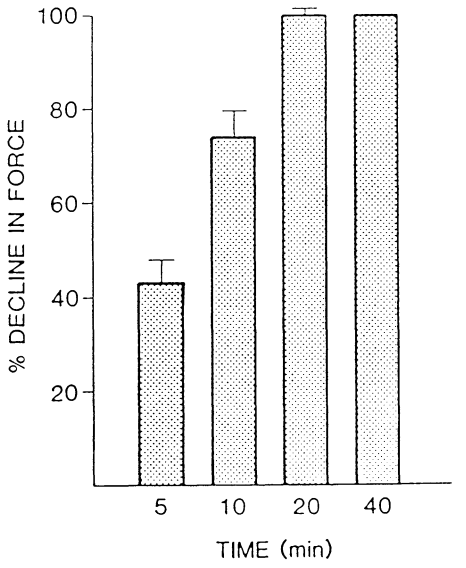

Fig. 1 : Effects of xanthine (2 mM) and xanthine oxidase (10 U/L), mixed in Krebs-Henseliet buffer (pH 7.4), on developed force in isolated perfused rat hearts. A resting tension of 2g was applied on the hearts. Hearts were paced at the rate of 300 beats/min and perfused at the rate of 8 ml/min.

have also demonstrated both structural and functional changes as well as protection with SOD and catalase. In addition, these workers reported a significant increase in coronary flow subsequent to PRFO exposure, discounting the possibility that ischemia may have been the cause of contractile failure and increase in resting tension. We have also reported that vascular changes in rat hearts due to PRFO are delayed events and require a prolonged exposure to radical species (20). In an isolated rabbit

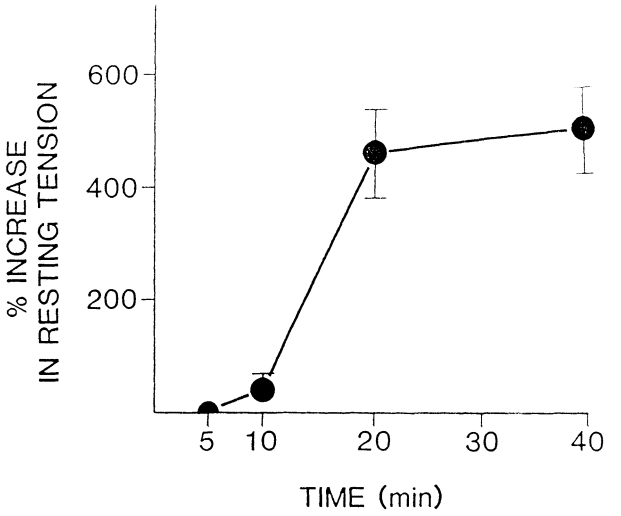

Fig.2 : Effects of x-xo on resting tension in paced rat hearts. All conditions are the same as in Fig.1.

ventricular septal preparation using purine-xo as a source of superoxide, only structural defects were reported in the vasculature with no significant change in the function (17). These differences in the rat (18-21) and rabbit (17) model remain unexplained. One possibility could be a poor or slower transfer of toxic species from the vascular space to the interstitium. However, in the same study when a hydroxyl radical generating system was substituted by adding iron loaded transferrin to purine-xo system, a significant decline in force along with more severe ultrastructural changes in the vasculature and myocytes were reported (17). Again SOD was protective against these radical-induced effects suggesting superoxide as the mediator of myocardial cell damage and

contractile failure.

In the isolated rat papillary muscle preparation, a combination of purine-xo was found to reduce active tension (24). However, catalase but not superoxide dismutase was protective against the radical induced effects. Failure of SOD to protect against the decline in mechanical function may suggest the importance of hydrogen peroxide as a mediator of myocardial cell damage in this preparation. The important point to be noted here is that unlike the rabbit preparation (17), a significant contractile depression could be produced without adding transferrin to the medium. The fact that in some studies catalase not SOD provided adequate protection against PRFO induced myocardial injury is consistent with the concept that hydrogen peroxide may play an important role in initiating myocardial damage, either through its direct cardiotoxic effects or through its ability to inactivate SOD (25-27).

In another approach PRFO were generated by passing a current through the physiological buffer (28,29). The advantage of this model system is that the perfusion medium is free of blood cell elements and enzyme proteins, thereby minimizing any potentially contaminating experimental artifact(s). In the study on isolated perfused rabbit hearts, current (20 mA) passed through the perfusion medium for 2 min resulted in a decline in the developed force which was accompanied by a significant rise in the coronary perfusion pressure (28). Addition of different antioxidants provided a varying degree of protection against the radical-induced contractile failure. However, none of the antioxidant treatments were effective in protecting the hearts with respect to the rise in coronary perfusion pressure (28).

Although the sources of PRFO employed in these studies were different, the contractile failure and structural alterations seen in these studies, clearly establish that PRFO induce contractile

dysfunction and may act as mediators of myocardial cell damage under a variety of conditions. Furthermore, the use of different antioxidants prior to or during exposure to PRFO appears to confer significant protection against radical-induced effects. There appears to be a significant difference between the rat and rabbit hearts in certain aspects of their response to PRFO.

REQUIREMENT FOR IRON IN EX VIVO SYSTEMS:

In rabbit myocardial septal preparations, the combination of purine and xo was found to cause a significant decline in force and myocardial cell damage only when iron loaded transferrin was added to the perfusion medium and it was suggested that presence of iron is required to bring about this effect (17). Since in this (17) and other studies (20,23) we did not provide any source of iron during the x-xo induced contractile failure (Fig.1), the data argue against a need for iron in using x-xo as a source of PRFO. Assuming that in our experiments iron may be present as a contaminant, we repeated the study in the presence of desferal, a known iron chelator. The results show clearly that desferal did not have any effect on the contractile failure pattern induced by x-xo (Fig.3). We also performed experiments in which x-xo was supplimented with 2.4 uM iron loaded transferin. Interestingly in these experiments, PRFO-induced contractile failure was significantly attenuated rather than excerbated (Fig.3). We attributed this protective effect to some transferrin-induced impedement in the transfer of toxic stimuli from the vascular to the interstitial space. In this regard, transferrin has been shown to deposit along the basement membrane as a layer of electron dense material between the endothelial and smooth muscle cells (30). It is possible that requirement for iron under certain conditions may not be an absolute neccesity (23).

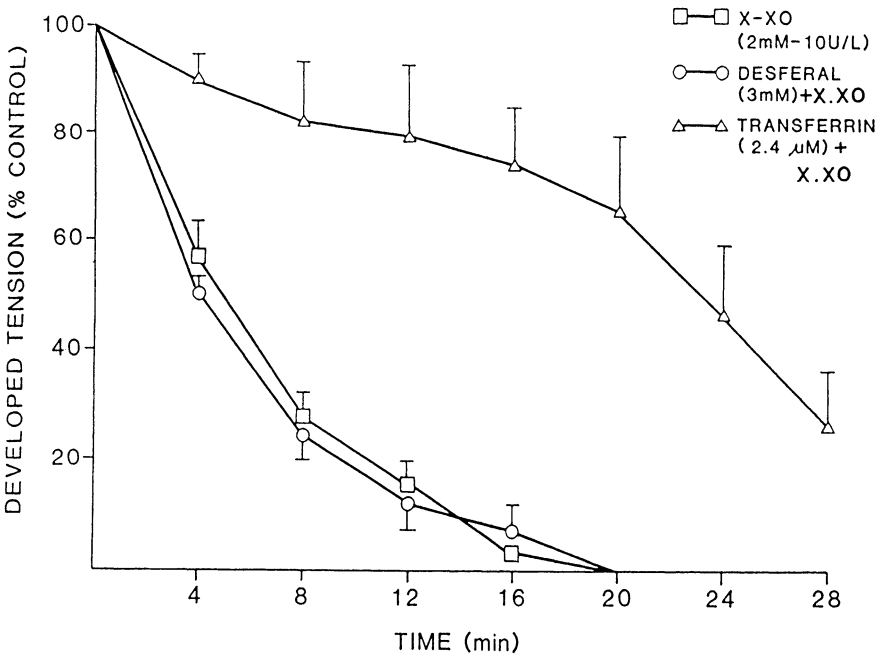

Fig. 3 : Effects of desferal and transferrin and x-xo induced contractile failure in paced rat hearts. All other conditions are the same as in fig.1.

VASCULAR CHANGES IN RESPONSE TO PRFO :

Studies of blood vessels in different model systems including pial microcirculation (31), cerebral arteries (4), dog, rabbit and rat coronaries (17,18,29) isolated rat tail artery (29),rabbit aorta (32) and rat cremastric arterioles (33) have

clearly shown that PRFO are vasoactive. However, there are still controversies with respect to both quantitative and qualitative nature of these effects. Concentration dependent reversible vasodilation on exposure to acetaldyde and xanthine oxidase was produced in pial circulation (31). Similarly, a biphasic response of vasoconstriction followed by vasodilation was seen in hamster cheek pouch subsequent to oxygen radical exposure (34). In rat hearts, a rise in coronary flow (18) as well as a fall and rise in coronary pressure (22) in response to PRFO has been reported. In rabbit hearts, however, only a significant rise in coronary pressure in response to PRFO was reported (28). It is apparent that coronary vessel response should be interpreted with caution and further work is required to clearly define coronary pressure changes due to PRFO.

Although the reported dilatory effects occurred before contractility decreased, it must be remembered that reduced contractility as well as rise in resting tension will also alter coronary flow through altered mechanical compression. In these studies, however, it is difficult to dissociate between myogenic vascular responses to PRFO as opposed to changes in flow due to mechanical compression. Although the mechanism(s) by which PRFO may influence vascular tone remain(s) unknown, in studies on pulmonary artery relaxation due to PRFO, it was suggested that there is an activation of a soluble guanylate cyclase which may mediate relaxation through c-GMP formation (35).

Increased permeability of the coronary vasculature indicating structural damage in rabbit heart has been attributed to hydroxyl radical and hydrogen peroxide in the perfusion medium (28). Vascular damage due to PRFO has been documented in many organs including heart (17,20,28). However in our studies vascular cell injury was observed to be a delayed event (20). At any rate it can be seen that PRFO have a significant effect on

blood flow during conditions of oxidative stress.

POTENTIAL MECHANISM FOR PRFO-INDUCED CONTRACTILE FAILURE:

Although the mechanism(s) responsible for the myocardial failure that occurs in response to PRFO remain(s) elusive, defects in energy production as well as calcium homeostasis secondary to membrane permeability changes have been postulated to be important factors operating at the cellular level (20,21). Since biomembranes are rich in polyunsaturated fatty acids, they are most susceptible to free radical induced lipid peroxide formation. Introduction of peroxidation products in the membranes as well as oxidation of sulfhydryl groups in important functional proteins and other molecules has been considered to effect the semi-permeable characteristics of the membrane and cellular function (36). In this regard, increased lipid peroxidation due to exposure to PRFO has been reported by many workers (18-21).

Membrane permeability changes in rat hearts exposed to PRFO have been documented by the intracellular localization of lanthanum, an electron dense tracer normally restricted to extracellular spaces (12,21). Oxygen radicals have been shown to influence, calcium transport in the sarcoplasmic reticulum (37). Inhibition of ATPase in the hamster cheek pouch (38) and membrane ATPases as well as calcium transport in the cardiac sarcolemma (39,40) on exposure to oxygen radicals and lipid peroxides have also been reported. Membrane permeability changes will promote Ca^{2+} influx and this coupled with defects in Ca^{2+} efflux/sequestration processes can be seen to cause Ca^{2+} overload which may also explain PRFO-induced rise in resting tension now reported by many workers.

Occurrence of Ca^{2+} overload has also been suggested to impair mitochondrial energy production (41). Exposure of hearts to PRFO results in mitochondrial swelling and loss of

cristae (12,17,20,21). In addition to morphological alterations, exposure of hearts to PRFO results in a significant loss of adenosine triphosphate as well as creatine phosphate (18,20,21). Thus mitochondrial function defects as well as loss of high energy phosphates due to PRFO is now established (18,20,21,42,43).

Many in vitro oxygen radical generating systems are commonly used to study the effects of PRFO on heart function. Although the source of PRFO as well as the relative proportion of species generated may differ from model to model, there appears to be a consensus with respect to deteriorating effects of PRFO on the contractile function and myocardial cell structure. Earlier, we have proposed a hypothesis to explain the genesis of myocardial dysfunction due to ex vivo perfusion with PRFO (19). It was suggested that initiation of oxidative chain reactions compromises subcellular membrane functions resulting in intracelluar increase in calcium as well as loss of high energy phosphates. These changes can explain the loss of contractile function, rise in resting tension and myocardial cell damage due to oxygen radicals. Application of this hypothetical scheme in in vivo conditions of cardiac pathology and dysfuntion will be established by further studies.

SUMMARY:

Partially reduced forms of oxygen (PRFO) very likely contribute in cardiac pathophysiology experienced under different clinical conditions. There are now variety of sources available for generating PRFO in ex vivo systems including oxidation of different substrates (purine, hypoxanthine, xanthine) by the enzyme xanthine oxidase, electrolysis of physiological buffers and many other chemical sources. Application of some of the ex vivo perfusion systems in understanding the pathogenic role of PRFO has been reviewed. Despite the diversity of sources

used to generate PRFO, a consensus is emerging with regard to their myocytic effects vis a vis contractile failure and cell damage. Different antioxidants such as superoxide dismutase, catalase, mannitol provide protection against these PRFO induced changes. However, vascular effects of PRFO require further clarification(s). Requirement for iron in PRFO induced myocardial contractile failure remains to be established. Our results suggest that presence of iron in the production of PRFO under certain conditions may not be absolutely essential. PRFO-induced myocardial cell damage and contractile failure is associated with the loss of high energy phosphates and probably also with an increase in intracellular calcium. These changes appear to occur secondary to an increase in lipid peroxidation as well as abnormalities of membrane functions.

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SUBCELLULAR BASIS OF CONTRACTILE FAILURE IN MYOCYTES:CALCIUM OVERLOAD OR ENERGY DEPLETION ?

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INTRODUCTION

The pathogenesis of contractile failure of heart muscle was investigated earlier in two major research areas, i.e., cellular energy generation and energy utilization. It became clear that overt disturbance in substrate metabolism, including that in oxygen consumption, was not found in contractile failure of the heart (1). However, localizing defective steps in the energy utilization process has been a more complicated task (2). The velocity of contraction and other mechanical parameters, as measures of intrinsic muscle contractility, were shown to be depressed in heart failure, while ischemic areas of ventricular muscle ceased to contract even before high energy phosphates were depleted.

Even more puzzling was the demonstration that release of intracellular enzymes from the myocardium was greatly accelerated when the previously hypoxic heart was reoxygenated (oxygen paradox). Likewise, ventricular function deteriorated dramatically when the heart was perfused with a solution containing a normal concentration of Ca (Ca^{2+}), following perfusion with a Ca-free solution (Ca paradox). In both cases, accumulation of Ca in the myocardium (Ca overload) was noted (3,4). The question is then whether Ca overload was the cause or the result of these pathologies.

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When isolation of adult heart cells was attempted some years ago, it was realized that isolated myocytes were intolerant to the normal concentration of Ca; cell contracture, occurring under these conditions, resembled the heart of Ca paradox, in which contraction bands were formed and the thick and thin filaments aggregated into a central mass (5). It was postulated that the permeability of the sarcolemmal membrane, particularly that to Na ions, was increased by perfusion with a Ca-free solution, which was required to separate cells from one another along the intercalated discs. Consequently, intracellular Na concentration was elevated, resulting in an increased Ca influx via Na/Ca exchange, when the extracellular fluid was changed to that containing Ca (6).

Accumulation of Ca in the cell was found also in the heart following ischemia-reperfusion (2). These results showing that increases in intracellular Ca concentration were associated with a number of pathological states were compatible with the view that maintenance of intracellular Ca homeostasis is critical to the normal cell function (3). The cytosolic Ca concentration is regulated precisely by subcellular membrane systems. The sarcolemmal membrane separates a concentration gradient of 10 000 fold. Ca ATPase of the sarcolemmal membrane is able to transport Ca ions against a gradient of this magnitude. The SR (sarcoplasmic reticulum) Ca pump can effectively modulate the cytosolic Ca during the cardiac cycle, while mitochondria can accumulate a large amount of Ca (7). Therefore, impairments in the function of subcellular organelles would result in perturbation of intracellular Ca homeostasis, leading ultimately to contractile failure. It is also possible that since the ion transport function requires an adequate supply of energy, defective energy generation could impair the transport activities, resulting in an elevation of the cytosolic Ca concentration. However, the measured Km values for ATP of the Ca pumps (0.03 - 0.18 mM) (7) indicate that energy depletion must be extreme, or inhomogenous within the cell.

Thus, intracellular accumulation of free Ca has for many years been attributed as the principal causative factor for cell damage. Elevated cytosolic Ca is believed to activate proteinases, phospholipases and other oxidative enzymes, probably altering membrane/cytoskeleton and causing cell lysis. Although Ca ATPase can reduce the increased concentration of free Ca in the cell, it requires the consumption of energy, thus diminishing ATP stores. When Ca handling by SR cannot cope with an increase in cytosolic Ca, the latter is taken up by the mitochondria (their Km value for Ca is higher), but the oxidative phosphorylation could be curtailed by the elevated matrix Ca concentration. Therefore, once the intracellular Ca concentration starts to rise, a cascade of adaptive reactions takes place, which may exceed cellular compensatory capacity, causing irreversible cell injury.

However, evidence in support of Ca overload as the subcellular basis of contractile failure was obtained predominantly from experiments of a prolonged duration. In fact, it has been difficult to follow an early time course of changes in intracellular Ca distribution and in energy depletion. This was overcome by the successful isolation of viable, Ca-tolerant heart myocytes, enabling investigators to look into the above problem in more detail. Moreover, several indicators for intracellular Ca ions became recently available (8), so that on-line measurements of cytosolic Ca can be performed. Further advantages of myocyte experiments are: i) the possibility of monitoring changes in a single cell and in subcellular compartments, ii) relative homogeneity of the cell population, iii) avoidance of diffusion and microvascular complications, etc. (9-14).

In order to deplete ATP stores in the cell, investigators previously found that an extreme degree of hypoxia (e.g., as low as 0.15 torr) was necessary (15). However, this difficulty can be avoided by achieving chemical "hypoxia", using FCCP (p-trifluoromethoxyphenylhydrazone) + rotenone,

iodoacetate + oligomycin, cyanide + 2-deoxyglucose, etc., as these combinations effectively suppress mitochondrial oxidative phosphorylation and glycolytic ATP production (11,12,14).

Therefore, the question of whether Ca overload or energy depletion is the primary cause of the subcellular basis of heart cell failure is far from settled. The present study was undertaken to evaluate the relationship between cellular free Ca and ATP concentrations in inducing contracture of isolated myocytes.

METHODS

Cardiomyocytes were isolated from rat hearts by a collagenase digestion procedure (5,16). Heparin (100 units) was injected intravenously into male Sprague-Dawley rats, weighing 250-300 g, 30 min prior to the heart perfusion. A retrograde perfusion of the heart was carried out for 5 min at 37°C with oxygenated Krebs-Henseleit buffer. The perfusate was changed to oxygenated Joklik Minimum Essential Medium (Gibco) containing taurine (60 mM), creatine (20 mM) and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (5 mM) at pH 7.3. Non-recirculating perfusion was followed by recirculating perfusion with a medium containing 100 units of collagenase (Worthington) per ml for 40 min. The ventricles were cut from the softened heart, minced, then transferred to fresh medium containing collagenase (250 units/ml), and shaken vigorously for 30 min at 37° C. The Ca concentration was increased to 50 μM and the suspension was shaken for another 30 min. Disaggregation was accelerated by pipetting. After digestion for a total of 60 min, the myocyte suspension was filtered through nylon mesh and purified by gravity settling in medium containing 1.5 % bovine serum albumin. The Ca concentration could be increased to 1.0 mM without untoward effects on cell viabilities. The myocytes were counted in a hemocytometer under a microscope. Their protein concentration was 4.60 ± 0.15

ng/cell (n = 15), and over 80 % of the cells showed rod-cell morphology. The number of cells harvested from one heart was 7 - 9 million.

For the preparation of permeabilized myocytes, the suspending medium was replaced with a "cytosolic" medium, which contained 100 mM KCl, 20 mM NaCl, 5 mM $MgSO_4$, 0.96 mM $NaHPO_4$, 2 mM EGTA (ethylene glycol bis[beta-aminoethylether] tetraacetic acid), 5 mM HEPES, pH 7.2, and 2 % bovine serum albumin. The cells were then treated with 60 μg saponin/ml (25-38 μg/mg protein) (17). The suspending medium was replaced by washing with cytosolic medium devoid of saponin and by centrifugation. Depending on the assay, 10 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone), 5 mM ATP, 10 mM glucose and 25 units hexokinase (Sigma) were added sequentially. Free Ca concentration was adjusted by varying the Ca/EGTA ratio.

RESULTS

In these experiments the free Ca concentration was maintained at an extremely low value by the addition of EGTA. During the isolation of myocytes approximately 50 μM of Ca ("essentially Ca-free") were maintained to help accelerate the action of collagenase. Subsequently, the extracellular fluid was changed to a "cytosolic" solution containing 2 mM EGTA, and then sarcolemmal membrane was permeabilized (chemically skinned myocytes). Therefore it is likely that SR and mitochondrial Ca contents were reduced to very low levels during these procedures (18). This is to minimize the possibility that Ca is released from intracellular sites, thus raising the cytosolic Ca level.

When myocytes were permeabilized with saponin in a solution containing EGTA, the morphology of the cell was essentially unchanged (Fig.1 and Table). Treatment with saponin was to remove the permeability barrier and hence to enable us to directly manipulate intracellular free Ca and ATP concentrations. It should be noted that low concentrations of saponin do not impair

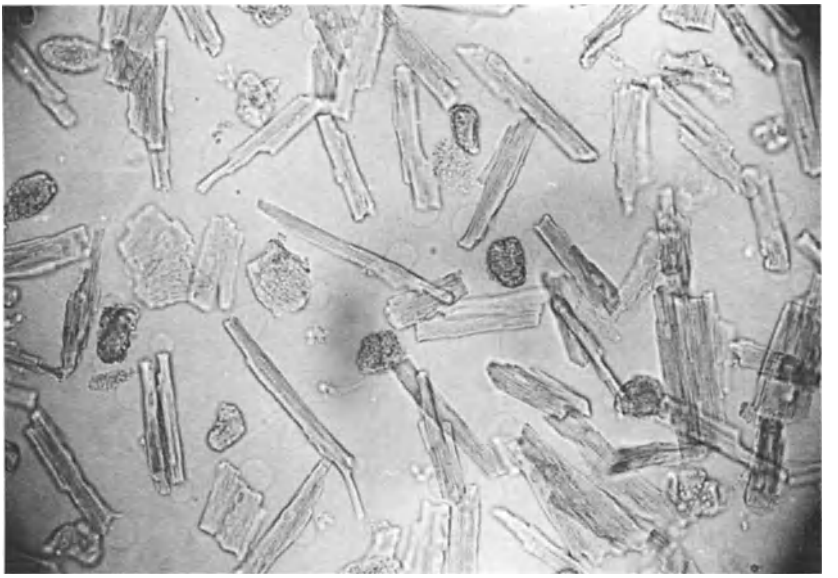

Fig. 1. Isolated adult rat heart myocytes after saponin treatment.

Table. Effect of energy level on contracture of myocytes.

Addition	Rod cells	Square cells	Round cells	n
+ EGTA (2 mM)	53.8 ± 13.7	12.4 ± 9.6	33.9 ± 8.1	10
+ Saponin (75 µg/ml)	51.2 ± 6.8	12.2 ± 0.9	36.5 ± 6.1	3
+ CCCP (10 µM)	4.5 ± 4.6	22.2 ± 6.6	73.3 ± 10.3	5
+ ATP (5 mM)	24.8 ± 1.3	22.9 ± 12.3	52.3 ± 12.0	4

Values are means + SEM in % of total cell population.
EGTA, saponin, CCCP and ATP were added consecutively in that order.

mitochondrial and SR functions (19). Addition of CCCP, which suppresses mitochondrial ATP production, caused contraction of cells as indicated by a diminution in the number of elongated cells and an increase in square and round cells (Fig.2 and Table). Contracture was reversed by bringing up the cytosolic ATP level to 5 mM (Table). Under a light microscope, it was possible to observe gradual elongation of the cell when ATP was added back to cell suspension. The results support the hypothesis that contracture of myocytes is induced by depletion of ATP and hence is relieved by raising the ATP concentration, when intracellular Ca concentration is kept low in the skinned heart cells.

The presence of glucose before addition of CCCP largely prevented cell contraction, indicating that glycolytic ATP production under these conditions was sufficient to maintain the ATP level required for rod cell morphology.

When hexokinase was added in addition to glucose to consume ATP existing in the permeabilized cell suspension, all myocytes went into contracture within a few minutes (Fig.3). Even in this instance, addition of ATP, immediately following hexokinase administration, temporarily blocked the appearance of contracture. Addition of Ca to permeabilized myocytes induced contracture, as is already known.

Our results indicate that contracture can be induced without a concomitant increase in intracellular Ca levels. In addition, in agreement with observations by other investigators (14,20), changes in morphology of myocytes in response to ATP depletion were inhomogenous. Since changes in overall population were followed in our studies, it is not possible to state with certainty whether or not round cells (cell contracture) were converted back to rod cells.

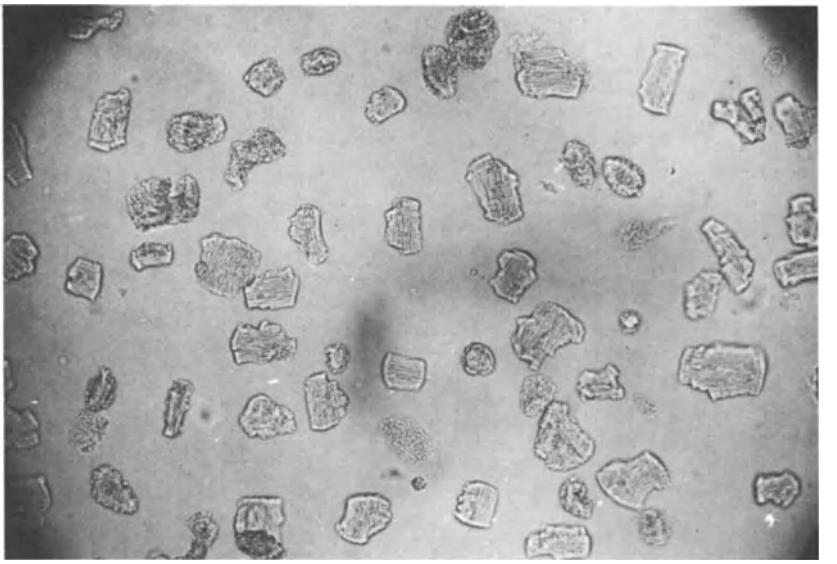

Fig. 2. Effect of addition of CCCP on rat heart myocytes permeabilized by saponin.

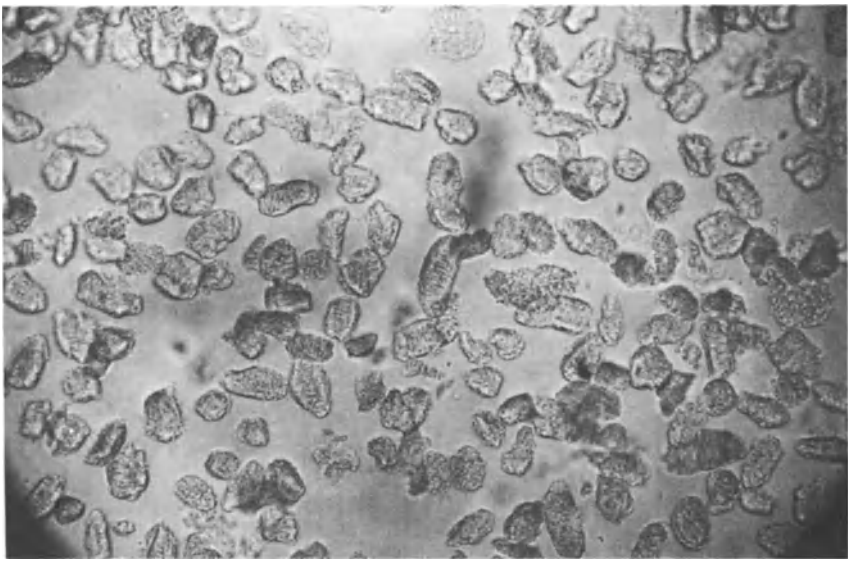

Fig. 3. Effect of addition of glucose and hexokinase on permeabilized myocytes.

DISCUSSION

These studies showed that contracture of isolated cardiomyocytes occurred dependent on the intracellular ATP and Ca concentrations. When the cellular energy supply is exhausted, contracture can take place even without "Ca overload", whereas excessive accumulation of Ca causes contracture of myocytes even if there is adequate generation of high energy phosphates. Therefore, the incidence of contracture is a function of the [free Ca]/[ATP] balance. In other words, both "Ca overload" as well as ATP depletion may be able to cause cell contracture and thus cell death. It was reported earlier that when the Mg ATP concentration is relatively high (10 mM), hypercontracture is a response to increasing Ca concentrations (Ca overload), whereas at μM ranges of Ca, myocytes are unable to maintain their rod cell morphology even at higher than physiological Mg ATP. In addition, the presence of 1 mM ATP caused 100 % of the cells in contracture at 320 nM free Ca, whereas at 10 nM free Ca the same ATP maintained 80 % of the cells in rod-cell morphology (19,20). Thus, the lower the ATP level, the lower the concentration of Ca which precludes myocytes from hypercontracture. Although these studies were carried out with chemically skinned myocytes, it is known that the sarcolemmal membrane contributes little to contracture (21).

Therefore, cell dysfunction can be induced by an increase in Ca concentration as well as by Ca-independent processes. Starke et al. showed this by studying the effect of oxidants on viability of hepatocytes. They reported that hydrogen peroxide caused cell death even when the cell Ca concentration was reduced by the EGTA treatment, while deferoxamine did not block the oxidant-induced elevation in Ca concentration despite its prevention of cell injury (18). Thus, cell death can occur independently of intra- as well as extra-cellular Ca concentrations. Oxidants affect not only Ca homeostasis but also other cellular metabolism, particularly redox states and mitochondrial

oxidative phosphorylation; membrane transport functions are critically linked to the former, whereas cell energy states depend on the latter. Therefore, the primary locus of action of oxidants in causing irreversible cell injury is as yet to be established (22).

Recent reports indicate that an increase in cytosolic Ca concentration was minimal when contracture of myocytes was induced by chemical anoxia. Only after cell contracture took place, did the cytosolic Ca start to increase (9,10,14). Indeed, changes in permeability of myocytes, with trypan blue and lactic dehydrogenase as indicators, were observed only after cell contracture (23). In other words, an increase in intracellular Ca concentration is the result of energy depletion, rather than the cause of cell contracture. The primary cause of the latter is not Ca overload but energy deprivation. Therefore, contracture of myocytes may be defined as rigor, requiring consumption of little energy. The rigor complexes form when levels of Mg ATP become insufficient to saturate the myosin binding sites. The concentration of Mg ATP needed to inhibit this Ca independent process in skeletal muscle was reported to be approximately 0.1 mM (23).

By contrast, some previous reports indicate that not only a decrease in cellular high energy phosphates, which occurs during anaerobiosis, but also the restoration of ATP production by mitochondrial oxidative phosphorylation contributes to the production of irreversible contracture of myocytes. This view is derived from the results showing that oxygen paradox was blocked by cyanide and an uncoupler (24).

Thus, an interrelationship between intracellular ATP levels and free Ca concentration in inducing contracture is complex. Although the bulk of evidence indicates that the permeability of sarcolemmal membrane of the isolated heart cell is within the physiological range, many previous experiments were performed by using quiescent myocytes. Consequently, the relationship between the

systolic-diastolic fluctuation of cytosolic Ca concentration and intracellular ATP level has not been delineated. Neither ATP nor Ca is distributed homogenously in the myocyte. In addition, the situation in the heart in situ is far more complicated due to its intricate geometry and tension development. Therefore, further studies are needed to elucidate these points.

SUMMARY

Subcellular basis of contractile failure of myocytes is briefly discussed. Both Ca overload and energy deprivation in the heart cell can cause myocyte damage, leading to contracture and death. Our studies as well as others support the view that contracture of myocytes ensues depending on the balance of intracellular free Ca to ATP concentrations, viz., the lower the energy level, the lower the concentration of Ca required to induce contracture. Therefore, ATP exhaustion can cause contracture even if there is no Ca overload.

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