creatine and creatine phosphate

scientific and clinical perspectives

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Contents

Section III: Magnetic Resonance Spectroscopy of Creatine Phosphate in the Cardiovascular System

Section IV: Therapeutic Aspects of Creatine and Creatine Phosphate Metabolism

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A Brief Review of the Sections

Section I: *Basic Biochemistry of Creatine and Creatine Phosphate*

This section covers aspects of the basic metabolism of creatine and creatine phosphate with particular emphasis on the enzyme creatine kinase, the creatine phosphate shuttle hypothesis, recent observations in animal and cellular models, on the high energy phosphate changes in physiological studies, β-GPA feeding, and some muscle disorders. The regulation of oxidative phosphorylation is discussed and findings relating to creatine kinase gene manipulation models are summarized.

Section II: *Biochemical Basis for a Therapeutic Role of Creatine and Creatine Phosphate*

In this section the mechanism underlying the mode of action by which creatine and creatine phosphate are thought to act as therapeutic agents is presented. A detailed description of the biophysical actions of creatine phosphate is given as well as *in vitro* observations on heart cells. This is a transition section between the basic and clinical science sections.

Section III: *Magnetic Resonance Spectroscopy of Creatine Phosphate in the Cardiovascular System*

Magnetic resonance spectroscopy is a non-invasive analytical technique for measuring chemical species, such as creatine phosphate, in humans, animals, cellular and other systems. This section outlines the current findings and requirements in NMR spectroscopy of the human heart and skeletal muscle in heart failure.

Section IV: *Therapeutic Aspects of Creatine and Creatine Phosphate Metabolism*

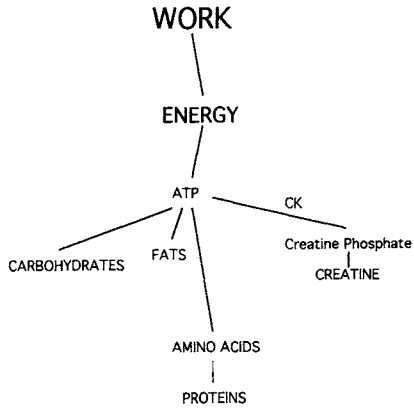
In the last section the uses of creatine phosphate as a therapeutic agent are introduced both for clinical and sports medicine application and in performance. This section finishes with a look towards future developments.

Appendix: *Assay for Creatine and Creatine Phosphate*

For the interested reader a detailed method for the assay of creatine phosphate and creatine has been summarized.

Preface

Creatine and creatine phosphate (CP, also called phosphocreatine, PCr) are important metabolites in high-energy phosphorus metabolism. They rank with dietary carbohydrates fats, proteins and other compounds as central components of the metabolic system involved in the provision of energy for work and exercise performance.



Over the past century numerous investigators have explored the basic biochemistry and in the past 20 years a whole science has developed around methods for measuring the molecules *in vivo* – proton and ^{31}P magnetic resonance spectroscopy. Some investigators have begun to examine the therapeutic role of the agents for the treatment of cardiovascular and other disease and in recent times much interest has developed in the potential of creatine (and PCr) for exercise and performance enhancement.

This book is designed to focus attention on creatine and creatine phosphate. The contributions have been invited from many of the key investigators in the field of high-energy phosphorus metabolism and, as such, represent an important distillation of the observations, ideas and theories which form the foundation for the exciting future of this science.

Each chapter represents the views of the individual authors and not necessarily those of the editors. Also, the book represents a reference and sourcebook and is not designed as a therapeutic manual. Hence, the administration of creatine and creatine phosphate must be performed according to the recommendations of the manufacturers and suppliers.

Michael A. Conway and Joseph F. Clark
1996

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Some of the work summarized in this book has depended on the efforts of several investigators and these are acknowledged at the end of the individual chapters where appropriate.

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1

Old and New Ideas on the Roles of Phosphagens and their Kinases

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Key words: ADP, phosphocreatine, muscle, energy kinetics

1. HISTORICAL INTRODUCTION TO PHOSPHAGEN KINASES

Lohmann is credited with the discovery of creatine kinase (CK) in 1934. He believed that the reaction of adenosine monophosphate (AMP) with two phosphocreatine (PCr) molecules gave adenosine triphosphate (ATP) and creatine (Cr) and that the observed orthophosphate (P_i) released from PCr in contracting muscle was due to ATP hydrolysis:

$$AMP + 2\,PCr \rightleftharpoons ATP + 2\,Cr$$
$$ATP \rightleftharpoons AMP + 2\,P_i$$

However, after the discovery of adenosine diphosphate (ADP), also by Lohmann in 1935, Lehmann (1936) described the now accepted CK reaction:

$$H^+ + PCr + ADP \rightleftharpoons Cr + ATP$$

Creatine kinase was first purified by Kuby *et al.* (1954) from rabbit muscle and its properties reported by Kuby and Noltman (1963).

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Meyerhof and Lohmann (1928) identified arginine phosphate in crab muscle and observed that, during contraction of iodoacetate-treated preparations its concentration decreased in a manner analogous to that demonstrated for PCr in vertebrate muscle (Lundsgaard, 1931). The arginine kinase reaction was demonstrated in crab muscle by Lohmann (1936) but, somewhat surprisingly, it was not reported from insect muscle until much later (Lewis and Fowler, 1962). Arginine kinase has been purified from a number of sources (e.g. Elodi and Szorenyim, 1956; Morrison *et al.*, 1957) and has been found to have a primary structure and properties very similar to those of CK.

The term phosphagen is used to describe those *N*-phosphorylguanidine derivatives which can transfer the *N*-phosphoryl group reversibly to ADP. Other phosphagens, and their kinases, have been identified from the muscles of annelids and echiuroids (Thoai *et al.*, 1953, 1963, 1972; Robin and Thoai, 1962).

In general, CK is characteristic of vertebrates and arginine kinase is characteristic of invertebrates. However, although arginine kinase has never been reported from vertebrates, CK is found in various invertebrate phyla (e.g. Porifera, Annelida, Echinodermata and Chordata) (Watts, 1971). In most cases a single species has one phosphagen but there are examples of more than one kinase occurring in some species of annelids and echinoderms. Phosphagens and their kinases are not restricted to muscle although it is in this tissue that their concentration is greatest. In vertebrates, phosphagens and their kinases are present in significant amounts in nervous tissues (Schmahl *et al.*, 1965) and to a lesser extent in liver, kidney, spleen, testis, and smooth muscle (Ennor and Rosenberg, 1952). However, liver, and spleen are richly vascularized and the phosphagen might be restricted to the muscular walls of blood vessels within these tissues. Phosphagen kinase activities have also been found in the gametes of invertebrates. In the echinoderm species, which possess both arginine and creatine kinases, CK occurs in the spermatozoa and arginine kinase in the eggs, whilst either may occur in the adult muscle (Moreland *et al.*, 1967). A similar situation appears to exist in the annelids, which have two phosphagens (arginine phosphate and creatine phosphate), but CK is always present in the spermatozoa (Thoai, 1968; Thoai and Robin, 1979).

The reason for the differences in distribution of the phosphagen kinases between invertebrates and vertebrates is still unclear. It is possible that the advantage of evolving a more complex and specific 'carrier' molecule for the 'energy-rich' phosphate group (that is, creatine rather than arginine) is related to the fact that arginine is used as an amino acid in processes other than protein synthesis. Thus, the use of the phosphagen to provide for regeneration of ATP means that the concentrations of arginine and creatine can change considerably in tissues that possess the phosphagen and therefore may be altered in the interstitial space. Since arginine has other roles in the body, such variations in concentration may have been disadvantageous in vertebrates.

One of these roles, which has been identified only recently, is the production of nitric oxide (NO) via the enzyme nitric oxide synthase as follows:

$$\text{arginine} \rightarrow \text{NO} + \text{citrulline}$$

Nitric oxide is known to have a number of important functions in the body – one of which is to control vasodilation via its production by endothelial cells. It is tempting to speculate that an increasingly important role of NO as a messenger molecule in vertebrates made it difficult for arginine phosphate to maintain its role as a phosphagen. Hence there was advantage in evolving phosphocreatine.

2. PHYSIOLOGICAL FUNCTIONS OF PHOSPHAGEN KINASES

A number of functions have been suggested for phosphagens and their kinases. It is generally presumed that different phosphagens will have the same function, whatever this might be. The possibility must be considered, however, that there is more than one role and that these functions could, in tissues with more than one phosphagen, be dependent upon the properties of various phosphagens. Furthermore, it is also possible that, in different tissues, phosphagens may have different physiological roles. Functions that have been proposed are considered below.

2.1. Fuel

Traditionally, phosphagens have been considered as a fuel available for the rapid rephosphorylation of ADP produced by the hydrolysis of ATP in processes such as muscle contraction. The significance of this role of phosphocreatine, for example to the athlete, is that, unlike ATP, it can be stored in the muscle, ready and available to rephosphorylate ADP in an instant! The quantities are small though greater than the ATP (enough in leg muscle for perhaps 5 s of sprinting) but it can be used very rapidly in sprinting races, while sprinting to the tape in longer races and when climbing hills during marathons and ultra-marathons. Lobsters move backwards amazingly fast by a "flick" of their tail. This flick is caused by the contraction of the abdominal muscles and, for the lobster, it represents an escape mechanism similar to the sprint in man. The fuel used to provide the ATP in the lobster is phosphoarginine. The abdominal muscles of the lobster have a very low capacity for anaerobic glycolysis but an enormous activity of the enzyme arginine kinase and at least four times more phosphoarginine compared to the concentration of phosphocreatine in human muscle. Indeed, it appears that *the lobster is totally dependent upon this fuel for its escape reaction.* Phosphoarginine (and phosphocreatine) stores generate ATP faster than glycolysis but the amount that

can be stored is strictly limited. It would be interesting to know if athletes who are very fast "off the mark" have a particularly high activity of CK (or [PCr]) for this "supercharging" boost. Does "speed off the mark" result from "lobster-like" energy generation?

Recent work from the laboratory of Eric Hultman in Stockholm has demonstrated that, in man, maximum sprinting can only be supported by using both glycogen and phosphocreatine simultaneously as fuels. This has led to considerable interest in providing creatine for athletes (see Newsholme *et al.*, 1994 and Chapter 13).

2.2. A buffer for ATP and ADP

Watts (1971) emphasized that ADP removal rather than ATP regeneration is the physiological function of phosphagens. They pointed out that ADP has an inhibitory effect on a variety of ATP-utilizing reactions so that any marked ADP increase could cause inhibition of the rate of vital processes in the body. This idea has been extended elsewhere (see Newsholme and Leech, 1983; Lewis and Fowler, 1962; Field *et al.*, 1994).

2.3. Transport of high-energy phosphate from mitochondrion to myofibril: the "phosphagen shuttle"

Hydrolysis of ATP occurs in the myofibrils, whilst ATP synthesis takes place within the mitochondria in aerobic tissues. Therefore, diffusion or specific transportation of ATP from its site of synthesis to the myofibril is required. Some propose that such a transport process involves CK (Gercken and Schlette, 1968; Nagle, 1970) and that PCr and Cr diffuse rather than ATP or ADP, respectively. Gercken and Schlette (1968) induced mechanical failure by perfusing rabbit hearts with fluorodinitrobenzene (FDNB). They found that failure occurred whilst the ATP content was high and presumed that this was due to compartmentation, i.e., the remaining ATP could not be used for contraction. Since the PCr content also decreased relatively little, they concluded that the CK system functions as an "energy-rich phosphate" transport or shuttle. The interpretation of experiments with FDNB is, however, complicated by the possibility that it will inhibit enzymes other than CK. It is known, for example, to be a powerful inhibitor of adenylate kinase (Mahowald *et al.*, 1962). Furthermore, a report by Newsholme *et al.* (1978) suggested, on the basis of measurement of maximum catalytic activation of phosphagen kinases from different muscles, that the activities were not sufficient to allow the "shuttles" provide all the ATP required by some aerobic muscles under *maximum* levels of work. Thus, the arginine kinase activities measured in the flight muscle of four insects were much lower than

the maximum calculated rates of ATP turnover during flight. Furthermore, when the CK activity in the direction of phosphocreatine formation was considered, the maximum activity in rat heart turned out to be lower than the calculated maximum rate of ATP turnover, and in hearts from other animals, the activities were similar to, or only slightly greater than, the maximum rate of ATP turnover. Since phosphagen kinase appears to catalyse a near-equilibrium muscle reaction (Beis and Newsholme, 1975), the net flux of "energy-rich" phosphate through the reaction, either at the myofibrillar or mitochondrial sites should be considerably less than the maximum activity *in vitro* of the enzyme (for theory, see Newsholme and Crabtree, 1973, 1976).

These results suggest that, in insect flight muscles, rat heart and possibly hearts from other animals, phosphagen kinase may not play a role in the transfer of "energy-rich" phosphate from mitochondria to myofibrils, at least under conditions of maximum rates of ATP turnover. It is possible that the role of PCr/Cr in the energy shuttle process depends upon the distances between the sites of ATP production and ATP utilization (see Chapter 5). With large distances as in spermatozoa, the creatine system functions as an important energy shuttle system (Juretschke and Kamp, 1990).

The role of phosphagens and phosphagen kinases in these aerobic muscles may be identical with that suggested for the more anaerobic muscles – it provides an energy reserve that decreases the extent of transient ATP and ADP concentration changes when the rate of ATP turnover is altered. However, the distribution of myocardial CK (i.e. in the intermembrane space and associated with the myofibrils) supports the role proposed by Watts (1971) that the phosphagen (and the kinase) prevents large transient changes in the ATP and ADP concentrations. This action may be important at the myofibrils since a large increase in the [ADP] could inhibit myofibrillar adenosine triphosphatase (Maruyama and Pringle, 1967). Also the phosphagen kinase present in the intermembrane space could prevent a large increase in the ATP concentration that might lead to inhibition of the adenine nucleotide translocase (see Klingenberg, 1970; Newsholme, 1976). Thus the role of the phosphagen kinase may be to provide stable ATP and ADP concentrations, particularly at certain sites within the muscle cell. This would minimize inhibition of key enzymes or enzyme systems that could result from large changes in the concentration ratio [ATP]/[ADP]. Accordingly what has been described as "kinetic efficiency" of metabolism should be maintained even during high rates of ATP turnover (Newsholme, 1976, 1977).

2.4. Inhibition of phosphofructokinase

Creatine phosphate inhibits phosphofructokinase, a regulatory enzyme in glycolysis (Uyeda and Racker, 1965; Krzanowski and Matschinsky, 1969).

Therefore, notwithstanding any other function, the decrease in PCr occurring during muscular activity could play a part in stimulating glycolysis at the onset of contraction. It may also play a similar role in control of pyruvate kinase activity.

2.5. Function in tissues other than muscle

The occurrence of phosphagen in tissues other than muscles suggests a more general function than that of ADP rephosphorylation. Jacobs *et al.* (1964) quote unpublished work suggesting a role in active transport and Ennor and Rosenberg (1952) speculate that "phosphocreatine has a function other than that of acting as a reservoir of readily available phosphate-bound energy for the adenylic acid system and that it may contribute energy to endergonic reactions directly and without the mediation of the latter system". Recently Hardin *et al.* found PCr utilization preferential over exogenous ATP in plasma membrane vesicles (1992).

3. USE OF CREATINE KINASE BY THE BIOCHEMIST AND PHYSIOLOGIST

There is good evidence that a large proportion of muscle ADP is bound to structural proteins and hence unavailable to ADP-utilizing enzymes such as CK. Thus ADP is bound structurally in actin so that it is not available for metabolism. But how much is bound and, therefore, how much is free? A problem arises since "classical" means of extracting muscle involves freeze-clamping and homogenization with perchloric acid: the latter releases the bound ADP so that it is measured along with the free ADP in subsequent assays. Thus, Seraydarian *et al.* (1962) found that approximately 80% of the ADP in frog sartorius muscle was not released using an extraction procedure that did not involve perchloric acid: they used an ethanol/citrate extraction medium. This work suggests that 20% of the total ADP was free and available to enzymes. Owing to doubts about the total ADP content and possible errors relating to the determination of bound ADP, it is possible that this concentration of free ADP is not accurate.

If one assumes that CK catalyses a near-equilibrium reaction and that the concentration of ATP, Cr, PCr and protons can be measured accurately, the free ADP concentration can be calculated from the CK equilibrium. The first evidence that CK catalyses near-equilibrium reactions was provided by Beis and Newsholme in 1975. This study provides the concentration of ATP, ADP, Cr (or arginine) and phosphocreatine (or phosphoarginine) in freeze-clamped muscle from different animals for use when calculating free ADP by the "crea-

Table 1.1. The free ADP concentration in some muscles based on the equilibrium catalysed by CK

Animal	Muscle	Total ADP (μmol/g)	Estimated free ADP (μmol/g)	Per cent free ADP (%)
Dogfish	White	0.73	0.282	38.6
Lizard	Abdominal	0.85	0.108	12.7
Frog	Gastrocnemius	0.56	0.032	5.7
Pigeon	Pectoral	1.02	0.86	8.4
Starling	Pectoral	1.15	0.051	4.4
Fowl	Pectoral	0.76	0.032	4.2
Mouse	Thigh	0.55	0.017	3.1
Rat	Thigh	0.72	0.023	3.2

The free ADP concentration was estimated using the creatine kinase equilibrium. The contents of ATP, ADP, PCr and creatine were obtained from Beis and Newsholme (1975).

tine kinase equilibrium method"; the level of free ADP was calculated from the data for eight different muscles. The results are presented in Table 1.1. The value varies from 3.1% to 38.6% of the total ADP.

4. THE PHOSPHOGLYCERATE KINASE–GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH) METHOD

If it is assumed that these two enzymes of glycolysis catalyse near-equilibrium reactions, then the free ADP concentration – that is, that concentration available to phosphoglycerate kinase – can be calculated using the following equation:

$$\left(\frac{NAD^+}{NADH}\right) = \frac{1}{K_{GAPDH} \times K_{3PGK}} \times \frac{[3\text{-phosphoglycerate}]}{[\text{glyceraldehyde-3-phosphate}]} \times \frac{[ADP]}{[ADP] \times [P_i]}$$

(3 PGK, 3 phosphoglycerate kinase; GAPDH, glyceraldehyde phosphate dehydrogenase)

Detailed systematic measurements of the 3-phosphoglycerate, glyceraldehyde-3-phosphate, ATP and P_i contents were made with the freeze-clamped body wall muscle of the pig roundworm. The cytoplasmic $NAD^+/NADH$ concentration ratio was also measured using the lactate dehydrogenase reaction (Williamson *et al.*, 1967). From these data, and this equation, the free ADP concentration in this muscle was determined. The results indicate that 0.28 mM is free ADP (the total ADP content is 0.8 mM).

4.1. Method for the determination of free ADP concentration based on the rate equation for pyruvate kinase

Both the above methods for determining the free ADP concentration involve enzymes that catalyse reactions near to equilibrium in the muscle. A different approach can be applied using the pyruvate kinase (PK) reaction. The available evidence suggests that this enzyme catalyses a non-equilibrium reaction in muscle (see Newsholme and Leech, 1983). The *in vivo* activity of PK in resting muscle is measured from the knowledge of glycolytic flux under these conditions. The PK activity is then calculated from knowledge of the *in vitro* kinetic properties of the enzyme and the measured contents of phosphoenolpyruvate (PEP), ADP and ATP in muscle at rest using the following rate equations:

$$v = \frac{V_{max}}{1 + \left(\frac{K_{PEP}}{[PEP]}\right) + \left(\frac{K_{ADP}}{[ADP]}\right) + \left(\frac{K_{PEP}K_{ADP}}{[PEP][ADP]}\right)\left(1 + \frac{[ADP]}{K_i}\right)}$$

In the three muscles investigated the calculated activities of PK are higher than the measured activities [derived from the knowledge of the glycolytic flux – since PK catalyses a non-equilibrium reaction its activity *in vivo* must be the same as the glycolytic flux (Newsholme and Leech, 1983)]. We assume that this difference stems from the fact that the ADP concentration used for the calculation of the estimated activity was obtained from the measured content of the whole cellular ADP. Therefore, the difference between the observed and expected muscle activities can be used to calculate the concentration of ADP available to pyruvate kinase, i.e. the free ADP. The calculation indicates that the free ADP concentration is 3% of the total in domestic fowl pectoral muscle and about 0.9% in the pheasant (Beis, 1973).

5. COMPARISON OF THE FREE ADP CONCENTRATION ESTIMATED BY DIFFERENT METHODS

The values obtained for the free ADP concentration in different muscles employing CK and PK for estimation are in reasonable agreement (see Table 1.2). However, there are only two muscles in which the two techniques can be compared. More results are required from a larger number of muscles in order to compare further the free ADP concentrations obtained from the two methods.

The method applied to the body wall muscles of the pig roundworm (i.e. a combination of the reactions catalysed by glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase) indicate that the free ADP

Table 1.2. Comparison of percentage of free ADP estimated by different methods

Animal	Muscle	Percentage of free ADP		
		Creatine kinase	GAPDH-3PGK	Pyruvate kinase
Round worm	Body wall	—	35	—
Frog	Gastrocnemius	5.7	—	—
Fowl	Pectoral	4.2	—	3
Pheasant	Pectoral	—	—	0.9
Pigeon	Pectoral	8.4	—	—

concentration is about 35% of the total ADP content. This estimation of the free ADP in this muscle is rather high compared with estimates obtained by the other method (see Table 1.2). A possible explanation could be that the structural proteins of this particular muscle bind less ADP than vertebrate muscle. Alternately, the values for the equilibrium constants employed in the calculations differ from those that apply to these reactions *in vivo*. The method needs to be applied to more muscles, particularly to those that have been used in the PK activity and CK methods, so that the results of all three methods can be compared in detail.

6. DETERMINATION OF THE AMOUNT OF ADP IRREVERSIBLY BOUND TO STRUCTURAL PROTEIN IN THE DOMESTIC FOWL PECTORAL MUSCLE

Muscle contains ADP in at least three different forms: irreversibly bound, reversibly bound and free. The methods described above (involving CK phosphoglycerate kinase and the technique based on the rate equation of pyruvate) estimate the free ADP concentration. However, another method has been developed to measure the amount of irreversibly bound ADP (i.e. the reversibly bound and the free ADP are removed prior to measurement of the bound ADP).

Preliminary experiments had established that powdered freeze-clamped muscle could be extracted at −15°C without any loss or gain of ADP or ATP (Newsholme and Taylor, 1970). If such an extract was incubated with an enzyme which could utilize ADP at this low temperature (such as PK) the free ADP and reversibly bound ADP would be removed. Thus incubation with PK and PEP results in ADP conversion to ATP. Any ADP remaining in the incubation mixture after a given incubation period could be described as irreversibly bound. In one method a piece of domestic fowl pectoral muscle was freeze-clamped and freeze-dried. The dried muscle was cut into very small

pieces and homogenized at −15°C with an extraction medium containing 16 mM triethanolamine, 1 mM ethylenediamine tetraacetic acid (EDTA), 6 mM $MgSO_4$, 30 mM KCl, 45% (w/v) glycerol at pH 7.5 (adjusted at 0°C). Immediately after homogenization, a volume of homogenate was transferred to a tube cooled to −15°C. A volume of PEP was added to give a final concentration of 3 mM and the enzymatic removal of ADP was initiated by addition of PK. Just before the addition of PK, a sample of homogenate was withdrawn and pipetted into an equal volume of 6% (w/v) perchloric acid, (PCA). After the addition of PK, samples were withdrawn at pre-determined intervals (usually at 1, 2, 5, 10, 15 and 30 min), pipetted into equal volumes of 6% (w/v) PCA and neutralized by 3 M $KHCO_3$ as soon as possible. The neutralized samples were then assayed for ADP. [Preliminary control experiments established that, under the conditions of the incubation, ADP added to the extract was converted stoichiometrically to ATP. Thus when ^{14}C-ADP was added to the extract at −15°C it was totally converted to ATP upon addition of PK. However, under these conditions only a very small amount of ^{14}C-ATP was converted to ADP (3–5%). (In these experiments ^{14}C-ATP and ^{14}C-ADP were separated by chromatography.)] The effect of pre-incubation at −15°C in the presence of PEP and PK on the content of ADP of the freeze-clamped, freeze-dried muscle is shown in Fig. 1.1. This content decreased to reach a plateau level after about 15–20 min of "pre-incubation". It was considered that this plateau represents the irreversibly bound ADP and that the proportion of total ADP bound in pectoral muscle is 60.5%. This value agrees reasonably with the results of Seraydarian et al. (1962), who estimated that about 80% of the total ADP in frog sartorius muscle is irreversibly bound to muscle structural proteins.

The significance of the finding that about 60% of the total ADP in some muscles may be irreversibly bound relates to the precise free ADP concentration in muscle and the question of the equilibrium between reversibly bound ADP and that which is free.

It is possible that phosphagens and phosphagen kinases have more than one role in muscle and perhaps other tissues. Arginine and creatine phosphate are probably important as fuels to replenish ATP in some if not most muscles. It is difficult to separate this role from that of buffering ATP and ADP concentrations, especially to prevent large changes in the latter which cause inhibition of key metabolic and contractile processes. The capacity of arginine or creatine kinase may not be sufficient for this system to function as an intracellular shuttle system for energy-rich phosphate when demand approaches maximum. If indeed the shuttle is important then one might ask why do some muscles possess it while others not – at least not at sufficiently active levels to satisfy maximum energetic requirements? Finally, the use of the CK equilibrium to calculate the free ADP concentration provides results that do not always agree with other calculations. The reasons for this raise further interesting questions.

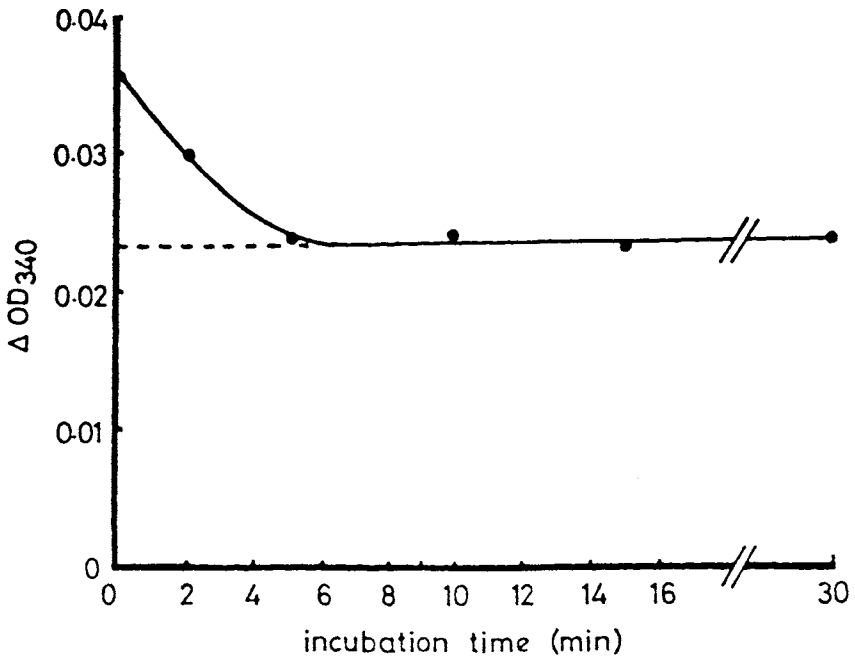

Figure 1.1 Determination of the irreversibly bound ADP. The percentage of the irreversibly bound ADP was estimated by extrapolating the linear portion of the graph to zero time. The zero time sample was taken to represent the total amount of ADP present in the muscle (ΔOD_{340}, change in optical density at 340). See text for details of the procedure.

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2

Creatine Phosphate Shuttle Pathway in Tissues with Dynamic Energy Demand

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1. CREATINE PHOSPHATE SHUTTLE HYPOTHESIS: CYTOSOLIC AND MITOCHONDRIAL CK SERVE AS COMPONENTS OF THE SHUTTLE

One of the central tenets regarding the regulation of mitochondrial oxidative metabolism is that ADP produced in the cytoplasm diffuses back into the mitochondria to stimulate the production of more ATP (i.e. respiratory control). Alternatively Jacobus and Lehninger (1973) proposed that ADP produced by high metabolic activity is immediately rephosphorylated to ATP through the action of creatine kinase (CK), using the phosphate from creatine phosphate (PCr). The respiratory signal to the mitochondria, in this

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hypothesis, is creatine. The creatine, produced at sites of high metabolic activity, diffuses back into the mitochondria to be rephosphorylated to PCr through the action of a different CK isoenzyme, the mitochondrial CK. The location of mitochondrial CK facilitates this reaction since it is bound to the outer surface of the inner mitochondrial membrane. Such a cycle, in which creatine and PCr serve as the diffusible intermediates connecting sites of energy utilization and energy generation (cellular ATPases and mitochondria, respectively), is referred to as the creatine phosphate shuttle (Bessman and Geiger, 1981; Bessman and Carpenter, 1985). Initial descriptions of the shuttle hypothesis were largely inferred from earlier studies showing the coupling of creatine as an acceptor for the phosphate group of ATP (Bessman, 1966, 1972) on the one hand, and the production of creatine by contracting muscle on the other. Experimental evidence from studies of isolated mitochondria (Jacobus and Lehninger, 1973) essentially demonstrated that all ATP produced in mitochondria, by oxidative metabolism, can be converted to PCr by the mitochondrial CK. The ADP produced at the expense of rephosphorylating creatine to PCr immediately becomes recycled into the mitochondria to be used for subsequent ATP production. Mitochondrial CK, purified to homogeneity (Roberts and Grace, 1980), was found to have slightly different enzyme kinetics from cytosolic CK (Basson *et al.*, 1985); mitochondrial CK favours ATP as substrate over PCr, while cytosolic CK favours PCr as substrate.

The shuttle hypothesis (Jacobus and Lehninger, 1973; Bessman and Geiger, 1981; Bessman and Carpenter, 1985) suggests that rephosphorylation of ADP to ATP at sites of energy demand involves "micro-compartments" of energy utilization, which are linked by the diffusion of creatine and PCr in mitochondria. There are several reasons why this energy shuttle system provides a more efficient supply of energy to cellular ATPases. Firstly, ADP and ATP do not diffuse freely within the cell (Baskin and Deamer, 1970) because they serve as the source of energy to all cellular ATPases (Basson *et al.*, 1985). As key regulatory compounds for many other metabolic enzymes, they bind to many proteins. In contrast, creatine and PCr bind only to CK within cells. The second reason is that ATP and ADP are bulkier and more highly charged molecules, when compared with creatine and PCr. Finally, through this shuttle process, ADP is rapidly removed from the micro-environment of the cellular ATPases, reducing the end-product inhibition produced by increasing concentrations of ADP, as rapid rephosphorylation of ADP to ATP occurs at the expense of PCr.

Components of the energy shuttle include "soluble" cytoplasmic CK isozymes and mitochondrial CK isozymes. The two soluble isoforms, MM-CK and BB-CK (Eppenberger *et al.*, 1967), are highly concentrated in muscle and brain tissue, respectively. Also, two different mitochondrial isoforms have been identified (Jacobs *et al.*, 1964; Jacobus and Lehninger, 1973; Roberts and Grace, 1980; Grace *et al.*, 1983) and shown to be encoded by separate nuclear genes (Hall and DeLuca, 1975; Perryman *et al.*, 1986; Villarreal-Levy *et al.*, 1987; Hossle *et al.*, 1988; Haas *et al.*, 1989, Klein *et al.*, 1991). The isoform

MB-CK exists primarily in cardiac muscle and to a much lesser extent in developing or regenerating skeletal muscle (Watts, 1973). In addition to their separate localization within the cytoplasm and the mitochondria, there is now ample evidence for tissue-specific and subcellular compartmentalization of the cytoplasmic ("soluble") CK isozymes that further supports the functional significance of a creatine phosphate shuttle.

2. CK IN SPECIALIZED CELLS: BIOCHEMICAL, PHYSIOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION

2.1. CNS neurons

Neurons of the central and peripheral nervous system are morphologically polarized cells, being highly specialized for the reception and transmission of (synaptic) information at the axon and dendrites, respectively. We have found that B-CK and mitochondrial creatine kinase are co-expressed in the same Golgi Type I (large) neurons, and not by the vast majority of central nervous system (CNS) interneurons and glia (Friedman and Roberts, 1994). Furthermore, these two isoforms of CK are expressed at morphologically distinct parts of the cell. The mitochondrial isoform of CK is localized primarily in the cell body of all Golgi Type I neurons. In contrast, B-CK mainly localizes within the cell processes, and to a lesser extent within the cell body. The distinct spatial arrangement of CK isoforms occurs throughout the brain including cerebral cortex, hippocampus, cerebellum and brainstem. Thus, the creatine phosphate shuttle of brain neurons serves to transfer metabolic energy from mitochondria enriched in the cell body to the cell regeneration processes of the ATP consumed during synaptic transmission (Friedman and Roberts, 1994). We have also shown that brain tissue expresses trace amounts of M-CK (Hamburg *et al.*, 1990).

2.2. Photoreceptors

Photoreceptor cells are among the most morphologically polarized cells. They subdivide into four distinct segments, each with different metabolic functions. The phototransduction events of the photic cycle occur in the outer segments. At the opposite end of the cell the highly elaborated nerve terminals synapse onto second-order neurons, between these two regions. The inner segments are packed with mitochondria and thus serve as the ATP-generating sites within the photoreceptors. In the retina of the chick, two different isoforms of CK are found which are especially concentrated (Wallimann *et al.*, 1986a) in photoreceptor cells. The energy produced within mitochondria of the inner segments

is converted to PCr, which then diffuses into the outer segments to regenerate ATP for visual cycle events (i.e. regeneration of cGMP and phosphorylation reactions). The creatine phosphate shuttle then serves to supply energy from the inner to the outer segments and nerve terminals for the rapid regeneration of ATP at the point of energy consumption. These results have been confirmed and extended by studies of bovine retina (Hemmer *et al.*, 1993). Using specific antisera and light and electron microscopy, two distinct isoforms of CK (BB-CK and mitochondrial CK) were shown to be separately compartmentalized in the outer and inner segments of the photoreceptors. Furthermore, the ATP-generating potential of the CK system was sufficiently large to regenerate the entire ATP pool during a photic cycle (Hemmer *et al.*, 1993).

2.3. Excretory epithelia

Creatine kinase immunoreactivity is most intense in the inner stripe of the outer medulla of the kidney. Both mitochondrial and BB-CK isoforms are specifically localized to the distal nephron, especially in the thick ascending limb. The pattern of renal expression of CK correlates with the region of greatest oxygen consumption, ATP utilization and sodium transport (Friedman and Perryman, 1991). In the very specialized sodium excretory (rectal) gland of the shark, there are also high levels of expression of multiple CK isoforms and PCr (Epstein *et al.*, 1981; Friedman and Roberts, 1992). Stimulation of this excretory epithelial gland with cAMP causes PCr levels to rapidly decrease with no change in the levels of ATP (Epstein *et al.*, 1983). Both the mitochondrial CK and BB-CK are expressed at high levels, equivalent to that measured in mammalian myocardium. Localization of the two isoforms is restricted to the basal but not the apical aspect of the tubules, consistent with the energy demands within such structures (Friedman and Roberts, 1994).

2.4. Intestinal epithelia

Intestinal epithelial cells are another example of polarized cells which express multiple CK isoforms. The brush border and the underlying supporting terminal web control the process of nutrient absorption, and contain structural proteins including myosins and actins (Mooseker, 1985). Mitochondria are excluded from the brush border. Immunological studies with specific antibodies show localization of BB-CK to the terminal web and within the cytoplasm (Keller and Gordon, 1991). Terminal web localization of BB-CK is retained in glycerol-permeabilized cells, suggesting that the CK may be structurally bound at this location. Since mitochondria are excluded from the brush border, the discrete subcellular localization of the CK on the one hand, and mitochondrial CK on the other, suggest the presence and functional signifi-

cance of an intestinal epithelial cell creatine phosphate shuttle. *In vitro* studies support the shuttle hypothesis and demonstrate that, at the brush border, locally CK generated ATP with PCr as substrate, is used selectively for myosin filament shortening at the same location (Gordon and Keller, 1992).

2.5. Cardiac and skeletal muscle

Myocardial gene expression of CK is developmentally regulated (Lamers *et al.*, 1989; Hasselbaink *et al.*, 1990). In the mouse BB-CK alone is expressed during prenatal development, until the last week, when MM-CK increases dramatically whilst the BB-CK isoform decreases concomitantly. Associated with the co-expression of M-CK and B-CK gene peripartum, and during the first 3 weeks postnatally, there is up to a maximum of 35% MB-CK activity (Hall and DeLuca, 1975; Ingwall, 1991). The mitochondrial isoform does not get expressed until several days postnatally (Hall and DeLuca, 1975; Ingwall, 1991). In several diseased states with altered cardiac function, alteration of CK gene expression also occurs. In pressure overload or volume overload induced cardiac hypertrophy of both humans (Jansson *et al.*, 1987; Ingwall, 1991) and experimental animals (Meerson and Javich, 1982; Younes *et al.*, 1984; Schuyler and Yarbrough, 1990; Field *et al.*, 1992), alteration of the CK isozyme expression develops. In the experimental diabetic rat, both M-CK and B-CK genes are downregulated, and in response to insulin, are upregulated (Popovich *et al.*, 1989, 1991). Doxorubicin, a widely used chemotherapeutic agent, produces a characteristic cardiomyopathy. Doxorubicin treated experimental animals produces selective inhibition of cardiac-specific genes including M-CK, which precedes ultrastructural changes (Ito *et al.*, 1990). Ischaemia also produces a reduction of the M-CK message (Mehta *et al.*, 1988). Human heart tissue (Ma *et al.*, 1995) and animal model studies indicate (Fontanet *et al.*, 1991) that different isoforms of CK may be concordantly regulated. Depleting the PCr pool by feeding a creatine analogue (Kapelko *et al.*, 1988; Zweier *et al.*, 1991), or by selective CK poisoning using iodoacetamide (Fossel and Hoefeler, 1987), decreases the capacity of the heart to perform stroke work. These results collectively support the hypothesis that energy reserve contributes to myocardial contractile reserve in the heart.

The role of the skeletal muscle CK system has been studied using both animals fed the creatine analogue β-GPA and the M-CK-deficient mouse produced by homologous recombination (van Deursen *et al.*, 1993; see below and Chapter 6). β-GPA inhibits creatine uptake such that the cellular creatine and PCr are reduced 10-fold (Shoubridge *et al.*, 1984; Meyer *et al.*, 1986). Both animal studies showed unaltered initial isometric twitch but they were accompanied by rapid deterioration of contractile tension. Furthermore muscle endurance performance was improved compared with control. Such findings indicate the power of cellular adaptation when the PCr/CK system is experimentally altered.

2.6. Spermatozoa

Spermatozoa are highly polarized cells with a large head and long and slender tail. The latter is the motor unit for locomotion and requires a high and constant energy expenditure. Two CK isoforms are spatially separated in spermatozoa (Wallimann *et al.*, 1986b; Tombes and Shapiro, 1987; Tombes *et al.*, 1987). The more apical form is the mitochondrial CK whereas the distal form is cytosolic CK (Wothe *et al.*, 1990; Wallimann *et al.*, 1992). The tail movement uses PCr as an energy source (Tombes and Shapiro, 1985) and can be inhibited by the CK inhibitor, fluorodinitrobenzene (Tombes *et al.*, 1987). These observations suggest that the creatine phosphate shuttle is responsible for the transfer of energy from the mitochondria of the sperm head along the tail to the distal end.

3. SUBCELLULAR LOCALIZATION OF CK

3.1 Myofibrils

Biochemical and morphological studies support the notion of a direct association of MM-CK with the contractile apparatus of skeletal and cardiac muscle. Many investigators have demonstrated the co-purification of CK with myofibrils (Ottaway, 1967; Wallimann *et al.*, 1977a,b). This association is reversible, as shown by the binding of CK to purified contractile proteins (Roberts and Grace, 1980; Elizarova *et al.*, 1987). Histological evidence that both CK activity (Sharov *et al.*, 1977) and immunoreactivity (Wallimann *et al.*, 1977a,b) are localized to myofibrils provides additional support for the specific association of CK with the contractile apparatus. Myofibrilar MM-CK associates specifically at the M-line (Wallimann *et al.*, 1977a,b).

Functional association of CK with muscle contraction suggests a close association of PCr levels with contractile function. The inhibition of CK *in vivo* with dinitrofluorobenzene produces rapid cessation of muscle contraction and a 50% reduction in ATP levels (Infante and Davies, 1965). During studies in which exercising muscle was fatigued or deprived of oxygen, ATP levels declined gradually whereas rapidly declining PCr levels correlated more closely with the loss of contractile function (Saks *et al.*, 1975, 1976).

3.2. Sarcoplasmic reticulum and plasma membrane

In addition to the association of CK with the contractile apparatus, electron microscopic histochemical studies also identify CK activity localized at the

sarcoplasmic reticulum (SR) and plasma membranes, where CK may serve to regenerate the energy necessary for ion transport (Levitski *et al.*, 1977; Sharov *et al.*, 1977). The co-purification of particulate CK activity with an SR membrane fraction provided the initial suggestion of a link between CK and calcium transport (Baskin and Deamer, 1970). The functional coupling of CK with ion transport was demonstrated by Levitski *et al.* (1977) in isolated heart SR. They found that significant amounts of MM-CK co-purify with SR vesicles, and that exogenous PCr is hydrolysed to creatine at a rate proportional to calcium pump activity. The maximal rate of calcium accumulation was greater with assay mixtures containing PCr and ADP alone as compared with the addition of equivalent amounts of ATP and a regenerating system of phosphoenolpyruvate (PEP) and pyruvate kinase (Levitski *et al.*, 1977). Essentially they obtained identical results using a highly purified SR preparation from chicken breast muscle, where exogenous PCr and ADP alone sustained a significant proportion (25%) of the maximal calcium transport activity, which was further increased (80%) with the introduction of CK to the uptake assay (Rossi *et al.*, 1990). Additionally, ATP regenerated by endogenous CK associated with SR vesicles was not in free equilibrium with the surrounding medium, but was used preferentially by Ca-ATPase for calcium uptake, indicating a functional coupling between SR-bound CK and Ca-ATPase (Korge *et al.*, 1993). The localization of CK activity at the plasma membrane of heart cells (Sharov *et al.*, 1977) suggests the association of CK with active sodium transport. Saks *et al.* (1977) subsequently showed that creatine production (from PCr) is tightly coupled to sodium pump activity using purified heart plasma membrane fractions. These results support a role for CK in the coupling of energy production with ion transport.

4. STRUCTURE–FUNCTION ANALYSIS OF CK

4.1. Catalytic and substrate binding domain

Studies with classical inhibitors identify CK as a thiol enzyme. Loss of catalytic activity paralleled closely the alkylation of a reactive cysteine side chain by iodoacetamide (Watts, 1973). The thiol group is thought to be cysteine-283 as evidenced by the reaction of CK with the substrate analogue epoxycreatine which shows affinity labelling at cysteine-283 (Buechter *et al.*, 1992). As well as cysteine, other amino acids are implicated at the catalytic site, including histidine, lysine and arginine residues.

Kinetic studies suggest separate but synergistic binding of MgATP and creatine. Recently, using site-directed mutagenesis, Lin *et al.*, (1994) and others (Furter *et al.*, 1993) tried evaluating further the role of cysteine-283. In Lin *et al.*'s study of mitochondrial CK expressed by the *Escherichia coli* system,

substitution of the cysteine residue drastically decreased enzyme activity. Some mutants, however, showed considerable activity when assayed in a modified system with low pH, low ADP, and high KCl. This suggests that cysteine-283, though important for optimal enzymatic activity, is not essential for enzyme catalysis. In contrast, using a COS cell expression system, we have concluded that cysteine-283 is essential for enzyme catalysis, and that arginine-292, with its positively charged side chain, is important for optimal enzyme activity. Previously, using affinity labelling, it was suggested that an aspartate residue may adjoin the substrate binding site, and may bind ATP (James *et al.*, 1990). We mutated this residue by site-directed mutagenesis (Lin *et al.*, 1994), but found the resultant mutant to have unchanged enzyme activity. This study also produced evidence for subunit interaction in CK such that a mutant monomer, devoid of intrinsic enzyme activity inhibited the dimeric enzyme when forming a heterodimer with a wild-type subunit. A model of dimerization has been proposed where the individual catalytic sites of the monomer are closely opposed in the dimeric structure.

4.2. Dimerization domain

The cytosolic MM-CK, BB-CK and MB-CK isozymes exist dimerically whereas the mitochondrial CKs exist both as a dimer and octamer. The cytosolic CKs isolated from different species retain the ability to form heterodimers under laboratory conditions (Watts, 1973), while *in vivo* there is no evidence that the monomeric CK subunit associates with other protein molecules. This suggests that the dimerization domain of CK is evolutionarily conserved. On the other hand the cytosolic and mitochondrial subunits do not dimerize with each other *in vivo*. Both the cytosolic and mitochondrial dimers are very stable and dissociate into the monomers only with denaturing agents. The mitochondrial octamer, however, can dissociate into dimers upon modification of the medium and represents the form of the enzyme which binds to the mitoplast (Meerson and Javich, 1982) and appears to be the functional *in vivo* quaternary structure of mitochondrial CK (Schlegel *et al.*, 1988; Wallimann *et al.*, 1992). The dimerization domain of the CKs may be important to the understanding of CK enzyme function and is an important area for future characterization.

4.3. Subcellular localization domain

Compartmentalization of cytoplasmic CK implies structural localization of the CK molecule and, indirectly, the presence of subcellular localization domains. The C-terminal half of MM-CK contains the epitope(s) for binding of the enzyme to the M-band in muscle (Schafer and Perriard, 1988). Whether

a specific domain exists for sarcoplasmic reticulum localization or association with other sarcoplasmic reticulum proteins has not been established. Recently, Rojo *et al.* (1991) showed association of the CK to model membrane preparation. The mitochondrial CK interacts with a phospholipid, cardiolipin of the inner mitochondrial membrane (Ottaway, 1967; Schlame and Augustin, 1985; Cheneval and Carafoli, 1988), and with a model phospholipid membrane (Rojo *et al.*, 1991). Further delineation of the subcellular localization domain is important to the understanding of the creatine phosphate shuttle and awaits analysis by site-directed mutagenesis through domain-deletion or domain-switching experiments.

5. TARGETED MODIFICATION OF CK

5.1. Transgenic animal model

Over-expression of B-CK by transgenic mice has been reported (Brosnan *et al.*, 1993). Under the control of a skeletal muscle actin promoter, the transgene was expressed and produced MB-CK (22–32%), which is normally absent in adult skeletal muscle. Over-expression of CK, however, did not alter ATP, creatine phosphate, or creatine levels, supporting the generally held belief that CK-catalysed skeletal muscle reactions are normally in equilibrium. A transgenic model with liver-specific expression of B-CK has also been reported (Brosnan *et al.*, 1990). The hepatocytes of the liver do not normally express any detectable CK.

5.2. Gene targeting

The gene targeting experiment of M-CK, using the technique of homologous recombination, created a strain of mouse which does not make M-CK mRNA or protein. No developmental abnormalities appear to develop. However, some parameters of contractile function such as "burst activity" are different amongst the mutant compared to the parental strain. The functional significance of this finding remains speculative. The important findings of this study are (1) PCr and ATP levels remained normal, and (2) active muscle hydrolysis continued to display PCr. This suggests that the PCr and ATP are close to an equilibrium level in the absence of MM-CK and furthermore it raises the interesting possibility that an alternative acceptor to MM-CK for muscle PCr. Interpretation of results from this study is limited by the absence of a quantitative analysis of the different isoforms of CK in order to evaluate completely the role of compensatory regulation of the other CK isoforms in the mutant strain.

5.3. Dominant negative modulation

Dominant negative modulation of gene systems, where the expression of a gene is inhibited through the interaction with a second factor, can be achieved at several levels; the transcription of messenger RNA, the translation level of mature message, or directly at the protein level. Antisense RNA is a proven modulating gene expression (Green *et al.*, 1986). However, the construct necessary to block the expression of the target gene is unpredictable and requires a trial and error approach to achieve the desired result. For B-CK, however, some investigations report that antisense RNA complementary to 3′ coding and non-coding regions blocks expression of the mRNA by inhibiting the translation (Chang *et al.*, 1989). The direct functional effects of this approach have not yet been studied.

We used site-directed mutagenesis to study the structure–function relationships of the CK enzyme (Lin *et al.*, 1994). In addition to finding that mutations at cysteine-283 or arginine-292 abolished the CK enzyme activity, the mutant monomer was found to inhibit enzyme activity of the wild-type monomer upon dimerization. This provides a new approach of dominant negative modulation of the CK isozyme at the protein level. With single amino acid replacement at the cysteine-283 or the arginine-292 site, the mutant heterodimer can be reduced to 25% of the activity of the wild-type heterodimer (Lin *et al.*, 1994).

6. FUTURE APPLICATIONS

There is ample ultrastructural, biochemical as well as physiological experimental evidence for the presence of the creatine phosphate shuttle pathway. It is also clear that during organ development and in certain disease states, the alteration of CK isoform expression occurs. The question, therefore, is no longer whether there is a functional role for CK in cell metabolism, but to what extent the compartmentalization of CK isozymes is important in the homeostasis of the specialized cells and in response to environmental cues. This question can be investigated by several of the newly developed recombinant DNA technologies, including "gene targeting" and "dominant negative modulation".

The homologous recombination experiment using M-CK demonstrates the ability of the mutant animal to adapt to this gene ablation by compensatory mechanisms, particularly by an increased mitochondrial mass and oxidative phosphorylation capacity. The functional changes of the myocardium have not yet been determined. It would be informative to examine the cardiac contractile function on the one hand and SR function on the other, since these depend principally on oxidative metabolism for energy and where compartmentalized CK is thought to have an important role for energy transfer.

Future gene targeting experiments may be directed toward a tissue-specific or development-specific regulatory element in order to assess more clearly the functional role of CK/PCr shuttle pathway of cardiac and other specialized tissues.

The finding that antisense RNA complementary to 3′ coding and non-coding sequences of CK potently inhibit translation *in vivo* (Chang *et al.*, 1989) is the first report to give support for a dominant negative modulation of CK at the message level. Our finding that the expression of a mutant CK monomer can inhibit the CK enzyme system produces another *in vivo* means of dominant negative modulation of this enzyme system. Using such a novel approach, it is theoretically possible to produce a mutant monomer that can selectively annihilate the CK enzyme system. If expression of the mutant monomer occurs under the control of a tissue-specific and/or development-specific promoter/enhancer, the functional role of a specific CK isozyme, such as MB-CK, in conditions such as pressure overload induced cardiac hypertrophy, can be explored.

7. SUMMARY

All cells use ATP as the immediate energy source. Cellular ATP is generated by oxidative metabolism and by glycolysis. Many specialized cells in an organism require fast and constant energy utilization to maintain specialized cellular function. However, most cellular ATPases utilize ATP and therefore rapid accumulation of end-product ADP and AMP will significantly alter the equilibrium kinetics around the cellular ATPase; it may be myosin ATPase, sarcolemmal ion-stimulated ATPase, SR calcium ATPase, or other regulatory ATPases. Compartmentalized isoforms of CK provide an adaptive mechanism which can rephosphorylate ADP to ATP, using creatine phosphate, thus maintaining the free ADP concentration at an appropriately low level. Another compartmentalized mitochondrial CK at the site of mitochondrial ATP generation catalyses the phosphorylation of creatine to creatine phosphate, thus completing the two components of a creatine phosphate shuttle pathway. It is now evident that many cells with constant and rapid energy demand have a compartmentalized CK system. New recombinant DNA methodology such as "site-directed mutagenesis" will facilitate the dissection of the CK molecule and demonstrate the molecular mechanism through which the CKs catalyse the enzymatic reaction as well as delineate the various features of the enzyme molecule which targets it to regions of diverse cellular ATPases. Furthermore future technologies such as "gene targeting", "transgenic models" and "dominant negative modulation" will provide the opportunity to alter the cellular CK level at selected tissue sites and at selected developmental or experimental time points. Understanding the contribution of the creatine phosphate shuttle pathway in cellular

homeostasis under conditions of stress, such as decreased energy supply (e.g. cardiac ischaemia) or increased energy demand (e.g. pressure or volume overload induced cardiac hypertrophy), will perhaps provide new insights and approaches for the management of diverse disease states.

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3

Experimental Observations of Creatine Phosphate and Creatine Metabolism

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Key words: heart, artery, creatine kinase, skeletal muscle

1. INTRODUCTION

Creatine (Cr) and creatine phosphate (PCr) metabolism have been studied by numerous groups over the past 20 years. In this chapter we present a brief summary of the current understanding of PCr metabolism using ^{31}P NMR analysis in experimental preparations of heart, skeletal and smooth muscle as well as cell culture techniques for measuring creatine transport.

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2. NMR STUDIES OF ATP AND CREATINE PHOSPHATE IN THE HEART

The heart uses most of its store of ATP for myofibrillar contraction and for the maintenance of ionic homeostasis. About 2% of cellular ATP is consumed in each heart beat in the rat (Jacobus, 1985), which means that the total stores of ATP would last only a few seconds in the absence of ATP resynthesis and regeneration. Under physiological conditions over 90% of the myocardial ATP is synthesized by mitochondrial oxidative phosphorylation (Harris and Das, 1991).

During cardiac stimulation, where the workload was increased five-fold, Jennings *et al.* (1981) showed that the ATP levels remain stable at 5 μmol/g wet wt. This indicates that under steady-state conditions, the rate of ATP synthesis and consumption are closely matched. ATP is directly synthesized from ADP and orthophosphate (P_i) in the mitochondria, but small or transient variations of cytoplasmic ATP are buffered by creatine kinase (CK) (as discussed elsewhere in this text and below). These observations derive from direct ^{31}P NMR measurements of ATP, P_i, pH and PCr, whereas the ADP levels are calculated using the equilibrium constant for CK.

In isolated heart mitochondria, the rate of respiration can be controlled by varying the concentration of ADP (Jacobus *et al.*, 1982). The concentration of free ADP *in vivo* is between 20 and 60 μmol/kg (Katz *et al.*, 1989) and near the K_m (LaNoue *et al.*, 1985; Heinemann and Balaban, 1990), and thus a doubling in [ADP] would significantly increase the rate of respiration. Adenosine diphosphate has been shown to be involved in the control of respiration in ^{31}P magnetic resonance spectroscopy (MRS) studies of normal human subjects (Radda, 1986). Orthophosphate also possibly regulates respiration (Harris and Das, 1991), as does the phosphorylation potential (the ratio of ATP over ADP+P_i). Both of these factors vary concomitantly with [ADP]. Seymour *et al.* (1987) have shown that increased perfusion pressure in isolated hearts can alter [ADP] when glucose or pyruvate are used as substrates. This does not occur when lactate or acetate are used as substrates (Seymour *et al.*, 1987), indicating that ADP is not the sole regulator of cardiac function. Balaban *et al.* (1986) report that the PCr/ATP ratio does not change with a five-fold increase in cardiac work during *in vivo* ^{31}P MRS studies (Fig. 3.1). If we assume that the total creatine pool and the intracellular pH remain constant with the increased work, the concentration of free ADP can also be considered constant. In support of these observations, a stable PCr/ATP ratio in normal human heart has also been reported with isometric and isotonic exercise (Conway *et al.*, 1988; Weiss *et al.*, 1990).

What then regulates ATP synthesis? One possibility is an increased delivery of substrates and subsequent changes in the NADH/NAD redox state. This is supported by some experimental data (Hansford, 1985) but not by others (Moreno-Sanchez *et al.*, 1990). Another possibility is activation of the mito-

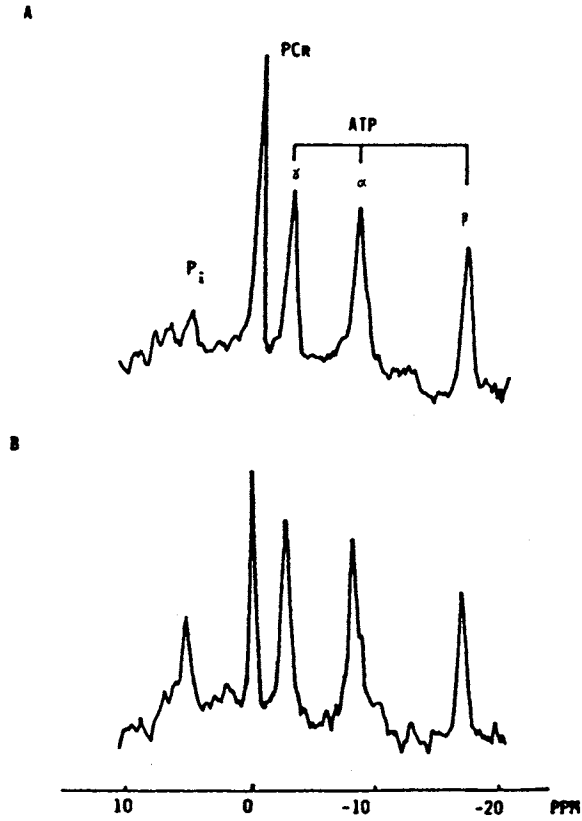

Figure 3.1. ^{31}P NMR spectra of isolated perfused rat hearts perfused with glucose at an isometric tension of 70 cm H_2O perfusion pressure. Spectrum A is from a control rat and spectrum B is from a hypertrophic rat. Hypertrophy was induced by hyperthyroidism. Spectra were collected with 200 transients using a 7 s delay and a 90° pulse width. As can be seen by comparing the PCr and ATP peaks, the hypertrophic heart has a lower PCr:ATP ratio. Reprinted from Seymour *et al.* (1990), with permission.

chondrial dehydrogenases by calcium (McCormack *et al.*, 1990) or of the ATP synthase itself (Harris and Das, 1991). Hearts perfused with Ruthenium Red (an inhibitor of mitochondrial calcium uptake) are incapable of maintaining constant ATP levels after isoprenaline stimulation (Unitt *et al.*, 1989). However, when calcium is used for stimulating respiration in isolated mitochondria, the observed increase in oxygen consumption depends upon the type and concentration of substrate (Denton *et al.*, 1979).

As discussed by Saks *et al.* (1991, 1993; see also Chapter 5), ADP amplification via mitochondrial creatine kinase represents another likely control mechanism. Amplification of transient changes must also be considered. It has been

shown that transient alterations in the PCr/ATP ratio take place during the first minute after increasing the workload (Paternostro *et al.*, 1992) and in hypertrophic hearts (which have a reduced PCr content) this change is accompanied by a decrease in [ATP].

Studies on immature lambs *in vivo* have shown that cardiac PCr, ADP and P_i vary with work (Portman *et al.*, 1989). This raises the possibility that the heart might regulate ATP synthesis with different strategies under various conditions. Such a phenomenon may be particularly relevant under pathological conditions.

3. CREATINE PHOSPHATE METABOLISM INVESTIGATED WITH β-GUANIDINOPROPIONIC ACID

3.1. Introduction

The role of the large amounts of CK and phosphocreatine in skeletal and cardiac muscle energy metabolism is unclear. The classical view of muscle energetics suggests that the CK reaction operates near equilibrium and functions to provide an energy store (or "buffer") via CK (Wallimann *et al.*, 1992).

Some investigators propose, however, that PCr may act as an energy shuttle between sites of production and utilization. Such a hypothesis (see Chapters 2, 4 and 5) is based in part on the observation that CK exists in the form of isoenzymes which are specifically associated with structures such as mitochondria and myofibrils. Functional interactions between ATP–ADP translocase and mitochondrial CK, and myosin ATPase and cytosolic CK, have been proposed to explain the role of these enzymes and molecules (Wallimann *et al.*, 1992). Low creatine and PCr are indeed found in various types of muscle pathologies such as muscular dystrophy and cardiac hypertrophy, but the causal relationship has not been determined. It appears possible therefore to relate deficiencies in these compounds to the compromised function of muscle, thereby contributing to the understanding of disease states.

3.2. β-GPA feeding

In an attempt to develop an animal model that might identify biochemical changes and pathology associated with creatine and PCr depletion, Chevli and Fitch fed rats an analogue of creatine: β-guanidinopropionic acid (β-GPA) (Fitch *et al.*, 1968b, 1974; Chevli and Fitch, 1979). This agent has been used to compete with creatine for entry into the skeletal muscle cells (1% of their diet w/w) as a means to study creatine transport (Fitch *et al.*, 1968b). Further work

by Shields and Whitehair (1973) showed that rats receiving diets containing β-GPA had abnormal creatine metabolism, with chronically reduced levels of muscle creatine combined with increased levels of excreted creatinine. Fitch *et al.* (1974) demonstrated that β-GPA feeding resulted in a decrease in muscle PCr with concomitant replacement by the phosphorylated analogue, β-GPA-P. Muscles from animals fed β-GPA were found to have decreased [β-GPA-P] during hypoxic contraction indicating that, like PCr, β-GPA-P could serve with CK in a system to regenerate ATP (Fitch *et al.*, 1974). β-GPA-P substitution led to a 1.5-fold increase in [lactate] and the use of high-energy phosphates was reduced by 32% (Fitch *et al.*, 1975). Both β-GPA and β-GPA-P were further shown to be poor substrates for CK, with an increased K_m and reduced V_{max} (Chevli and Fitch, 1979).

Shoubridge *et al.* (1985a,b) performed studies on both skeletal and cardiac muscle, using β-GPA. Rats fed β-GPA for 6–10 weeks showed a 90% reduction of [PCr] in both muscle types. Skeletal muscle [ATP] was also reduced by 50%. Adaptations were seen in skeletal muscle with Type II muscle fibres demonstrating a decrease in diffusion distances, increased aerobic capacity and decreased glycolytic potential. Some of these changes are discussed in more detail in Chapter 6.

Cardiac muscle showed no enzyme changes or variations in performance criteria following chronic β-GPA feeding, even though a four-fold decrease in flux through the CK reaction was seen. β-GPA feeding for 6–8 weeks by Kapelko *et al.* (1989) led to a decrease in left-ventricular (LV) pressure, lower maximal coronary flow at maximal resistance, and a 32–40% decrease in maximum oxygen consumption. Cardiac failure resulted with an associated increase in LV diastolic end-pressure. This effect depended on the workload and on the extent of PCr replacement. Creatine phosphate was thus identified as important for cardiac contractile function and Zweier *et al.* (1991) propose that the mechanism may relate to optimizing the thermodynamics of high-energy phosphate utilization.

Although chronic β-GPA administration does significantly lower skeletal muscle Cr and PCr (see Table 6.1), a number of cellular modifications result from such interventions which cast doubt on the usefulness of this strategy for investigating the effects of just PCr and Cr depletion. In Type II muscle, β-GPA administration caused a decrease in fibre diameter, increased citrate synthase activity and reduced glycogen phosphorylase activity (Shields *et al.*, 1975). The soleus muscle, which is predominantly an oxidative type muscle, showed increased Type I fibres (Moerland *et al.*, 1989). Recent studies on cardiac muscle after 13–19 weeks of β-GPA feeding exhibit impaired mechanical performance, a decreased rate of cross-bridge cycling, changed myosin isozyme expression from fast V1 to slower V2 and V3, as well as Bi-ventricular hypertrophy (Mekhfi *et al.*, 1990). These changes lead to an improved economy of cardiac contraction, but are not sufficient to maintain maximal force. The changes seen with β-GPA administration may reflect adaptive

responses to β-GPA, and not just to depletion of creatine and/or PCr. Heart muscle mitochondria were not, however, able to adapt and phosphorylate β-GPA. Indeed, Clark *et al.* (1994) found that mitochondrial CK cannot phosphorylate β-GPA under all experimental conditions studied.

3.3. Acute β-GPA administration

Chronic administration of β-GPA appears to create a number of technical problems, including conflicting results from different groups, and possible adaptive and pathological responses that may or may not be directly related to the depletion of Cr and PCr. Comparisons between studies are also complicated as there are differences in both the diet and the age of the animals under investigation. To investigate the effects of PCr depletion without the adaptation and pathology, Unitt *et al.* (1993) investigated the acute effects of β-GPA perfusion and its phosphorylation on the energy metabolism and function of isolated rat heart. The administration of 150 mM β-GPA led to the accumulation of β-GPA-P, which was accompanied by a 30% decrease in PCr. This was concomitant with acute energetic changes. It remains to be determined if these changes are related to the pathological and adaptive changes seen in the chronically fed animals.

Following on from this work, Boehm *et al.* (1995) have examined the acute energetic effects of β-GPA on the energetics of porcine carotid artery vascular smooth muscle (Boehm *et al.*, 1995). Compared with striated muscle, smooth muscle employs different strategies for the provision of ATP to meet energy demands. Oxidative phosphorylation is primarily linked with contractile energy demands, whilst glycolysis is used (even under fully aerobic conditions) to fuel membrane energetics (Paul *et al.*, 1979; Paul, 1983; Campbell and Paul, 1992). Contraction in vascular smooth muscle is characterized by only a two- to three-fold increase in ATP utilization. Since the ATP breakdown can be matched by aerobic resynthesis there is no apparent decrease in ATP+PCr content (Paul, 1980).

In recent studies (Boehm *et al.*, 1995) observed that at a perfusion concentration of 50 mM, β-GPA rapidly enters vascular smooth muscle and becomes phosphorylated by CK, leading to the accumulation of β-GPA-P. β-GPA phosphorylation occurs with a concomitant decrease in [PCr], [ATP] and [lactate]. The fall in lactate may be due to an increase in oxidative metabolism stimulated by elevated ADP. Phosphorylation is carried out by cytosolic CK, though the mitochondria are important in the phosphorylation potential. The rate of β-GPA phosphorylation is substrate dependent, being more rapid with glucose compared with pyruvate. These observations indicate that vascular smooth muscle has a greater [ATP] when perfused with glucose and that this may enable more rapid phosphorylation of β-GPA.

4. CREATINE PHOSPHATE IN SKELETAL MUSCLE

4.1. Normal skeletal muscle

Hoult *et al.* (1974) were the first to apply ^{31}P NMR to the observation of PCr and ATP in rat skeletal muscle. Numerous other investigators have since applied the technique to the study of muscle metabolism and it can now be used to differentiate red and white muscle (Meyer *et al.*, 1985); for example, biceps (white muscle) has 31% more PCr compared with soleus (red muscle) and 40% more ATP. As discussed previously, [PCr] decreases towards zero during exercise while [ATP] remains relatively constant in both muscle types (see Fig. 13.1). Meyer *et al.* (1986) demonstrated that if PCr is depleted by chronic β-GPA administration the tissue is unable to utilize glycogen stores effectively. This study did not however address the possible inhibition of glycogen phosphorylase by β-GPA-P. β-GPA-P was utilized during muscle stimulation, though to a lesser extent than PCr. From these results they concluded that normal PCr hydrolysis via CK (which is decreased with β-GPA-P) is important for maximizing activation of glycogenolysis and/or glycolysis.

4.2. Abnormalities in dystrophic skeletal muscle

An example of diseased muscle with decreased PCr concentrations is duchenne muscular dystrophy (DMD). It is one of the most common and serious of the fatal genetic disorders affecting man (Emery, 1988). It arises in approximately 1/3500 boys and primarily affects skeletal muscle and to a lesser extent smooth muscle, cardiac muscle and brain (Gardner-Medwin, 1980). The genetic defect causes a lack of expression of the 427 kDa protein, dystrophin, in all such tissues (Hoffman *et al.*, 1987; Nudel *et al.*, 1988). Extensive skeletal muscle necrosis develops with regeneration during the early stages of the disease, probably resulting from this lack of dystrophin. Eventually muscle necrosis exceeds regeneration and the patient usually succumbs to respiratory failure aged 17–20 years (Gardner-Medwin, 1980; Webster *et al.*, 1988). The role of dystrophin in the muscle cell remains speculative. One theory suggests that dystrophin has a structural role within the cell or cell membrane and may act to help to maintain plasma membrane integrity (Mokri and Engel, 1975). If the lack of dystrophin destabilizes the membranes, leading to disruption, the cell will die due to the uncontrolled influx of ions through the leaky membranes. The second theory proposes that dystrophin may partake in ionic regulation across the cell membrane. This could account for the increased $[Ca^{2+}]_i$ known to occur in dystrophic muscle cells (Bertorine *et al.*, 1982; Glesby *et al.*, 1988). The increased calcium is thought to cause calcium

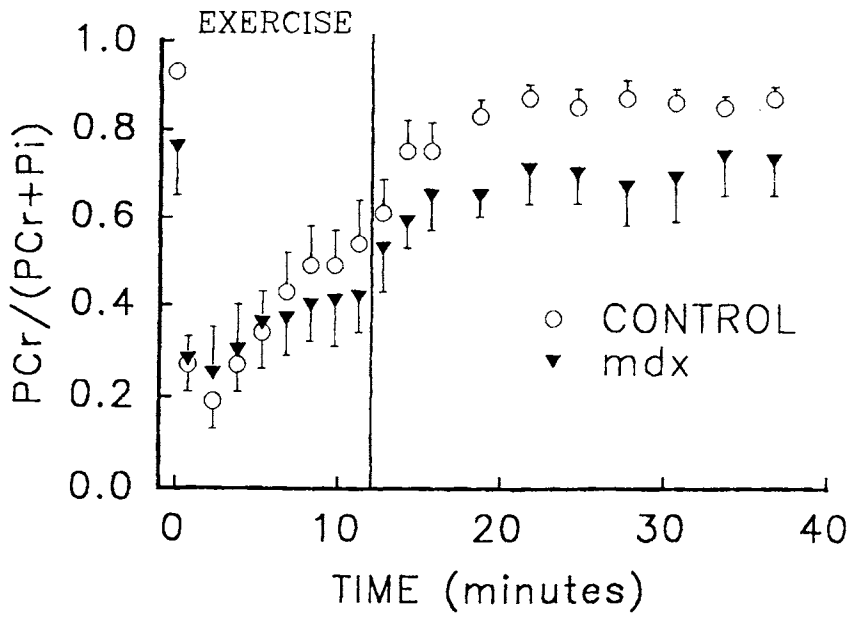

Figure 3.2. Time course of PCr and P_i levels as measured by ^{31}P NMR in the mouse skeletal muscle. PCr/(PCr+P_i) change in control and *mdx* skeletal muscle at rest, during exercise and recovery at 10 Hz stimulation (±SD; $N = 6$).

overload in the mitochondria, thereby affecting mitochondrial function (Wrogemann and Pena, 1976; see above). In the most severe case this mitochondrial damage might lead to ATP and PCr depletion and eventual cell death.

We studied mitochondrial function in dystrophic mouse muscle compared with control in order to determine whether mitochondrial abnormalities exist due to the lack of dystrophin. The *mdx* mouse, an accepted model of DMD with a genetic and biochemical defect similar to the human condition (Bullfield *et al.*, 1984), was compared with the control strain C57B1/10ScSn. A gastrocnemius muscle exercise protocol was developed which allowed stimulation of the mouse muscle *in vivo* at 1, 10 and 100 Hz with simultaneous recording of tension generation and metabolite changes visualized by ^{31}P NMR (e.g. PCr, P_i and ATP: $N = 6$). At all the stimulation frequencies used the PCr/PCr+P_i decreased significantly in *mdx* muscle at rest, during exercise and upon recovery compared with control (Fig. 3.2; Dunn *et al.*, 1992, 1993). Total [creatine] determined fluorimetrically from excised and rapidly frozen gastrocnemius muscle was not significantly different between *mdx* and control muscle (20 ± 1.8 and 21 ± 0.8 μmol/g wet weight, respectively). This normal [creatine] in muscle cannot, therefore, account for the decreased PCr levels. Since there is extensive muscle fibre regeneration in *mdx* muscle (unlike the human

DMD condition), the decreased PCr level cannot simply be explained by a decreased muscle bulk in muscular dystrophy.

These findings raise the question as to what is the cause of the decreased PCr and what effect it has on pathogenesis? The results of the current study indicate that increased intracellular calcium levels affect *in vivo* mitochondrial function of *mdx* muscle to lower the PCr levels. The decrease of important energy-containing metabolites such as PCr and ATP ([ATP] was also significantly decreased in *mdx* muscle compared with control: from 5.2 ± 0.4 to 6.8 ± 0.3 μmol/g wet weight) could lead to the extensive muscle cell necrosis characteristic of this condition. Further research into the possible mechanism by which calcium is increased might help elucidate the role of dystrophin in the cell. Methods designed to replace a functional dystrophin protein into dystrophic muscle cells can, therefore, be tested *in vivo* for their efficacy using this stimulation protocol which measures the changes in $PCr/(PCr+P_i)$. Other methods where intracellular or extracellular PCr could be adjusted may be used to determine the role of PCr in this pathology. A decrease in Cr such as that seen in other animal models of DMD may also exacerbate the mitochondrial deficiency due to creatine-stimulated respiration (see Chapters 4 and 5). This has yet to be investigated.

5. CREATINE TRANSPORT

5.1. Introduction

The influx and efflux of creatine from the cell are key determinants of cellular creatine concentrations. Cytosolic [creatine] is decreased in several pathological conditions, notably muscular dystrophy (Fitch, 1977) and after myocardial infarction (Rix *et al.*, 1993). Furthermore, dietary supplementation with creatine in humans leads to elevated intracellular creatine levels that correlate with an increase in maximal voluntary muscle contraction (Harris *et al.*, 1992; Greenhaff *et al.*, 1993b).

Despite the importance of creatine transport, relatively little work has been done to characterize these processes. The transporter that mediates influx has only recently been cloned and the regulatory factors and cellular mechanisms that control these fluxes are as yet unclear.

5.2. Creatine uptake

Most studies on creatine transport have focused on creatine influx and several tissues have been examined (Ku and Passow, 1980; Loike *et al.*, 1986a; Möller and Hamprecht, 1989; Odoom *et al.*, 1993). This saturable active

transport is highly specific for creatine, sodium dependent, concentrative and sensitive to metabolic inhibitors (Fitch *et al.*, 1968b; Fitch and Chevli, 1980; Ku and Passow, 1980; Loike *et al.*, 1986a, 1988; Möller and Hamprecht, 1989; Bennett *et al.*, 1991). Two sodium molecules are transported for every creatine molecule and the K_m for sodium is 55 mM (Möller and Hamprecht, 1989) thus favouring Cr transport. The uptake of creatine appears to be little affected by extracellular pH over the range pH 6.9–7.9 (Syllm-Rapoport *et al.*, 1980).

The electrogenic nature of creatine transport is important in the mechanism of uptake of the molecule. The transporter exploits the negative membrane potential as well as the inwardly directed sodium gradient in order to maximize creatine uptake. The K_m for creatine uptake ranges from 40 to 90 μM (Seppet *et al.*, 1985; Loike *et al.*, 1988; Möller and Hamprecht, 1989). In rat blood plasma the concentration of creatine is 140 μM (Syllm-Rapoport *et al.*, 1981) compared to 500 μM (Marescau *et al.*, 1986) and so muscle transporters will be close to saturated with substrate. Earlier research indicated a high value for the apparent K_m in isolated rat extensor digitorum longus, 0.5 mM; (Finch and Shields, 1966 and Fitch *et al.*, 1968) and so the possibility exists that there is a difference between the uptake in isolated tissue as opposed to that in whole cells. However, this seems unlikely as more recent rat heart experiments also yield the lower K_m (Seppet *et al.*, 1985).

A K_m value for the transporter in human muscle has not yet been published. Some data for human monocytes and macrophages (Loike *et al.*, 1986a) show the normal cell K_m to be approximately 30 μM. The K_m for saturable uptake in human erythrocytes is not dissimilar at 20 μM (Ku and Passow, 1980) or 87 ± 32 μM (Syllm-Rapoport *et al.*, 1980, 1981). The physiological serum human creatine concentration is 10–60 μM (Loike *et al.*, 1986a; Bennett *et al.*, 1991). Thus, the transporter in human cells can respond to physiological fluctuations in creatine by altering the total activity of the transporter.

Several groups report a second non-saturable uptake component (Fitch and Shields, 1966; Ku and Passow, 1980; Loike *et al.*, 1986b) which is sodium independent (Loike *et al.*, 1986b). Under physiological conditions this represents at most 10% of the uptake (Syllm-Rapoport *et al.*, 1981). There is, however, evidence for a smaller component of passive flux (Ku and Passow, 1980; Syllm-Rapoport *et al.*, 1980, 1981).

5.3. Inhibition of creatine uptake

Inhibitors of active creatine uptake are valuable in demonstrating the specificity of the transporter (Fitch *et al.*, 1968a; Fitch and Chevli, 1980; Ku and Passow, 1980) and allow study of creatine depletion (Fitch and Chevli, 1980; Shoubridge and Radda, 1984; Seppet *et al.*, 1985; Otten *et al.*, 1986). The most widely used inhibitor, β-guanidinopropionate (β-GPA), has a K_i for

competitive inhibition of approximately 0.2 mM (Fitch *et al.*, 1968a; Möller and Hamprecht, 1989). β-GPA is, however, a poor kinetic substitute for Cr, as discussed above and in Chapter 6. A number of other inhibitors have also been identified (Fitch and Chevli, 1980).

Inhibition of the creatine transporter has shown that the rate of efflux is low and measurements on rat skeletal muscle *in vivo* indicate that the cell loses 1.7–2.5% of total creatine per day on average (Bloch *et al.*, 1941; Otten *et al.*, 1986; Meyer, 1989). The similarity to the rate of loss of creatine from the cell as creatinine (2% of the total cell creatine is lost per day) led Bloch to propose that creatine efflux from the cell occurs almost exclusively as creatinine (Bloch *et al.*, 1941). Similarly creatine efflux from the porcine carotid artery is negligible after 72 h in a creatine-free medium. If creatine leaves the cell as *creatine*, then the rate is so slow as to make it difficult to measure (Fitch and Sinton, 1964; Fitch and Shields, 1966). The efflux of creatinine from erythrocytes occurs passively through a non-protein dependent, non-active transport process (Ku and Passow, 1980).

5.4. Modulation and regulation of creatine transport

Little effect on creatine uptake rates has been observed with PCr, creatinine, ornithine, glycine, glutamic acid, histidine, alanine, arginine, leucine, methionine or cysteine (Fitch, 1988; Loike *et al.*, 1988; Möller and Hamprecht, 1989). The sodium-dependent uptake of creatine in culture is sensitive to extracellular creatine concentration (Loike *et al.*, 1988). Myoblasts maintained for 24 h in a medium containing 1 mM creatine exhibited one-third of the uptake activity of cells bathed for the same duration in a medium lacking creatine. The downregulation was slowed by inhibitors of protein synthesis. From this observation one can conclude that extracellular creatine regulates the induction of the expression of a protein that decreases the sodium-dependent uptake activity only after the creatine enters the cell.

No alteration in the efflux rate for creatine was observed in studies of the effects of changes in extracellular creatine concentrations (Loike *et al.*, 1988). If creatine was leaving mainly by passive or facilitated diffusion, an alteration in efflux rates would be expected. Thus, it seems probable that the creatine is leaving mainly as creatinine, possibly with a small creatine component as they both move down their concentration gradients.

Adding thyroxine (T4) to mimic hyperthyroidism increases the uptake and efflux of creatine in the rat heart (Seppet *et al.*, 1985). This group found a 20-fold increase in V_{max} and questioned the earlier findings which indicated that T3 induces inhibition of creatine uptake (Dinking *et al.*, 1959, Fitch *et al.*, 1960). Support for the findings of Seppet *et al.* (1985) comes from more recent studies in which the exposure of cultured mouse myoblasts to T3 produced increased creatine uptake (Odoom *et al.*, 1993). This observation may result

from the increase in the Na/K pump activity caused by T3 (Brodie and Sampson, 1988), an effect supported by the sensitivity of uptake to oubain (Möller and Hamprecht, 1989).

5.5. Creatine loading

Creatine loading can be accomplished by incubating tissues with high concentrations of creatine. Total tissue creatine and PCr increase dramatically, probably because of creatine flooding across the membranes. This was performed in porcine carotid artery by Clark and Dillon (1995) to examine the flux through the CK system in the presence of increased metabolites. They found that despite increased PCr in the porcine carotid artery the CK reaction remained at equilibrium.

If porcine carotid arteries are incubated with 50 mM creatine substituted for sodium, in the absence of phosphate, the vessels tend to die. Figure 3.3 illustrates two ^{31}P NMR spectra from two different porcine carotid arteries. Spectrum A is the control spectrum that was incubated for 12 h in a P_i free Krebs buffer with glucose as the substrate in the absence of creatine. The PCr/ATP ratios are consistent with those reported by Clark and Dillon (1995) and show healthy carotid arteries. Spectrum B is from a porcine carotid artery that has been incubated with the same buffer but in the presence of 50 mM creatine. Interestingly, the PCr peak is greatly enlarged, while the ATP peaks are decreased. These results indicate that high levels of creatine in the presence of active CK will act as a phosphate sink, leading to the loss of ATP and possibly the nucleotide pool. Consistent with the equilibrium nature of CK, nucleotide levels appear to fall, but this occurs to the detriment of the cell.

5.6. Future studies

Much of the research on creatine transport has been performed with a view towards therapeutic intervention in pathological conditions of the muscular system. Exercise combined with creatine ingestion enhances the increase in cell creatine (Harris *et al.*, 1992). Thus, it may be possible to increase this effect by combining similar protocols with stimulatory hormones such as T3 or adrenaline (the latter because isoproterenol appears to enhance creatine uptake) (J.E. Odoom, unpublished data). As the hormones or other factors that affect transport become known, therapeutic interventions for particular disease states and individual patients can be devised.

The control of creatine transport may also have relevance for the physiology of adaptation to exercise. We know that there is a rapid influx of creatine compared with a much slower net efflux and this suggests that each bout of exercise may cause an increase in cell creatine content that persists and is cumulative

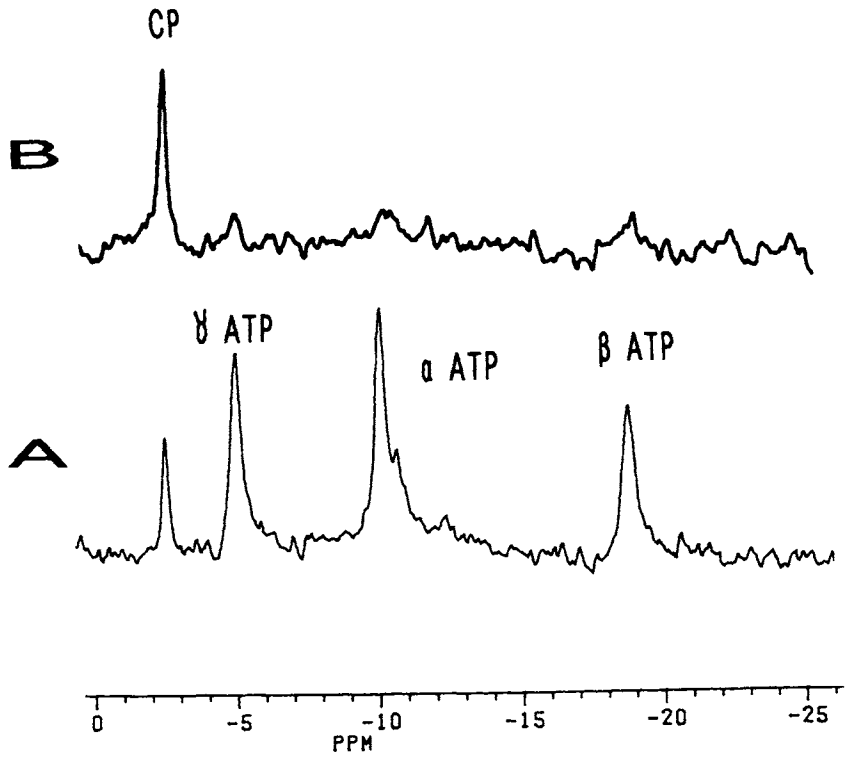

Figure 3.3. Stacked plot of two ^{31}P NMR spectra from two isolated perfused porcine carotid arteries. Spectrum A is from a porcine carotid artery incubated with a control Krebs buffer in the absence of P_i for 12 h. Spectrum B is from porcine carotid arteries that have been incubated with the same buffer but with 50 mM creatine added in equimolar substitution with sodium. The resultant increase in PCr is due to increased creatine in the artery and is caused by the rapid phosphorylation from CK. There has been a decrease in the ATP peaks which may be due to the cellular phosphate effectively being lost to the cell in the form of PCr.

with subsequent exercise, thereby leading to a concomitant increase in [PCr]. The studies of Harris *et al.* (1992) support such a proposal. The major limiting factor may be the circulating plasma creatine concentration, a factor which is determined by the balance between ingestion and *in vivo* production by the kidney/pancreas and liver.

In using creatine as a supplement to enhance sporting performance via increased intracellular [Cr] and [PCr] (see Chapter 13), the circulating level of creatine is less likely to limit the desired energetic and performance benefits than the activity of the transporter itself. It is becoming established that under normal physiological conditions the maximum intracellular creatine concentration is of the order of 150 mmol/kg of cell protein. Any increase in

intracellular creatine will lead to an increase in [PCr] and thereby enhance muscular performance.

6. SUMMARY

In this chapter we have seen that ^{31}P NMR allows measurement of changes in [PCr] during muscle stimulation and metabolic manipulation as well as in diseased muscle. The ratio of PCr to ATP reflects the status of cellular energetics. This ratio changes from tissue to tissue under normal healthy conditions and is therefore an indication of tissue type. Skeletal muscle has the largest ratio followed by heart, whilst the ratio is lowest in vascular smooth muscle. The PCr/ATP ratio also changes within tissues to give an index of tissue function or disease.

Creatine analogues can affect the muscle [PCr] as was shown above. The chronic and acute changes seen in the energetic profile of the tissue exposed to β-GPA further demonstrate the importance of PCr in muscle function.

We conclude that the PCr metabolism is important to normal cellular function and that as such it is tightly regulated in muscular tissue. This is not exclusively limited to the intracellular biochemistry; we will see in Chapters 9, 11 and 12 that PCr has potential clinical and therapeutic applications.

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4

An Introduction to the Cellular Creatine Kinase System in Contractile Tissue

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Key words: creatine kinase, energy transduction, smooth muscle, skeletal muscle, heart

1. INTRODUCTION

Extracellular creatine (Cr) is sequestered into the cytosol by a sodium-driven co-transport system from where it is phosphorylated by the intracellular isoenzymes of creatine kinase (CK) (see Chapters 1, 2 and 3). Discussion of extracellular creatine use for improving cellular energy transduction requires an understanding of the role of this system. To this end, a brief review of the contractile tissue intracellular creatine kinase system follows.

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2. THE CREATINE KINASE SYSTEM IN DIFFERENT MUSCLE TYPES

Creatine kinase functions as an important cellular enzyme facilitating energy transduction in muscle cells by catalysing the reversible transfer of a phosphate moiety between ATP and creatine phosphate (PCr):

$$PCr + ADP + H^+ \rightleftharpoons ATP + Cr \quad (1)$$

CK is composed of two subunit types (M and B monomers) giving three isoenzymes, MM-CK, MB-CK and BB-CK. In addition, there is a fourth CK isoenzyme located on the outer side of the inner mitochondrial membrane which differs biochemically and immunologically from the cytosolic forms. The M and B isoenzymes are so named because they were first characterized in muscle and brain, respectively. These isoenzymes appear to be integral components of the contractile apparatus energy metabolism because they have been localized to skeletal, cardiac and smooth muscle contractile proteins (Ventura-Clapier *et al.*, 1987b; Wallimann *et al.*, 1992; Clark, 1994). Their presence and distribution depends on muscle type (Table 4.1). Significant amounts of CK are also found in the nervous system and its distribution corresponds to tissues with dynamic energy demand (see Ma *et al.*, Chapter 2). Clinically, CK is used to aid in the diagnosis of acute myocardial infarction and has been proposed as a marker of cardiac pathology.

Skeletal muscle CK exists almost exclusively in the MM form and is responsible for temporal ADP and ATP buffering by virtue of the near-equilibrium CK reaction in this tissue (Shoubridge *et al.*, 1984). The enzyme is present in cardiac muscle at lower activity compared to skeletal muscle, but with a high diversity in isoenzymic profile. Studies with subcellular fractionation and histochemical localization revealed that all four CK isoenzymes are present in heart muscle, and are bound to intracellular structures at the sites of energy production and energy utilization, such as plasma membranes, sarcoplasmic reticulum, nuclei, myofibrils and mitochondria (Sharov *et al.*, 1977). In addition to the traditional role of CK as a temporal ATP/ADP buffer, several investigators have proposed that the presence of the CK isoenzymes in cardiac muscle facilitates the transduction of high-energy phosphate throughout the cell and acts to fine tune the regulation of energy production and utilization (Saks *et al.*, 1987, 1995; Wallimann *et al.*, 1989), i.e. spatial energy buffering. Smooth muscle has low CK activity and a diversity of isozyme composition and localization that depends on the smooth muscle type (Clark, 1994). As with cardiac muscle, the relative role of CK as a temporal ATP/ADP buffer or a spatial energy buffer remains controversial (Fig. 4.1, on page 54).

Mitochondrial CK (Mi-CK) is thought to form complexes with voltage-dependent anion channels (VDAC) in the outer mitochondrial membrane and with the adenine nucleotide translocase in the inner mitochondrial mem-

Table 4.1 Creatine kinase and its isoenzymes

		Isoenzymes			
	Activity[a]	MM-CK	MB-CK	BB-CK	Reference
Aorta	8	20	36	43	Boehm *et al.* (1992)
Aorta	26			++++	Ishida *et al.* (1991)
Carotid artery	2	56	2	42	Tsung (1976)
Stomach	23	3	6	91	Lang (1981)
Colon	125	4	0	96	Tsung (1976)
Vas deferens	329		++	+++	Ishida *et al.* (1991)
Uterus:					
Gravid	196	22	8	70	Clark *et al.* (1993)
Non-gravid	74	20	10	70	Clark *et al.* (1993)
Heart:					
Ventricle	700	50	20	10	Saks *et al.* (1994)
Ventricle	800	52	46	2	Lang (1981)
Atrium	402	78	22	0	Tsung (1976)
Brain:					
Cerebrum	90			100	Lang (1981)
Cerebellum	87			100	Lang (1981)
Skeletal muscle	3281	100			Tsung (1976)

[a]Activity is in IU/g wet weight.

Mi-CK, though present in muscular tissue, is present in relatively low levels in smooth and glycolytic muscles, so it is not always reported (see Wallimann *et al.*, 1992). Saks *et al.* (1998) reported Mi-CK to be about 20% of the total CK activity in ventricle.

++, +++, ++++, relative activities.

brane (Wallimann *et al.*, 1992). These are known as contact sites and are thought to permit channelling of energy (Bakker *et al.*, 1994).

VDACs are reported to be associated with hexokinase and glycerol kinase in certain tissues (McCabe, 1994), suggesting that the contact site may also include these two enzyme systems. However, this supposition awaits further evidence and clarification in contractile tissue. Along with adenylate kinase found in the intermembrane space of mitochondria, Mi-CK hexokinase and glycerol kinase may play an important role in regulating the concentration of ADP in mitochondria (Fig. 4.2). The functional contribution of these enzymes in the control of oxidative phosphorylation *in vivo* has yet to be defined.

3. SKELETAL MUSCLE

Skeletal muscle is the tissue with the greatest CK activity (1000–3000 IU/g wet weight (Wallimann *et al.*, 1992)). The PCr concentration is also very high ($20 \leqslant$ mM) compared with that in cardiac and smooth muscle and this enables

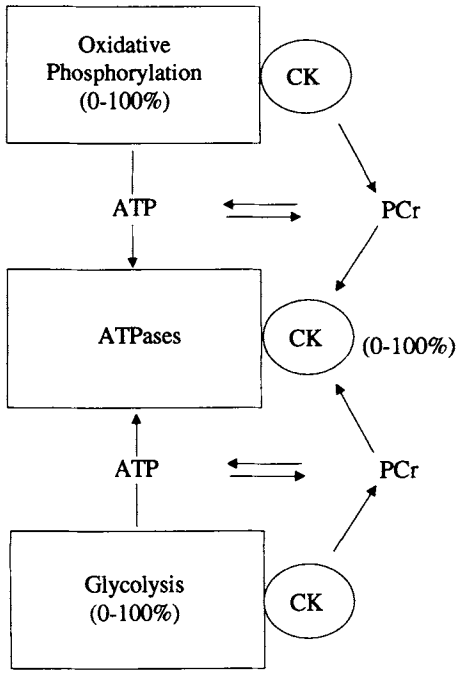

Figure 4.1. Schematic representation of the role of CK in cellular high-energy phosphate transduction. Oxidative phosphorylation and glycolysis supply ATP to cellular ATPases, the relative contributions depending on the cell type. Localized CK at the inner mitochondrial membrane, glycolytic enzymes and cellular ATPases allow a contribution of high-energy phosphate transduction through PCr. The importance of this route is dependent on tissue type and the demand for high-energy phosphate.

the CK system to have a large temporal ATP buffering capacity (see Chapter 13). The role of CK as a temporal ATP buffer in skeletal muscle is discussed in detail elsewhere (Wallimann *et al.*, 1992). There are, however, well-characterized fibre type differences in skeletal muscle CK activity. Fast-twitch glycolytic fibres have greater CK activity than slow-twitch oxidative fibres and the latter have a higher percentage of Mi-CK. Some relationship appears to exist between the CK activity and PCr concentration but, whereas [PCr] can be manipulated by creatine loading, the [PCr] remains largely unchanged when CK is absent (Wallimann *et al.*, 1992; see Chapter 6).

4. CARDIAC MUSCLE

4.1. Mitochondrial creatine kinase

The interrelationship between Mi-CK on the outside of the inner mitochondrial membrane and the local myocyte environment has been the subject of

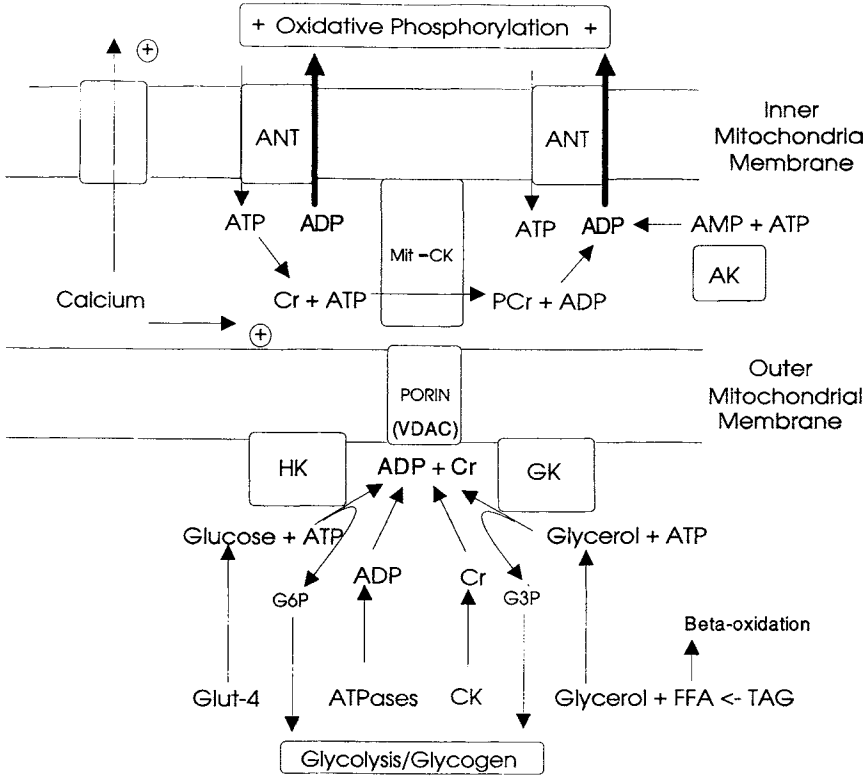

Figure 4.2. Putative arrangement of mitochondrial CK adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), hexokinase (HK), glycerol kinase (GK) and adenylate kinase (AK) at a mitochondrial contact site. This complex of ADP-regenerating enzymes may play a key role in controlling the K_m of ADP with respect to oxidative phosphorylation. Evidence suggests the arrangement is sensitive to $[Ca^{2+}]_i$, insulin and disease.

much research by Valdur Saks (Saks *et al.*, 1976, 1984, 1987, 1991a) and Theo Wallimann (Wyss *et al.*, 1991, 1992). Mitochondrial CK is an octameric protein found on the outside of the inner mitochondrial membrane (Wallimann *et al.*, 1992). Saks *et al.* (1991a and b) have found that Mi-CK is functionally coupled to the control of oxidative phosphorylation through its close proximity to the ATP/ADP translocase on the inner mitochondrial membrane. Because of this co-localization, nascent ATP from the adenine nucleotide transporter is preferentially available to Mi-CK to phosphorylate Cr. The resultant ADP does not readily diffuse out of the mitochondrial intermembrane space, but rather is transported back into the mitochondrial matrix via the adenine nucleotide translocase to stimulate respiration. Functional coupling of Mi-CK to oxidative phosphorylation

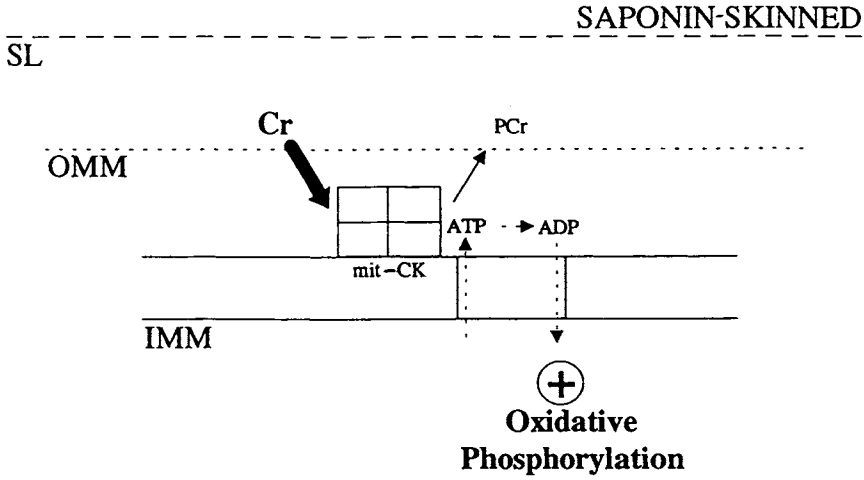

Figure 4.3. Schematic representation of creatine-stimulated respiration at the inner mitochondrial membrane of a saponin-skinned cardiac fibre. Excess creatine (Cr) shifts the local Mi-CK reaction in favour of an increase in local ADP. ADP is then transported via the adenine nucleotide translocase into the mitochondrial matrix to stimulate respiration. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; SL, sarcolemma mit-CK, mitochondrial creatine kinase bound to the IMM.

causes an increased sensitivity of mitochondrial respiratory control to changes in ADP. Through this mechanism Cr stimulates respiration (Veksler *et al.*, 1988, 1991; Saks *et al.*, 1991a, 1993). Creatine shifts the local Mi-CK equilibrium (Eqn 1, page 52) towards the production of PCr and ADP (ADP in turn stimulates respiration). Thus creatine and Mi-CK can lower the K_m, or increase the sensitivity, for respiratory control of oxidative phosphorylation with respect to [ADP] (Fig. 4.3). For further details concerning Cr-stimulated respiration see Chapter 5, Wallimann *et al.* (1992) and Ingwall *et al.* (1980).

4.2. Myofibrillar creatine kinase

Bound to the myofibrils, MM-CK is the isoenzyme present and localized to the A-band as well as being distributed across the entire filament (Saks *et al.*, 1976; Veksler and Kapelko, 1984; Wallimann and Eppenberger, 1985). In α myofibrillar preparations, endogenous creatine kinase is functionally coupled to myosin ATPase (Saks *et al.*, 1984; Ventura-Clapier *et al.*, 1987b). Similar results were found by Clark *et al.* (1992, 1993, 1994) using Triton X-100 skinned smooth muscles. The local high activity of CK in heart myofibrils (2

IU/mg protein) arises mostly due to reversibly bound enzyme with an apparent K_m of 0.14 mg/ml. This activity represents 5–10 times the myosin ATPase activity (Saks *et al.*, 1984; Ventura-Clapier *et al.*, 1987; Krause and Jacobus, 1992). Using the Triton X-100 skinned fibres, the presence and role of myofibrillar CK with regard to tension development and heart function has been studied. By manipulating calcium movements and metabolite concentrations, this technique allows the mechanical properties of the myofilaments to be studied without interference from the excitation–contraction process (and its control mechanisms). Under such conditions, the intrinsic properties of the contractile proteins can be examined. Creatine kinase, bound to myofibrillar structures, can rephosphorylate enough Mg-ADP to ensure optimal contractile capacities, normal calcium sensitivity, and adequate cross-bridge cycling rate in the absence of Mg-ATP (Ventura-Clapier *et al.*, 1987a and b). These results show that CK has the capacity to generate ATP from ADP at a sufficient rate and in sufficient amounts to fulfil contractile protein energy requirements.

Further studies show that when PCr is absent, contractile properties are altered, such that relaxation is impaired and the kinetics of contraction are slowed (Ventura-Clapier *et al.*, 1987c). Relaxation becomes greatly impeded owing to the decrease in [Mg-ATP], thus leading to rigor tension development (tension without calcium). These findings suggest that there is restricted access of nucleotides to the intramyofibrillar space and that myofibrillar CK is necessary to ensure maximal activity of the myosin ATPase and a high local Mg-ATP/Mg-ADP ratio.

Myofibrillar CK is functionally coupled to Mi-CK via intercommunication of substrates (Cr:PCr as well as ADP:ATP). Thus it acts as an energy transducer at both the mitochondria and the contractile proteins. This energetic transduction is consistent with the energy shuttling discussed elsewhere (Chapters 2 and 5). It seems therefore, that the CK bound to the contractile proteins has the dual function of energy transduction and maintaining the Mg-ATP/Mg-ADP ratio. The relative importance of CK as a temporal or spatial buffer under different conditions and the obligatory functional dependence on PCr remain controversial.

4.3. Creatine kinase in acute and chronic pathology

Contractile dysfunction is one of the earliest consequences of myocardial ischaemia. The force of contraction becomes depressed leading to a gradual rise in diastolic tension and ischaemic contracture. This occurs in parallel with a rapid and substantial decrease in PCr and accumulation of inorganic phosphate while the CK reaction keeps Mg-ATP levels nearly constant as long as PCr is present. Though calcium is the normal mechanism controlling myofibrillar tension, these events are not directly linked to changes in cytosolic

calcium (Ventura-Clapier *et al.*, 1987a; Lee and Allen, 1991). Early contractile failure is usually attributed to inorganic phosphate or ADP accumulation, or to acidification, all of which decrease maximal force and the calcium sensitivity of contraction. The effects of inorganic phosphate are modulated by the concomitant decrease in PCr (Mekhfi and Ventura-Clapier, 1988; Saks *et al.*, 1995).

The mechanism underlying the rise in end diastolic pressure and the increased tone seen during contractile failure is not clearly understood. This tension is not due to calcium accumulation but rather to Mg-ATP depletion and the formation of slowly cycling rigor cross-bridges (Ventura-Clapier and Vassort, 1981). Rigor tension develops in the millimolar range of [Mg-ATP] when PCr is absent or very low. In the presence of PCr, owing to the ADP-rephosphorylating capacity of CK, rigor tension develops in the range of 0.1–0.9 mM Mg-ATP. In the presence of Mg-ADP and PCr, no rigor tension develops owing to active endogenous CK. When PCr is exhausted, the development of rigor tension is increased by rapid accumulation of Mg-ADP. Some investigators report that the ischaemic contracture can be mediated through decreased [PCr] and local Mg-ADP accumulation (Ventura-Clapier and Vassort, 1981; Veksler and Kapelko, 1984; Veksler *et al.*, 1988). Therefore, myofibrillar CK and PCr are important factors controlling cardiac contractility by ensuring optimal functioning of myosin ATPase and its finely tuned regulation via excitation–contraction coupling. It has been shown that myofibrils of acutely ischaemic heart do not exhibit functional alterations in either myosin ATPase activity or CK, nor in contractile properties; thus it may be concluded that ischaemic failure arises mainly due to altered intracellular compounds, e.g. PCr and/or Mg-ADP (Ventura-Clapier *et al.*, 1987a and b). Hence, the CK system seems to represent a specific energy supply or transducing system for contraction.

There is much interest in the role of alterations in the CK system on energy transduction in myocardial hypertrophy and cardiomyopathy. Veksler *et al.* (1988) were the first to report that creatine-stimulated respiration is reduced in skinned fibres of the cardiomyopathic hamster. Khuchua *et al.* (1989) later correlated this with diminished Mi-CK activity. Khuchua *et al.* (1989) suggested that the efficacy of cellular regulation of respiration by [ADP] may be altered by changes in CK isozyme expression. In support of this, Saks *et al.* (1991b) have found that reduced cardiac output and elevated end diastolic pressure in human cardiomyopathy correlate with reduced Mi-CK activity and a loss of creatine-stimulated respiration in skinned fibres obtained from biopsy samples. This observation agrees with results of Clark *et al.* (1994a), who used β-GPA fed rats. Interestingly, it has been shown that Mi-CK activity per mitochondrion correlates with increasing creatine-stimulated respiration (Field *et al.*, 1994). Some suggest that decreased Mi-CK activity may be an important marker for determining the point of transition between a physiological hypertrophy and pathological hypertrophy or "failure state" (Ingwall *et al.*, 1980;

Seppet *et al.*, 1985). The myofibrillar end of the CK shuttle is generally less or not at all affected. However, a switch in the isozyme composition of mysoin from V_1 to V_3 is seen in rat hearts (Mekhfi *et al.*, 1990).

4.4. Creatine kinase and cardiac development

Foetal development is accompanied by a marked increase in total CK activity and the relative content of the different CK isoenzymes varies during perinatal development. The major isoenzyme present in heart and skeletal muscle during foetal life is the BB isoform. Foetal differentiation and maturation of the muscle cell is characterized by a decrease in the BB isoform and an increase in MM-CK. This phenomenon takes place at an earlier stage of development in heart than in skeletal muscle. During perinatal life, Mi-CK appears later in development (Ingwall *et al.*, 1980) but is species dependent. Thus, the two main isoforms that are specifically linked to the sites of energy utilization and energy production do not appear at the same time. However, expression of specific isoforms does not imply localization or a precise function. The perinatal organization of the functional activity of CK in myofibrils and mitochondria has been studied by Ventura-Clapier *et al.* (1992). They observed in rabbit heart that although the isoforms are expressed at different stages, their compartmentalization appears in parallel during postnatal development (Hoerter *et al.*, 1991, 1994). In the guinea-pig, both isoforms are present and functionally active in myofibrils and mitochondria before birth (Ventura-Clapier *et al.*, 1992). This is consistent with the greater degree of maturation at birth of guinea-pig versus rabbit heart. CK isoenzyme compartmentation plays a part in the complex organization of the highly differentiated mammalian cardiac cell during perinatal maturation.

5. SMOOTH MUSCLE

Creatine kinase activity in smooth muscle is generally quite low when compared with striated muscles, such as heart and skeletal muscle (Table 4.1). The role and function of smooth muscle CK is poorly understood and the current state of knowledge in relation to this topic is described in detail by Clark (1994). This section will focus on how CK is involved in intracellular smooth muscle PCr metabolism.

5.1. Localization of creatine kinase

The four major isoenzymes of CK that have been identified are found in smooth muscle (Ishida *et al.*, 1991; Clark, 1994). There is strong evidence for

subcellular localization of CK in smooth muscle (Ishida *et al.*, 1991; Clark *et al.*, 1992, 1993). Mitochondrial CK is found in the mitochondria while BB-CK binds to the contractile proteins (Clark *et al.*, 1992, 1993), and Mi-CK can generate PCr via nascent ATP from oxidative phosphorylation (see Chapter 5). As described earlier, PCr is then able to diffuse out of the mitochondria to other cellular locations and serve as an energy source. Recently more evidence has been reported where the PCr concentration was found to be dependent upon oxidative metabolism (Boehm *et al.*, 1995). One plausible site where PCr might be utilized is at the contractile proteins, where BB-CK is found to be localized in Triton X-100 treated smooth muscle fibres (Clark *et al.*, 1993). Creatine phosphate can then be used by cytosolic CK to phosphorylate ADP to ATP which then functions in cross-bridge cycling and other energy-requiring processes. It should be noted that a secondary effect of this system is to keep ADP concentrations low at the contractile proteins. Indeed, the presence of ADP at the contractile proteins of smooth muscle significantly slows relaxation (Nishiye *et al.*, 1993).

Further evidence comes from Clark (1990), who found that the BB-CK has a high K_m for ATP. This would favour PCr conversion to ATP at the contractile proteins. Thus, PCr could be used as an energy source for the cross-bridges. Nishiye *et al.* (1993) reported that the K_d of ADP for inhibiting cross-bridge cycling during relaxation is 1.3 μM. Assuming this to be true and that [ADP] plays a role in cross-bridge regulation, the CK system, with its ATP/ADP interconversion, may be important in normal cross-bridge function.

The locations of MM-CK and MB-CK have yet to be determined, but there is evidence that CK is associated with glycolytic metabolism (Dillon and Clark, 1990). Furthermore, there are reports that CK is coupled to membrane pumps (Wallimann, 1984; Hardin *et al.*, 1992). Despite the apparently important role of CK, low levels of PCr (1–7 mM) are found in smooth muscle (Clark, 1994), compared with 15 mM in striated muscle. These relatively low PCr concentrations are adequate for smooth muscle to maintain its energetics during stimulation. Ishida and Paul (1989) report that vascular smooth muscle cannot maintain tension in the absence of PCr, even with ATP still present. Interestingly, relaxation was not affected by excess PCr (Dillon and Clark, 1995).

5.2. Role of creatine kinase in energy transduction

Two types of smooth muscle have been characterized: phasic smooth muscle has contractile actions typical of the rhythmic contraction of the intestines, while tonic smooth muscle is represented by the protracted tension maintenance seen in the artery. Vascular smooth muscle also tends to have lower PCr and ATP concentrations and lower specific CK activity compared with other smooth muscle types (Clark, 1994; see Table 5.1). The greater intra-

cellular [PCr] may be related to the activity of the smooth muscle, with more energetically active smooth muscle requiring greater energetic reserves. Smooth muscle, loaded with PCr by incubation with extracellular creatine (Scott and Coburn, 1989), was found to show no change in contractile function (Clark and Dillon, 1995). The investigators concluded that the control of smooth muscle function was not affected by the CK system activity on the myosin ATPase. However, the ability of the contractile system to perform work is increased by the elevated [PCr] owing to the added energy reserve. Triton X-100 skinned smooth muscle displays no difference in tension generation with ATP in the absence of PCr (Clark *et al.*, 1992). These results required the activity of CK to be bound to the contractile proteins. Luvgren and Hellstrand (1985) found a correlation between [PCr] and force in the rat portal vein. It thus appears that PCr is not essential for smooth muscle contraction but that it may act as a regulatory mechanism for energetic homeostasis/metabolism/supply. Thus the relationship between PCr and contractile force in smooth muscle remains unclear.

Signal amplification by the CK system derives from the ability of creatine to stimulate mitochondrial oxygen consumption at intermediate [ADP]. This occurs by means of increased local [ADP] at the mitochondrial intermembrane space, thereby decreasing the apparent K_m for ADP as described earlier. The so-called creatine-stimulated respiration does not occur in smooth muscle (Clark *et al.*, 1994b). Results from isolated guinea-pig uterus mitochondria showed neither direct coupling of mitochondrial CK to oxidative phosphorylation, nor restricted ADP diffusion (Clark *et al.*, 1993, 1994b). Nonetheless, the integral components of the CK circuit are present and operable in smooth muscle. It appears that this muscle can maintain its energetic and contractile function by a functional coupling between the sites of energy production and energy utilization. This coupling is realized through the subcellular localization of specific CK isoenzymes, implying that PCr/Cr is an important part of smooth muscle metabolism and homeostasis.

6. SUMMARY

The findings of numerous investigators since the mid-1970s support the role of the CK circuit as an integrated system of energy supply and energy consumption in cardiovascular tissue. It is clear that when Cr enters the cell via the specific Cr transporter (see Chapter 3) rapid phosphorylation by the CK reaction takes place. The resultant PCr is utilized by the cell for essential metabolic processes. The CK system plays a role in both energy transduction and signal amplification in cardiovascular tissue (see Chapter 5).

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5

Cardiac Energetics: Compartmentation of Creatine Kinase and Regulation of Oxidative Phosphorylation

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Key words: adenine nucleotides, cardiomyocytes, compartmentation, creatine kinase, diffusion, free energy exchange, mitochondria, oxidative phosphorylation, regulation

1. INTRODUCTION

The creatine kinase (CK) isoenzymes function, *in vivo*, coupled to intracellular structures (mitochondria, myofibrils and cellular membranes). They operate at equilibrium in the cytoplasm as part of the intracellular creatine phosphate (PCr) pathway, or "phosphocreatine circuit" for energy channelling (Saks *et al.*, 1978, 1991a; Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Jacobus, 1985; Wallimen *et al.*, 1992; Wegmann *et al.*, 1992; Wyss *et al.*, 1992; Aliev and Saks, 1993). Chapters 1, 2 and 4 provided an introduction to the CK system. We will now see how compartmentalization of CK enzymes are involved in cardiac energy metabolism.

Very recent experimental work with membrane permeabilized (or "skinned") cells has shown that besides an energy transport function the CK system may also regulate cellular respiration as a result of the retarded or

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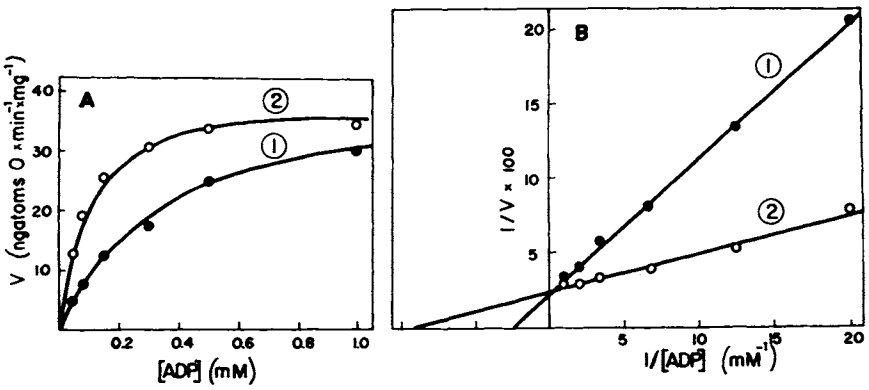


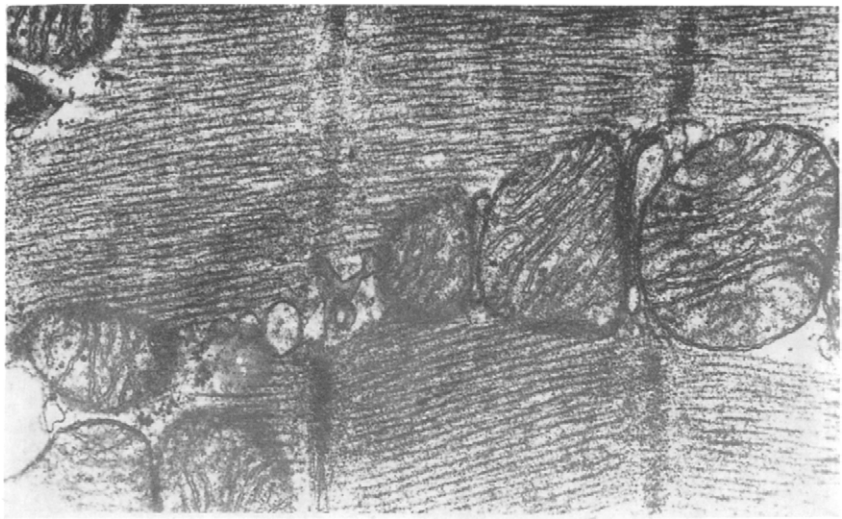
Figure 5.1. (A) The dependence of the rate of respiration of the skinned cardiac fibres on the external ADP concentration in the absence (curve 1) and in the presence of 25 mM creatine (curve 2). (B) Linearization of the dependency shown in Fig. 5.1A in the double reciprocal plots. Note the change in the value of apparent K_m for ADP by creatine at constant V_{max}.

restricted diffusion of ADP inside the cells (Saks *et al.*, 1991a,b, 1993). These data are outlined and explained in this chapter. An hypothesis based on local oscillating changes of ADP and creatine concentrations and a feedback signal between contraction and respiration in heart and skeletal muscle is also introduced.

2. THE ROLE OF THE OUTER MITOCHONDRIAL MEMBRANE IN RESTRICTING ADP DIFFUSION AND CONTROLLING RESPIRATION

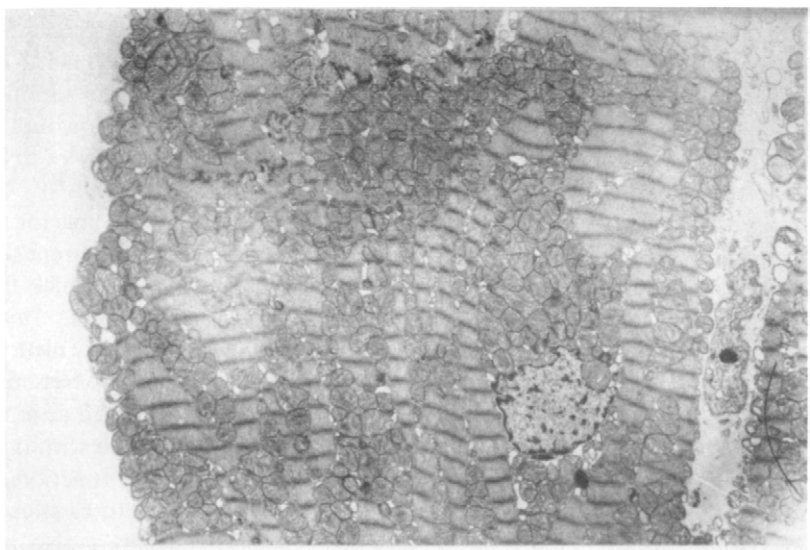
Experimental results using saponin-skinned fibres (where all the cells have had their sarcolemma removed but intracellular structures preserved) are shown in Fig. 5.1. The respiration rate of the mitochondria is measured with increasing ADP concentrations (Saks *et al.*, 1991a,b, 1993) and the maximal rate of respiration is achieved only at millimolar external ADP concentrations, as shown by curve 1 of Fig. 5.1A. However, the rate of respiration at any ADP concentration accelerates significantly in the presence of creatine (curve 2 of Fig. 5.1A) owing to mitochondrial CK activation (Saks *et al.*, 1991a,b, 1993).

The stimulation of respiration is due to a significant decrease in the apparent K_m for ADP at constant V_{max} (Fig. 5.1B). However, when these experiments are repeated in the presence of 125 mM KCl (which is known to release mitochondrial CK from the inner membrane), the stimulating effect of creatine on respiration disappears completely (Saks *et al.*, 1995). Such stimulation by creatine depends on functional coupling of mitochondrial CK to the adenine



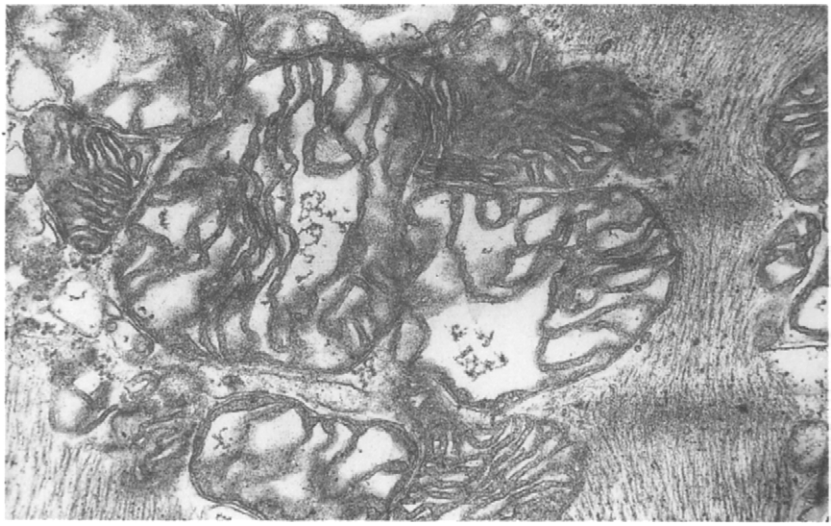
A

Figure 5.2. (A) Electron micrographs of the cardiac fibres. Control, magnification 50000 ×.



B

Figure 5.2. (B) After saponin treatment. The phospholipid bilayer of the sarcolemma is destroyed and vesicularized, but intracellular structures – mitochondria and myofibrils – are normal and have an appearance characteristic for these structures in the hyperosmotic physiological salt solution.



C

Figure 5.2. (C) Fibres after 30 min in the 40 mOsM solution, 54000 ×. For control before osmotic shock, see Fig. 5.3B. Mitochondrial outer membrane rupture is obvious, but the mitochondrial population with the well-preserved outer membranes is seen.

nucleotide translocase. We have shown that detachment of mitochondrial CK from the inner mitochondrial membrane abolishes the functional coupling between mitochondrial CK and adenine nucleotide translocase (Saks *et al.*, 1993). Thus, the stimulating effect of creatine on the respiration of saponin-skinned cardiac fibres seems to be directly related to tight functional coupling between these two proteins. This coupling involves some restriction of diffusion of ADP – or substrate channelling.

In order to determine which cellular structures might be responsible for the retarded diffusion of ADP and the cause of this very high apparent K_m, saponin-skinned fibres were treated with 0.8 M KCl to solubilize thick filaments (myosin) and to obtain so-called "ghost" fibres (Saks *et al.*, 1993). Using conditions of mitochondrial swelling in a hypo-osmotic medium (Stoner and Sirak, 1969; Kuznetsov *et al.*, 1989), disruption of the outer mitochondrial membrane was achieved in isolated mitochondria as well as in the saponin-skinned cardiac fibres. Thus, experiments were performed with thick filaments removed **or** with the outer mitochondrial membrane functionally removed. It was then possible to determine which of these structures affected ADP's ability to stimulate respiration. Further experiments were also performed with morphological and biochemical analysis to determine the extent of outer mitochondrial membrane disruption (Saks *et al.*, 1991a,b, 1993).

Incubation of skinned heart fibres in a 40 mOsM solution produces marked outer mitochondrial membrane disruption (Fig. 5.2) as shown in electron

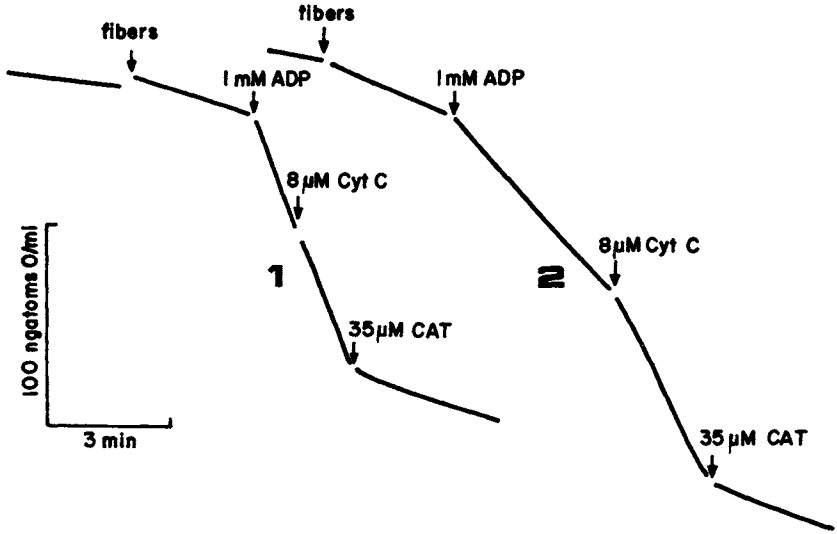


Figure 5.3 The oxygraph traces of recording of the respiration of skinned cardiac fibres before (1) and after (2) osmotic shock in the 40 mOsM solution. The respiration rates were recorded in the medium containing 125 mM KCl to detach the cytochrome *c* from the membrane and in this way to test the intactness of outer mitochondrial membrane. CAT, carboxyatractylaside.

micrographs. However, significant areas of preserved outer membrane can also be observed. After hypo-osmotic treatment there are several populations of mitochondria in the skinned fibres – those with intact and those with interrupted membranes. In order to get a rough estimate of the ratio of these populations the cytochrome *c* test was used; the results are shown in Fig. 5.3. It shows that before hypo-osmotic treatment (trace 1), addition of exogenous cytochrome *c* has no effect on the respiration of skinned fibres at maximal ADP concentrations. This relates to the intact outer membrane (endogenous cytochrome *c* is not released and conversely the exogenous cytochrome *c* cannot enter). However, after hypo-osmotic shock, the maximal rate of respiration is significantly decreased (trace 2) but can be restored by adding exogenous cytochrome *c* (8 μM). This shows that about half of the mitochondrial population has disrupted outer membranes under these conditions.

A kinetic analysis of ADP-dependence of respiration in "ghost fibres" (after removal of myosin) (Fig. 5.4) illustrates no difference between the experimental K_m before (curve and line 1) and after (curve and line 2) myosin thick filament dissolution. In both cases, the value of the apparent K_m for ADP is high (381±67 μM). Thus, the binding of ADP to myosin ATPase fails to explain the retarded ADP diffusion because the presence or absence of myosin did not change the affinity (K_m) for ADP (Kuznetsov *et al.*, 1989; Saks *et al.*, 1991a,b, 1993).

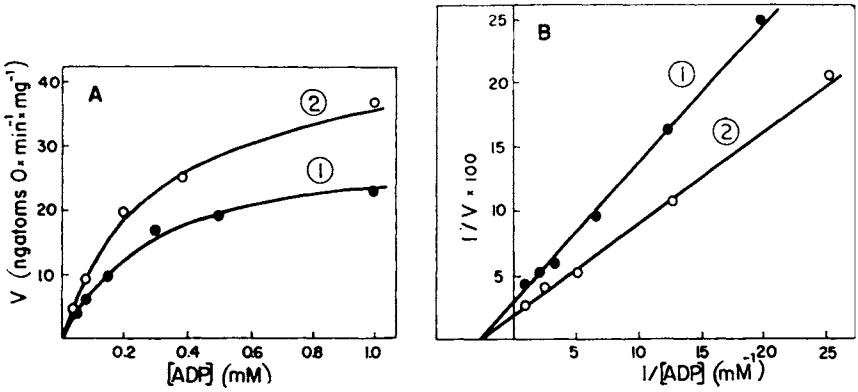


Figure 5.4 (A) The dependence of the respiration rate of the skinned and "ghost" fibres on the external ADP concentration. 1, control, skinned fibres; 2, ghost fibres, obtained after treatment of skinned fibres with 800 mM KCl solution.

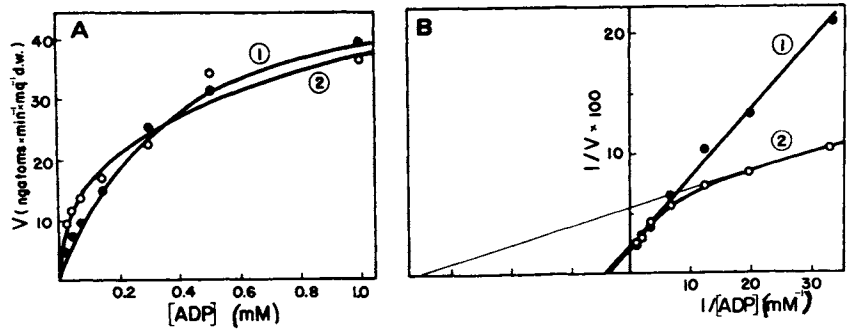


Figure 5.5 (A) The dependency of respiration rate on the external ADP concentration of the skinned cardiac fibres before (curve 1) and after (curve 2) osmotic shock in the 40 mOsM solution. (B) Linearization of the dependency shown in Fig. 5.5A in the double reciprocal plots. 1, control; 2, after osmotic shock in the 40 mOsM solution. In the latter case two different kinetics are seen corresponding to two populations of mitochondria with disrupted and intact outer membranes (Figs 5.3A and C respectively).

As shown in Fig. 5.5A, osmotic shock destroys the outer mitochondrial membrane and significantly changes the dependence of the rate of respiration upon external ADP concentration. At low [ADP], respiration is higher after osmotic shock (Fig. 5.5B). In this figure two lines are shown, reflecting two regulatory processes in these fibres, one characterized by a high apparent K_m for ADP equal to that of fibres before osmotic shock, and a second process with an apparent K_m for ADP of 35 μM (which is close to that for isolated mitochondria or for those lacking the outer membrane, see Table 5.1).

The results demonstrated in Figs 5.4 and 5.5 have led to the conclusion that

Table 5.1 Apparent K_m values for ADP in regulation of respiration in different preparations at pH 7.2.

Preparation	K_m^{app} for ADP (μM)	
	−Creatine	+Creatine
1. Skinned cardiac fibres	263.7±57.4	79±8
2. Skinned cardiac fibres in KCl, 125 mM	225±25	354±7.5
3. Skinned skeletal muscle fibres	334±54.2	105±14.9
4. Ghost cardiac fibres (without myosin)	381±67	—
5. Skinned cardiac fibres with swollen mitochondria[a]	I: 315±23; II: 32.3±5	—
6. Isolated mitochondria		
Heart	17.6±1.0	13.6±4.4
Liver	18.4	—

[a]I, control; II, osmotic shock.

the outer mitochondrial membrane is an important intracellular structure which retards ADP diffusion out of the intermembrane space (Kuznetsov *et al.*, 1989; Saks *et al.*, 1993). In the isolated mitochondria (with morphologically intact outer membrane), however, the outer mitochondrial membrane barrier function is less evident (see Table 5.1).

The high outer membrane permeability of isolated mitochondria *in vitro* to low molecular weight substances is well recognized. When first observed, it was assumed that this was just a "leaky" membrane phenomenon. Detailed studies since the mid-1980s, from several laboratories, have shown that the high permeability is due to the existence of protein pores, or voltage-dependent anion selective channels (VDAC) with pore size in the range 2 μM (Colombini, 1987; de Pinto *et al.*, 1987; Mannella and Tedeschi, 1987; Tedeschi and Kinnally, 1987; Zimmerberg and Parsegian, 1987; Kayser *et al.*, 1988; Kottke *et al.*, 1988; Benz *et al.*, 1990; Liu and Colombini, 1991). The 35 kD protein forming these pores has been isolated from different sources and characterized and cloned. In a series of publications from the laboratories of Brdiczka and Wallimann (Kottke *et al.*, 1988; Brdiczka, 1991; Wallimann *et al.*, 1992), the mitochondrial porin channels have been shown to be involved in forming dynamic contact sites between the inner and outer mitochondrial membranes. Particularly interesting is the participation of the cubic mitochondrial CK octamers at these contact sites. Such a supramolecular complex of porin–CK–adenine nucleotide translocase assumes direct channelling of substrates (ATP and ADP) and very efficient creatine phosphate (PCr) production from nascent mitochondrial ATP and cytoplasmic creatine (Saks *et al.*, 1978; Wyss *et al.*, 1992).

These results (Kuznetsov *et al.*, 1989; Saks *et al.*, 1991a,b, 1993) demonstrate the role of the outer mitochondrial membrane in regulating the diffusion of

ADP from the cytoplasm into the intermembrane space *in vivo*. Even though isolated mitochondria have morphologically well-preserved membranes, they have a high permeability to ADP (Kuznetsov *et al.*, 1989; Saks *et al.*, 1993). *In vivo*, however, mitochondrial membrane permeability is low. Thus, it is apparent that there are important regulatory factors acting on the VDAC permeability to ADP inside the cells. It is most probable that there exists an as yet unknown intracellular factor associated with the outer membrane *in vivo*, which controls permeability.

In living cells, the oncotic pressure which develops as a result of the high cytoplasmic protein concentration might be an additional factor decreasing the VDAC permeability for ADP. This means that at physiological ADP concentrations the outer membrane may be a significant barrier for ADP and thus cause diffusion limitations. This striking difference *in vitro* compared with *in vivo*, between the functional behaviour of the mitochondria with respect to ADP (see Table 5.1), supports Sjostrand's observations (1978). He studied the mitochondrial membrane system *in vivo* and *in vitro* under carefully controlled conditions using tissue fixation. Our recent work indicates that a very limited amount of cytoplasmic ADP crosses the outer mitochondrial membrane of the cells *in vivo* to activate oxidative phosphorylation [remember oxidative phosphorylation is coupled to the CK reaction (Kuznetsov *et al.*, 1989; Saks *et al.*, 1993; and see Chapter 4)]. This reaction is a powerful amplification mechanism regulating and controlling the rate of oxidative phosphorylation (Fig. 5.1). To act as a control mechanism, mitochondrial CK resides at the outside of the inner membrane to ensure direct substrate channelling (Wyss *et al.*, 1992).

3. METABOLIC OSCILLATIONS IN CYTOPLASM AS A FEEDBACK SIGNAL

The estimated concentration of free ADP in the cardiac cell cytoplasm of the normal heart is around 30–50 μM (Heineman and Balaban, 1990; Balaban *et al.*, 1986; Balaban, 1990; Katz *et al.*, 1987, 1989; Veech *et al.*, 1979). Several investigators (Balaban *et al.*, 1986; Balaban, 1990; Katz *et al.*, 1987, 1989; Veech *et al.*, 1979) have pointed out that this ADP concentration exceeds the K_m for ADP found in isolated mitochondria (about 17 μM) (Chance and Williams, 1955, 1956; Kuznetsov *et al.*, 1989). If this K_m applied *in vivo*, then the calculated ADP would keep the rate of respiration constantly close to maximum. However, the respiration rate is not this rapid *in vivo* (Balaban *et al.*, 1986; Katz *et al.*, 1987, 1989; Heineman and Balaban, 1990; Balaban, 1990). If the value of the K_m for ADP found in this work for cardiomyocytes is used (300 μM), the cytoplasmic concentration of 50 μM ADP gives a respiration rate of not more than $\frac{1}{12}$ of V_{max}. Obviously, if the ADP flux is an order of magnitude slower than that required for maximal respiration, then even

activation of the Krebs cycle and of the respiratory chain by calcium (Hansford, 1985; Katz *et al.*, 1987; Balaban, 1990; McKormac *et al.*, 1990) will not be sufficient to reach maximal respiration. Therefore, under these conditions, ADP supply will become the rate-limiting factor during periods of increased work, providing there is no system for regenerating ADP in the intermembrane space

If the K_m were to be 300 μM a dilemma arises with respect to ADP production, because in order to supply enough ADP to the adenine nucleotide translocase for maximal respiration (remembering the very low permeability of the outer membrane for ADP), ADP would have to be generated between the outer and inner membranes, in the so-called intermembrane space. This would occur in response to some stimulus from the cytoplasm and candidates for this role are CK and adenylate kinase (Saks *et al.*, 1978; Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Kottke *et al.*, 1988; Brdiczka, 1991; Wallimann *et al.*, 1992). In muscle cells, the dominating ADP-producing system may belong to the CK system.

The PCr pathway (Saks *et al.*, 1978; Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Wallimann *et al.*, 1992) [("circuit" according to Wallimann *et al.* (1992) and Wyss *et al.* (1992) and "shuttle" according to Bessman (Bessman and Geiger, 1981; Bessman and Carpenter, 1985)], requires CK to be at sites of energy production and energy consumption. We know that the mitochondrial CK reaction is driven by the translocase running unidirectionally producing PCr (Saks *et al.*, 1978; Jacobus, 1985; Wallimann *et al.*, 1992). In myofibrils it is driven in the forward direction **only** by the steady state rate of myosin ATPase in the direction of PCr utilization (Clark *et al.*, 1995). This is a kinetically favourable reaction and close spatial localization of these two proteins within the same cellular compartment makes their interaction very efficient (Ventura-Clapier *et al.*, 1987a,b; Hoerter and Ventura-Clapier, 1993). Exactly the same is true for subcellular membranes such as the sarcoplasmic reticulum and the sarcolemma (Saks *et al.*, 1978). In the cytoplasmic compartment CK operates at equilibrium (Meyer *et al.*, 1984; Bessman and Carpenter, 1985; Jacobus, 1985; Kushmerick *et al.*, 1992; Wegmann *et al.*, 1992; Wyss *et al.*, 1992; Aliev and Saks, 1993; see also Chapter 6). However, this equilibrium reaction may occur in a highly structured medium within the cytoplasm. This may be necessary because several components of the reaction (adenine nucleotides and associated energetic enzymes) exist in multiple microcompartments and so concentration gradients may exist (for review, see Glegg, 1984; Yee and Jones, 1985; Jones, 1986; Wallimann *et al.*, 1992). We may assume, however that the **total** reaction is at equilibrium throughout the cytoplasm as a result of multiple equilibrium states of cellular PCr-creatine with ATP–ADP pools.

In the cytoplasm, CK concentration (activity) is high (Ventura-Clapier *et al.*, 1987b) and the enzyme may be involved in complexes with other cytoplasmic proteins (Dillon and Clark, 1990; Clark *et al.*, 1995) (cytoskeleton,

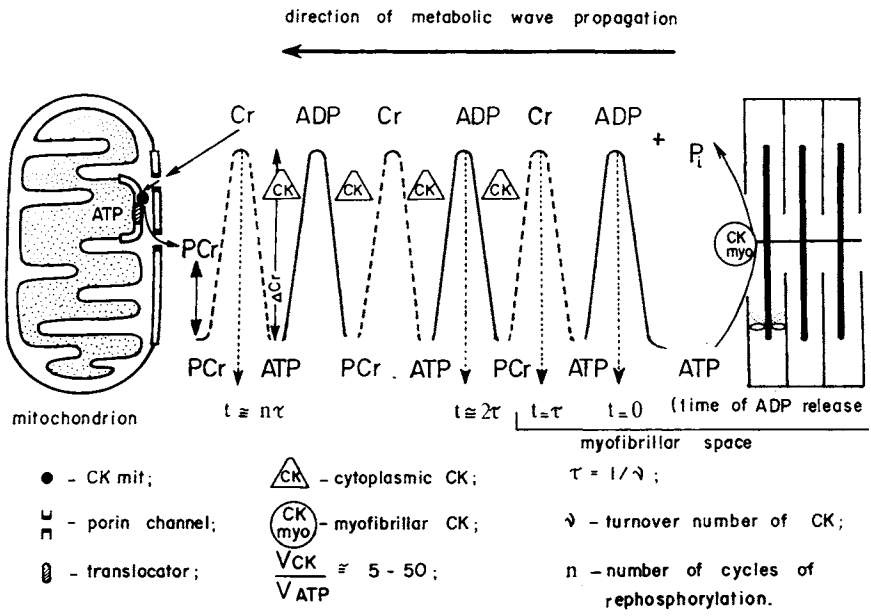


Figure 5.6 Consecutive ADP and creatine concentration changes in microcompartments associated with cytoplasmic CK result in a shift from equilibrium. This is induced by cyclic ADP liberation during contraction. The result is a wave-like feedback acting as a signal from myofibrils to mitochondria. For further explanation, see the text. CK myo, myofibrillar CK; CK (enclosed in triangle), cytoplasmic CK; $V_{CK}/V_{ATP} \cong 5-50$; ν, turnover number of CK; $\tau = 1/\nu$; n, number of cycles of rephosphorylation.

glycolytic enzymes, etc.), leading to the formation of dynamic microcompartments for ATP or ADP in the cytoplasmic space between two neighbouring CK molecules. The proposed PCr concentration changes induced by ADP production during the contraction cycle in myofibrils are illustrated (Fig. 5.6). In agreement with, and in addition to, the concept of Nagle (Nagle, 1970a; Koretsky *et al.*, 1985), this figure shows that within the myofibrillar space local ADP increases could be quickly replaced by a change in creatine concentration due to ADP rephosphorylation at the expense of PCr via the coupled CK reaction – this is the first step for signal transformation (Ventura-Clapier *et al.*, 1987a,b; Hoerter *et al.*, 1993). The next steps are sequential local cyclic changes of ADP and creatine concentrations due to phosphoryl group transfer by cytoplasmic CK (facilitated diffusion, or vectorial ligand conduction; Meyer *et al.*, 1984). Since each step brings the previous one back into equilibrium and quenches the deviation from equilibrium at that step, a single metabolic stimulus alone – alternating local changes in ADP and creatine concentrations – would propagate from myofibrils to mitochondria. The frequency of this signal is that of contraction (heart rate), but the amplitude may

decrease in the direction of the stimulus wave owing to high creatine and creatine phosphate mobility (Nagle, 1970a,b). The amplitude may also be a function of the energetic demands. In cardiomyocytes, the sarcomere contractions are synchronized and the stimuli described are distributed in all directions to all mitochondrial populations. On reaching the mitochondria, the local change in creatine concentration would stimulate the reactions leading to rephosphorylation. If the mitochondria receive a signal as a local ADP concentration change, then this signal is amplified by the coupled mitochondrial CK enzyme system. This would induce rapid PCr production and signal quenching. The result would represent a rapid and effective response to energetic demand.

It is very important that metabolic signal transduction occurs with a rate exceeding the rate of ATP turnover by an order of magnitude (Koretsky *et al.*, 1985; Jeremy *et al.*, 1993). The use of NMR magnetization transfer has given values for the rate of cardiac ATP synthesis (phosphate-ATP transfer) *in vivo* which range from 1–3 μmol/g dry weight/s, depending upon the workload (Nunnally and Hollis, 1979; Zahler *et al.*, 1987; Zahler and Ingwall, 1992; Jeremy *et al.*, 1993). This rate is close to the rate of ATP synthesis calculated from rates of oxygen uptake in isolated mitochondria (Jeremy *et al.*, 1993). The net rate of the CK reaction (ATP–PCr or PCr–ATP transfer) is, however 20–30 μmol/g dry weight/s (Nunnally and Hollis, 1979; Zahler *et al.*, 1987; Zahler and Ingwall, 1992; Jeremy *et al.*, 1993). Because magnetization transfer experiments are not able to distinguish between subcellular populations, the above results include the rates of the unidirectional mitochondrial or myofibrilar flux (Zahler and Ingwall, 1992), and cytoplasmic equilibrium CK reactions which have a value of about 20–25 μmol/g dry weight/s. In the ("simple") mathematical analysis of facilitated diffusion of ATP and PCr in cells, Meyer *et al.* showed that with CK at or near equilibrium, the PCr flux is dominant, representing more than 99% of high-energy phosphate flux (Kushmerick *et al.*, 1992). From this observation, we can conclude that the equilibrium of CK is required for rapid signal transduction between two (or more) functionally coupled CK systems in myofibrils (and at membranes) and in mitochondria, respectively.

Concerning the feedback signal transduction, it is possible that there is another similar system functioning in the muscle cells – the adenylate kinase system. Against the background of a high ATP concentration this reaction ($2ADP \rightleftharpoons ATP + AMP$) may contribute to the feedback signal which reaches mitochondria as local changes in ADP concentration (Zelenznikar *et al.*, 1990). However, because of the low permeability of the mitochondrial outer membrane for ADP, creatine is needed for signal amplification and for myocardial PCr production. This new concept is considered further by Saks *et al.* (1995).

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6

The Role of the Creatine Kinase/Creatine Phosphate System Studied by Molecular Biology

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Key words: creatine, creatine kinase, creatine phosphate, β-guanidinopropionic acid, mutation, NMR

1. INTRODUCTION

Many functions have been assigned to the creatine phosphate (PCr)/creatine kinase (CK) system and some of these are described in Chapters 2, 4, 5 and 7. Little work has been performed, however, to determine how essential the CK system is in the normal function of the entire organism. Basically, there are two ways in which one can evaluate the requirement for a functioning CK system *in vivo*. The first method involves altering the intracellular substrates in order to short-circuit the system. This has been done using various creatine analogues such as β-guanidinopropionic acid (β-GPA), cyclocreatine and β-guanidinobuteric acid (Zweier *et al.*, 1991; Clark *et al.*, 1994; Boehm *et al.*, 1995). Creatine analogues act as competitive CK inhibitors owing to their different kinetics and affinities (Wallimann *et al.*, 1992; Clark *et al.*, 1994). Another technique for examining the CK system *in vivo* is to genetically manipulate the tissue isoenzymes. This has been achieved by Koretsky and co-workers (Koretsky *et al.*, 1990; Miller *et al.*, 1993) who **added** brain-type CK to

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liver (Brosnan *et al.*, 1990) and skeletal muscle (Brosnan *et al.*, 1993), while van Deursen *et al.* (1993) made deletions of the CK gene itself and studied the changing metabolism. These are fruitful avenues for basic science research and are addressed here.

Recent reports show that there are two sub-isoforms of the B monomer (Soldati *et al.*, 1990; Wirz *et al.*, 1990). Interestingly, the chicken brain-type CK appears to have multiple isoforms produced by different initiation codons. This gives rise to distinct amino termini of the B monomer. Alternate splicing of the *N* terminus can also produce different sub-isoforms of the B isoenzyme (Wirz *et al.*, 1990). It is unclear if the different *N* termini produce different subcellular localizations of the enzymes or an alteration in function, but subcellular compartmentalization of isoenzymes of CK has been proposed by many authors (Clark *et al.*, 1992, 1993; Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Boehm *et al.*, 1995).

When BB-CK is added to the livers of mice, the livers are able to synthesize and utilize PCr in a manner similar to muscle (Miller *et al.*, 1993; Mason and Quistorff, 1994). The phosphagen pool is doubled and the livers are better able to buffer ATP via CK and PCr. In contrast, overexpression of BB-CK by muscle did not change the mouse muscle ATP or free creatine concentration. However, increased molecular exchange between PCr and ATP occurs, as shown by magnetization exchange experiments (Brosnan *et al.*, 1993).

2. TRANSGENIC KNOCK-OUT

The technical details relating to genetic knock-out of a gene coding for a specific enzyme are discussed at length in the works of van Deursen *et al.* (1993, 1994b). Using genetic manipulation, these authors completely removed all of the M-CK present in mouse muscle. This mutation was characterized using NMR and exercise experiments as well as feeding the creatine analogue β-GPA. van Deursen and co-workers also developed a series of mutations with graded expression of M-CK.

After confirming that they had indeed produced a benign null mutant lacking the M-CK gene, van Deursen *et al.* (1993) characterized the metabolic changes which occurred in these M-CK-deficient animals. Such animals are healthy and upon visual observation develop (Wallimann, 1994) and reproduce normally.

3. MUSCLE PERFORMANCE

Though there was no change in absolute force generated, the skeletal muscles were found to lack burst activity. Thus despite being able to generate maximal tension, they were unable to generate or maintain normal force. However, no

Table 6.1. Metabolites and enzymes in mouse muscle.

	Control	M-CK-deficient	β-GPA–	β-GPA+
P_i[a]	0.058±0.017	0.051±0.009		
PCr[a]	0.449±0.029	0.466±0.022		
ATP[a]	0.158±0.013	0.158±0.009		
pH[a]	7.18±0.08	7.19±0.09		
Cr	9.31±0.68	6.84±0.58[b]	2.1±0.5	3.0±0.9
PCr	16.22±1.4	21.67±1.8[b]	9.0±1.2	3.3±1.6
ATP	7.88±0.5	6.95±0.64	5.8±0.38	6.34±68
COX	604±28	1094±230-[b]	1379±208	836±190
CS	127±32	231±27[b]	349±41	230±41
NADH oxred	47±11	71±11[b]		
Succ C oxred	68±10	111±22[b]		
Glycogen	26.1±5.8	42.3±7.2[b]	57.6±12.3	74.7±16
Lactate			3.1±1.0	2.7±0.7

[a] NMR values of peak areas in arbitrary units.
[b] Significantly different from wild-type.

Enzyme activity in mU/mg protein. Metabolites are given in μmol/g wet weight.
COX, cytochrome *c* oxidase; CS, citrate synthase; NADH oxred, NADH-O_2 oxidoreductase; Succ C oxred, succinate-cytochrome *c* oxidoreductase. β-GPA + and – are M-CK-deficient mutants with and without β-GPA feeding, respectively (van Deursen, 1994, with permission).

change developed in the size or distribution of Type I and II fibres. Also, Type IIa and b fibre types were unaltered and so the lack of burst activity does not appear to result from altered fibre type. One possible explanation for the lack of burst activity is that the maximal force falls because of a lowered capacity to maintain ATP owing to the lack of M-CK. This observation supports a role of M-CK as an energy buffer for short-term skeletal muscle activity (see Chapter 1).

The Type II fibres had an increased intermyofibrillar mitochondrial value and an increased capacity for glycolysis and glycogenolysis. This is shown by the changes in key metabolic enzymes and metabolites which are summarized in Table 6.1.

The increased cytochrome *c* oxidase, citrate synthase, NADH-O_2 oxidoreductase and succinate-cytochrome *c* oxidoreductase is evidence for increased mitochondrial volume in the cell. Interestingly, PCr and ATP levels were found to be normal in M-CK-deficient muscle while the glycogen content was elevated. The increased glycogen was associated with an increase in consumption during exercise but with **normal** lactate production. Elevated [PCr] may result from synthesis by the Mi-CK, but an absence of PCr degradation would be due to the lack of cytoplasmic M-CK.

NMR spectroscopy of the mouse muscle was performed, and it was found that the M-CK-deficient muscle was indistinguishable from control (Fig. 6.1). During magnetization exchange experiments, however, there were striking

differences. No exchange was seen between PCr and ATP in the deficient mice whereas exchange was observed in the controls. Despite the lack of NMR observable exchange, there was a clear fall in [PCr] during exercise confirming that hydrolysis of PCr takes place to maintain ATP. This "normal" fall in [PCr] during exercise was concomitant with a rise in orthophosphate (P_i) and an apparent buffering of [ATP]. Thus PCr utilization appears to occur normally even without M-CK. This indicates that Mi-CK produces ATP from PCr and ADP upon demand. Normally, however, Mi-CK functions to produce PCr (Chapter 4).

It is not known why no exchange was detected with magnetization exchange experiments while [PCr] hydrolysis was normal. The hydrolysis of PCr may be due to Mi-CK which is present and active in the mitochondria. This hydrolysis may occur only during periods of energetic demand, such as exercise. It explains the lack of observable NMR exchange but would be contrary to the observation of Saks as discussed in Chapter 5, who proposes that Mi-CK is functionally coupled to oxidative phosphorylation. Functional coupling to oxidative phosphorylation is observed under normal conditions and is abolished in some pathologies. Therefore uncoupling Mi-CK from oxidative phosphorylation in this case remains plausible.

Another observation from the M-CK-deficient mice was found with the administration of the creatine analogue β-GPA. In the M-CK-deficient muscle (which did have Mi-CK activity), there was no accumulation of β-GPA-P after 10 weeks of β-GPA feeding. Previous studies have shown that such feeding produces 90% replacement of PCr with β-GPA-P (Shoubridge and Radda, 1984, 1987). The β-GPA did cause a 70% and 60% decrease in PCr and Cr, respectively and a 15% fall in ATP. The muscles from these animals demonstrated decreased force, but were resistant to fatigue. Key oxidative enzymes also increased (see Table 6.1). Thus the M-CK-deficient mouse skeletal muscle is capable of adapting to the severe creatine and PCr depletion caused by β-GPA administration. Whilst the failure of Mi-CK to phosphorylate β-GPA has been reported (Clark *et al.*, 1994), the importance of the transgenic experiment is that the CK-deficient and Cr-depleted animals were still capable of adapting to severely compromised energy metabolism (van Deursen *et al.*, 1994a).

4. GRADED CK KNOCK-OUT

In one study, the content of M-CK which was present and active in mouse muscle was manipulated in a graded fashion and the effects of the graded M-CK content studied (van Deursen, 1994). The resulting five mutations had M-CK activities at levels of 100%, 50%, 34%, 16% and 0% compared to normal. Table 6.2 summarizes the metabolite and enzyme changes seen.

There was a decrease in the ability of the muscle to perform burst activity

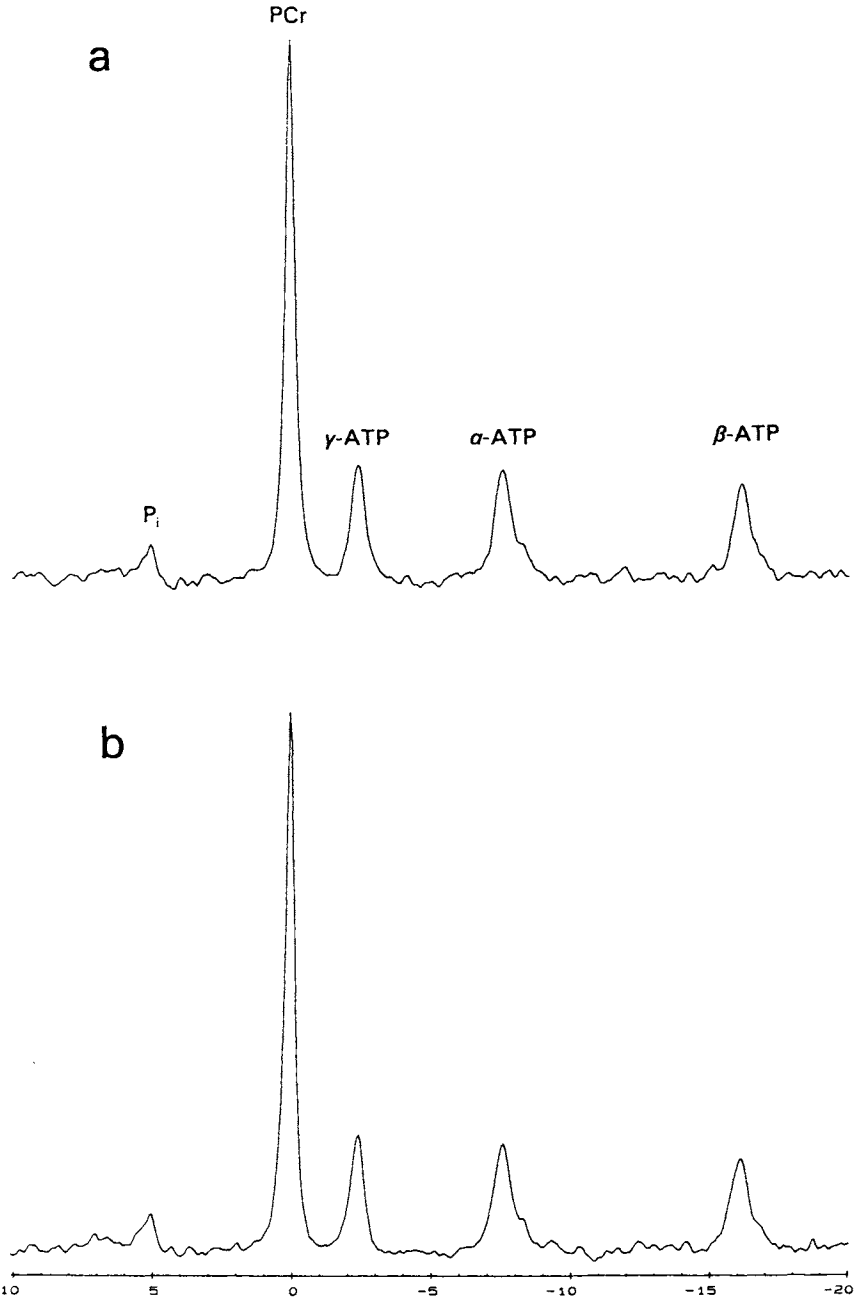

Figure 6.1. (a) Spectrum from the wild-type mouse muscle at rest obtained with ^{31}P NMR. The peak position (chemical shift) of the P_i peak is used to determine the intracellular pH. (b) Spectrum from the M-CK-deficient mouse muscle at rest. Peak areas are reported in arbitrary units in Table 6.1. The ADP concentration can be calculated using the equilibrium constant for CK, the total creatine concentration, and by obtaining the pH, [ATP] and [PCr] from the NMR spectra.

Table 6.2. Enzymes and metabolites in graded M-CK-deficient mouse muscle.

	100% M-CK	50% M-CK	34% M-CK	16% M-CK	0% M-CK
P_i[a]	5.8±1.7	4.9±0.5	4.6±1.3	4.6±0.8	5.1±0.99
PCr[a]	44.9±2.9	47.0±1.6	46.7±0.6	44.2±1.2	6.6±2.2
ATP[a]	16.4±1.6	16.0±2.3	16.4±1.6	17.0±2.1	16.1±1.4
ATP	7.9±0.5	7.7±0.7	7±0.4	7.0±0.6	7.0±0.6
COX	604±28	551±96	730±118	776±112	1094±230
CS	127±32	148±23	153±12	181±20	231±27
Glycogen	26.1±5.8	nd	18.0±7.6	19.7±5.4	42.3±7.2
Glycogen[b]	17.3±4.1	nd	12.7±3.8	14.0±4.0	25.3±3.5
K_f(1/s)	0.95±0.12	0.79±0.05	<0.05	<0.05	<0.05
$Flux_f$[c]	7.51±1.06	6.08±0.67	<0.38	<0.38	<0.38

[a] NMR values of peak areas in arbitrary units.
[b] Glycogen concentration after 3 min of muscle stimulation at 5 Hz.
[c] Flux in μmol/g wet weight/s.

Enzyme activity in mU/mg protein, N=6 or more for all values (van Deursen, 1994). Metabolites are given in μmol/g wet weight. COX, cytochrome *c* oxidase; CS, citrate synthase.

which correlated with the level of M-CK expression. This close correlation of burst activity and M-CK levels indicates the importance of M-CK for maximizing muscle activity. The homodimer MM-CK is localized at the contractile proteins and may act to maintain a high ratio of ATP/ADP (see Chapter 4). In the M-CK-deficient mouse, local ATP levels are prone to fall while the ADP levels rise. The effect on the contractile apparatus could therefore be seen as decreased burst activity. Contractile function may be altered by either a poor supply of ATP and/or high [ADP] slowing cross-bridges.

The other striking observation was that the flux of energy through the CK reaction is not visible with magnetization exchange in the mutants with 0–34% of the M-CK activity. Figure 6.2 demonstrates a typical magnetic exchange experiment (using inversion transfer) from wild-type and M-CK-deficient mouse muscle. No exchange is detectable between PCr and ATP as seen in Fig. 6.2F, because upon PCr inversion, the γ-ATP resonance amplitude does not decrease. But, at 50% of M-CK activity, essentially 100% exchange is observed. One possible explanation for these findings is that the ^{31}P NMR might be detecting the flux through a pool of CK which is free or soluble in the cytoplasm. Low levels of CK mean that a larger percentage of M-CK would be bound to contractile proteins and other sites and such bound M-CK might be less able to contribute to NMR visible exchange between PCr and ATP. Therefore soluble M-CK levels may be too low to catalyse exchange between PCr and ATP at a rate which is rapid enough to be visible with magnetic exchange experiments. Such a hypothesis is supported by the results of Koretsky and co-workers (Brosnan *et al.*, 1993) with mouse muscle where CK activity was increased to 150% of normal. In their mice, the observable

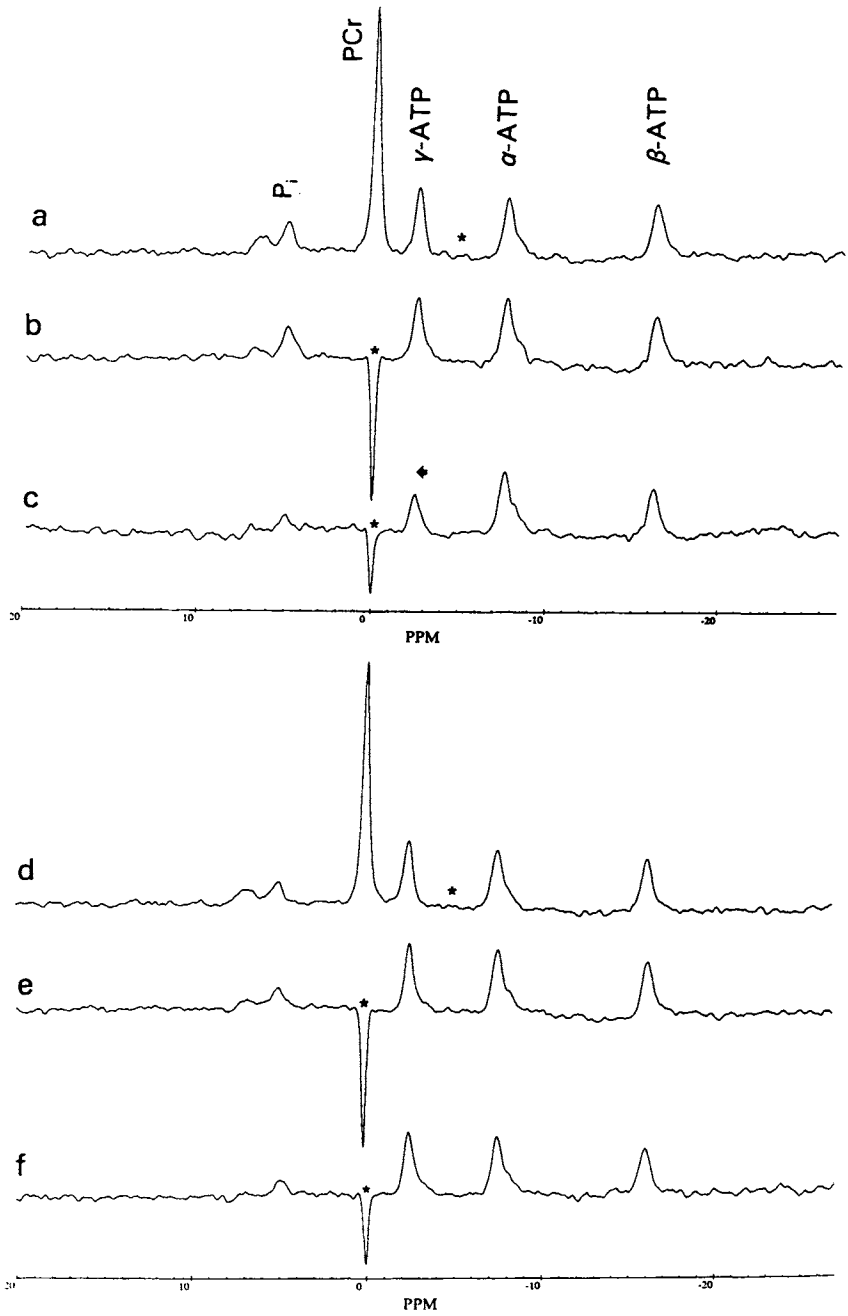

Figure 6.2. (a–c) Inversion transfer experiment from wild-type mouse muscle. (e–g) Inversion transfer experiment of M-CK-deficient mouse muscle. (a) and (d) are control spectra with selective inversion centred at *. (b) and (e) are obtained 0.5 ms after inversion of PCr. (c) and (f) are obtained 500 ms after inversion of PCr. Exchange between PCr and ATP is seen in the wild-type as a decrease in the intensity of the γ-ATP and is indicated by the arrow. No exchange is seen in (f), indicating no significant exchange between PCr and ATP in the M-CK-deficient mouse muscle.

magnetic exchange was twice that of controls. These results indicate that the relationship of bound to unbound CK may affect the ^{31}P NMR measurements of enzyme-catalysed exchange between PCr and ATP since increased free CK increased the NMR visible exchange.

5. TRANSGENIC OVEREXPRESSION

In the study by Brosnan *et al.* (1990, 1993), CK was overexpressed in the mouse muscle. These experiments were performed by adding BB-CK to a plasmid vector and incorporating it into the mouse to increase CK activity as well as adding an isoenzyme that is not normally expressed in healthy muscle. They found that muscle containing 150% of the control CK activity displayed 200% of the normal flux through the CK reaction (as measured with saturation transfer). In control muscle, 100% of the CK activity was MM-CK, while the transgenic mice had 60% MM-CK, 32%, MB-CK and 8% BB-CK (none of their studies reported Mi-CK). Increased CK did not change muscle metabolites but did increase the exchange observed between the metabolites. The altered isoenzyme distribution is consistent with a random interaction and association of the isoenzymes in the cytoplasm. There was no increase in total MM-CK activity, hence all the additional activity was due to B-CK being distributed randomly with M-CK. ADP was calculated with the CK equilibrium and was also found to be unchanged, as were ATP, PCr, total creatine and pH.

6. SUMMARY

Mouse skeletal muscle which is deficient in M-CK lacks burst activity but maintains the ability to hydrolyse PCr as an energy source to buffer ATP during periods of muscle activity. Tissue lacking M-CK is able to respond to creatine depletion and shows some adaptation. These results indicate that the CK system and PCr metabolism have functions that cannot be removed by deletion of the M-CK gene. Future experiments are indicated on muscle deficient in Mi-CK accompanied by (if the mutant is viable) M-CK deletion so as to gain insight into the importance of PCr and CK in muscle metabolism.

ACKNOWLEDGEMENTS

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7

Molecular and Cellular Mechanisms of Action for the Cardioprotective and Therapeutic Role of Creatine Phosphate

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Key words: ADP, PCr, ATP, membrane, heart

1. INTRODUCTION

In the era of thrombolytic therapy and reperfusion, the development of new strategies for cardioprotection is an important therapeutic goal (Hearse 1980, 1990; Reimer and Jennings, 1986; Julian *et al.*, 1989; Jennings *et al.*, 1990; Piper, 1990). Such strategies are also applicable in cardiac surgery for intra-operative mycardial protection and for the protection of the allograft during

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transplantation (Hearse, 1980; Semenovsky *et al.*, 1987). One agent which shows promise in this respect is creatine phosphate (Neoton).

Creatine phosphate (PCr) was discovered by Eggleton and Eggleton in 1927 (see Ennor and Morrison, 1958, for review), and the discovery predated that of ATP. It has taken more than 50 years of intensive study to understand various aspects of the physiological function of this molecule. It is now accepted that PCr has at least a dual role in cells with high energy turnover such as in the heart, skeletal muscle, brain, retina, spermatozoa and nerve endings (Saks *et al.*, 1978; Bessman, 1985; Jacobus, 1985; Wallimann *et al.*, 1989). The intracellular PCr content is high and ranges from 2 to 30 mM (Dawson, 1983; Gard *et al.*, 1985). Evidence to date suggests that it acts as an energy (ATP) buffer (Mommaerts, 1969) and also as an intracellular energy carrier (Saks *et al.*, 1978; Bessman, 1985; Jacobus, 1985; Wallimann *et al.*, 1989).

In pathological states related to energy deficiency (ischaemia, anoxia or toxic cardiomyopathies) PCr becomes depleted well before there is a fall in ATP (Neely *et al.*, 1973; Reimer and Jennings, 1986; Jennings *et al.*, 1990). Rapid changes of PCr content under these conditions have led to the proposition that extracellular PCr may be able to compensate, at least to some extent, for the energy deficiency (Breccia *et al.*, 1985). The impermeability of membranes to the charged PCr molecule would appear to argue against such a proposal. However, the results of the studies on administered PCr demonstrate significant effects and have led to the concept that the efficiency of exogenous PCr is not related to its participation in the energy metabolism of the cell but to membrane-stabilization and to an effect on the microcirculation (Marshall and Parratt, 1974; Rosenshtraukh *et al.*, 1979; Fagbemi *et al.*, 1982; Sharov *et al.*, 1986, 1987; Saks *et al.*, 1987a; Ruda *et al.*, 1988; Tronconi and Saks, 1989; Parratt and Marshall, 1990; Perepetsh *et al.*, 1990).

2. PHARMACOLOGICAL EFFECTS OF EXOGENOUS PCr

In experiments *in vitro*, the main effect found with exogenously administered PCr is significant protection of contractile function of the isolated ischaemic heart as estimated both by better recovery of developed tension and by a rapid decrease in end diastolic pressure (Fig. 7.1). This effect was first discovered by Parratt and Marshall (Marshall and Parratt, 1974; Parratt and Marshall, 1990) and confirmed by Robinson *et al.* (1984) and by others (Sharov *et al.*, 1986; Saks *et al.*, 1987a; Afonskaya *et al.*, 1989; Borgoglio *et al.*, 1982; Seppet *et al.*, 1988; Courtois *et al.*, 1990; Robinson *et al.*, 1988).

Figure 7.1 shows the data reported for the isolated perfused rat heart (Saks *et al.*, 1988). In the control preparation, only 20% of the contractile force is restored upon reperfusion after 60 min of ishaemia, whereas in the presence of 10 mM PCr the functional recovery is more than 80%. Thus, PCr provides a

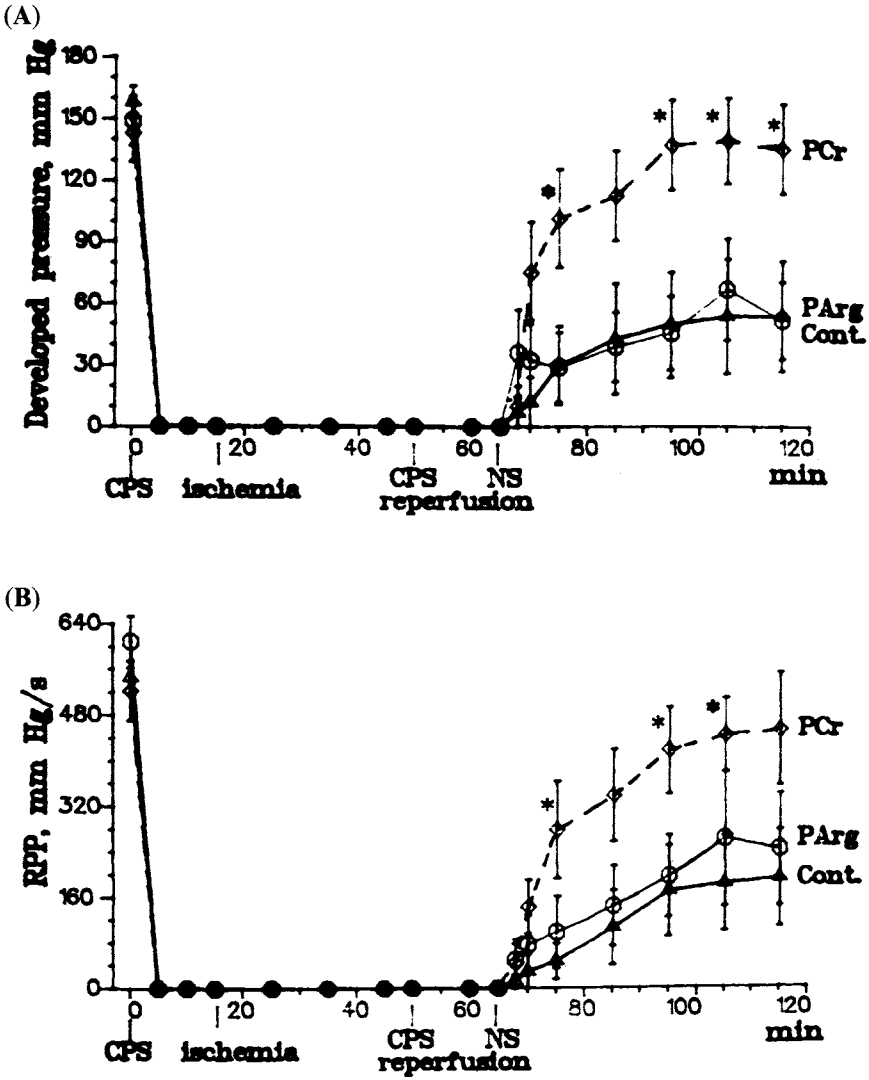


Figure 7.1. Recovery of developed tension (A) and resting tension (B) of rat heart after 60 min of ischaemia in the presence of PCr. From Konorev *et al.* (1991) with permission.

striking protective effect under such controlled conditions (Saks *et al.*, 1987b; Sharov *et al.*, 1987; Seppet *et al.*, 1988). Of particular importance in relation to cardiac function is the complete relaxation observed (decrease of resting tension to the preischaemic values) and also the absence of reperfusion contracture (Saks *et al.*, 1988; Seppet *et al.*, 1988; Konorev *et al.*, 1991). This beneficial effect of PCr on diastolic function is especially worthwhile since the

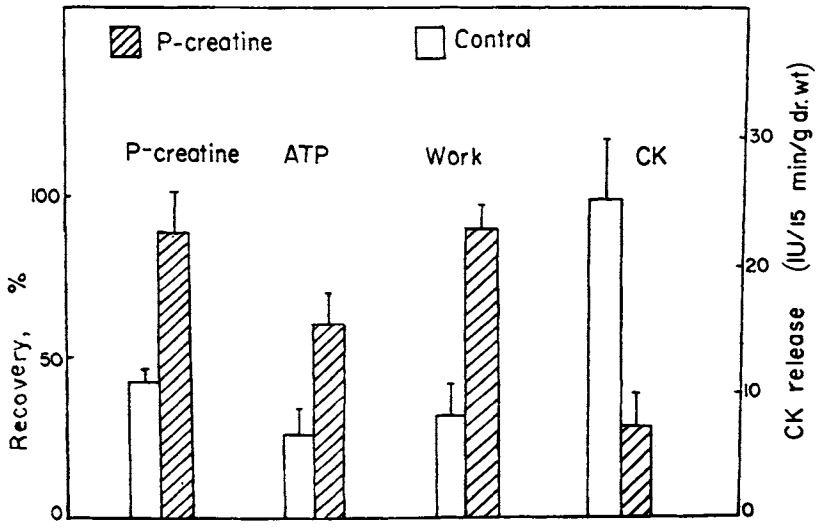


Figure 7.2. The effect of exogenous PCr on the high-energy phosphate content, contractile function of perfused rat heart and release of CK after 35 min of total ischaemia at 37°C and 30 min of reperfusion. From Sharov *et al.* (1987) with permission.

relaxation phase determines cardiac filling and cardiac work (Robinson *et al.*, 1984).

The second effect of exogenously administered PCr is metabolic protection and intracellular ATP and PCr preservation (Fig. 7.2). There is a several-fold decrease in creatine kinase (CK) release during postischaemic reperfusion which is similar to that documented by Robinson *et al.* (1984). This is consistent with cardioprotection due to maintenance of membrane structure and cellular integrity (Saks *et al.*, 1987a; Sharov *et al.*, 1987) because release of CK results from membrane damage. More information on cardioprotection with PCr is discussed in Chapter 12.

3. CALCIUM DEPENDENCE OF THE ANTI-ISCHAEMIC EFFECT OF PCr

Robinson *et al.* (1988) proposed that an anti-ischaemic effect of PCr may be linked to its ability to bind Ca^{2+} ions in perfusion media and so the role of Ca^{2+} ions in the cardioprotective effect of PCr was recently studied by Konorev *et al.* (1991). This report demonstrated that PCr had a particular protective advantage in this respect since the structural analogue of PCr, phosphoargenine (PArg) (which possesses a similar Ca^{2+} binding property as PCr) **did not** exert any cardioprotective effects (Fig. 7.1; Saks *et al.*, 1988).

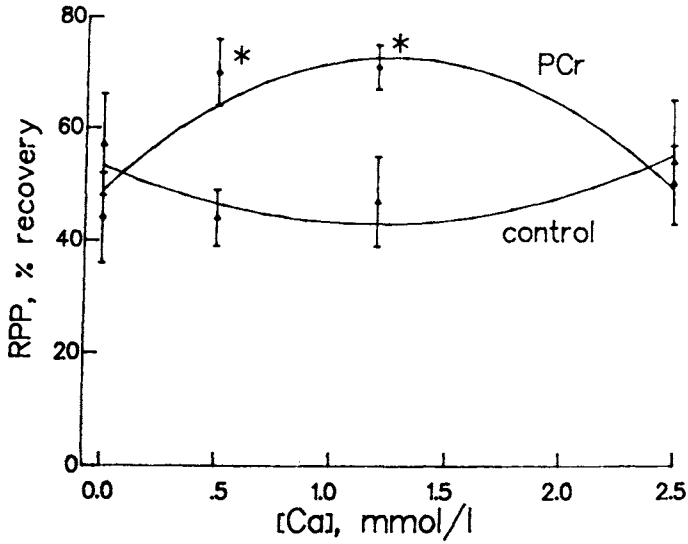


Figure 7.3. Dependence of postischaemic recovery of rate–pressure product index RPP upon concentration of $CaCl_2$ in modified K-H solution. RPP, rate–pressure product; $p<0.05$ compared with control group. Curves are for experiments in the presence and absence of exogenous PCr.

In studies on the effect of PCr on contractile recovery of the isolated rat heart subjected to ischaemia in the presence of varied Ca^{2+} concentrations (Konorev *et al.*, 1991), PCr failed to exert a positive effect in a Ca^{2+}-free medium. But when 0.5 mM $CaCl_2$ was added to hearts subjected to ischaemia for 30 min, the postischaemic contractile performance of these hearts recovered much better in the PCr group than in controls (Konorev *et al.*, 1991). Experiments using modified Krebs Hensleit (K-H) solutions containing 1.2 and 2.5 mM $CaCl_2$ were also performed. The degree of protection [represented as a percentage of rate–pressure product (RPP) recovery upon reperfusion] was plotted against the Ca^{2+} content in modified K-H buffer (Fig. 7.3). The measurements of the RPP recovery in the PCr groups were not different from controls at 0 and 2.5 mM Ca^{2+}, but were significantly different at 0.5 and 1.2 mM Ca^{2+}. This indicates that there is an optimum Ca^{2+} concentration (around 1.2 mM) at which the pronounced protective effect of PCr is observed (Fig. 7.3).

Two conclusions are drawn from these results. First, chelation of some Ca^{2+} by PCr does not explain the phenomenon because at the optimal Ca^{2+} concentration (1.2 mM) chelation of Ca^{2+} diminishes the protective effect. Also, PArg (which chelates Ca^{2+}) does not protect the ischaemic heart (Saks *et al.*, 1988). The chemical structure of the PCr molecule may be key to the protective effect since in control experiments it was demonstrated that inorganic phosphate and creatine (which are the separate constituents of PCr) did not

afford **any** protection on the ischaemic myocardium (Robinson *et al.*, 1984; Paratt and Marshall, 1990). The second conclusion, however, is that Ca^{2+} is important for the molecular mechanism of PCr protection on the ischaemic myocardium and this is considered in detail below.

4. PROTECTION OF THE HEART FROM OXIDATIVE DAMAGE

Ronca *et al.* (1989), Zucchi *et al.* (1989) and also Conorev *et al.* (1991) have shown that functional protection is afforded by PCr against another type of injury – oxidative damage caused by perfusion of isolated heart with H_2O_2 (90–190 μM). This perfusion solution simulates damage caused by lipid peroxidation due to active oxygen free radical formation during postischaemic reperfusion (Piper, 1990).

The oxidative stress first induces an elevation of diastolic pressure associated with lipid peroxidation by H_2O_2-generated radicals (Fig. 7.4). H_2O_2 perfusion of the isolated rat heart results in a gradual elevation of resting tension. It appears to be irreversible since the contracture is not abolished upon H_2O_2 withdrawal (Fig. 7.4A). Creatine phosphate, however, completely prevents the increase in resting tension during 40 min of H_2O_2 perfusion. After 20 min, control heart contractility decreases rapidly and this effect coincides with an elevation of the diastolic pressure. H_2O_2 withdrawal did not lead to contractile recovery amongst controls while in the PCr group the maximal rates of contraction and relaxation were fully restored, and the developed pressure and the RPP slowly returned to their initial values (Fig. 7.4B). These findings agree with those reported by Zucchi *et al.* (1989), who also showed that PCr prevented the rapid formation of malondialdehyde, a lipid peroxidation product. This effect was not reproduced by creatine or inorganic phosphate used separately. Also, PCr delayed the leakage of cardiac enzymes (Zucchi *et al.*, 1989). Such observations favour the proposal that PCr protects cell membranes against lipid peroxidation.

The potent protective effect of PCr against oxidative damage raises the question as to whether PCr itself may possess antioxidative activity, or whether this effect is indirect owing to changes in membrane properties induced by PCr. To examine this question, studies were performed on the combined effect of PCr and of the conventional antioxidant t-tocopherol phosphate (TPP) (Conorev *et al.*, 1991). The combined action of PCr and TPP on the isolated rat heart subjected to ischaemic arrest resulted in an additive protection (Fig. 7.5). From these experiments it is concluded that the two agents exert their effects through different mechanisms and that PCr is not acting solely as an antioxidant (Fig. 7.5B).

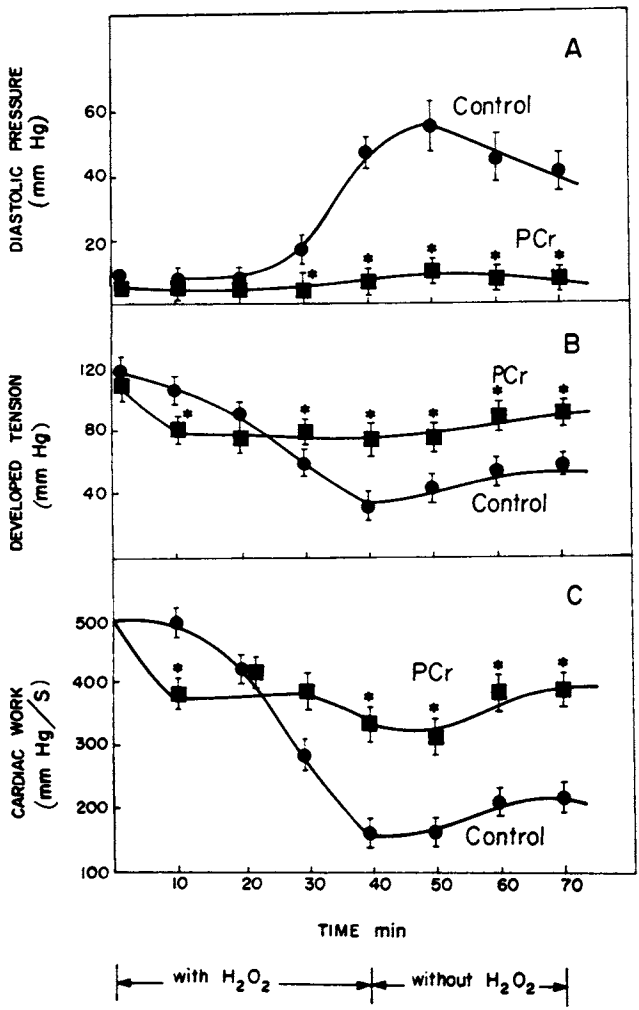
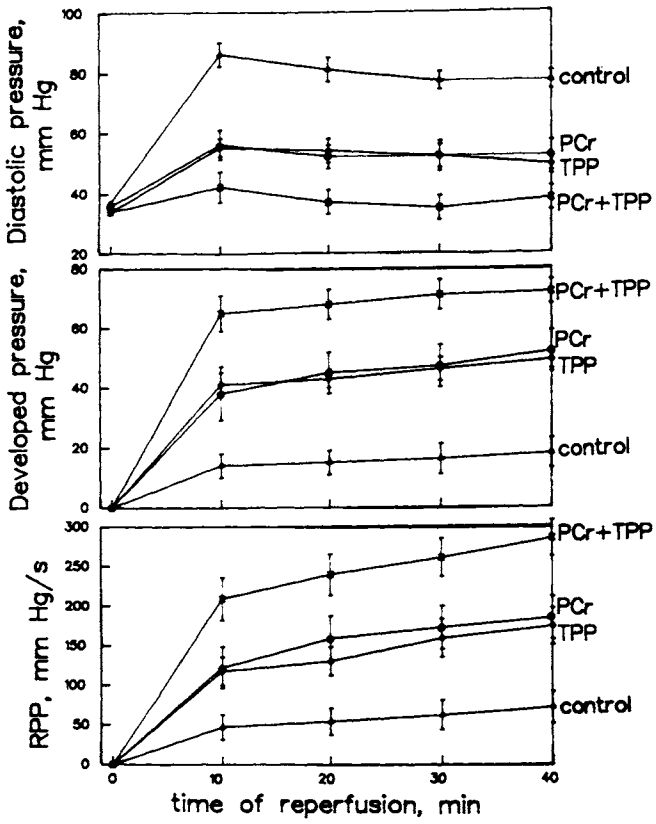


Figure 7.4. Effect of exogenous PCr on contractility of isolated rat heart (isovolumic model) during and after perfusion with 90 μM H_2O_2 in Krebs–Henseleit solution. From Conorev *et al.* (1991) with permission.

5. EFFECT OF PCr ON PHOSPHOLIPID METABOLISM

The products of phospholipid degradation, lysophosphoglycerides (LPG) [mostly lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE)], are rapidly elevated in the ischaemic zone of the rabbit heart (Conorev *et al.*, 1991). Such accumulation (Fig. 7.6) may be one of the major factors contributing to electrical instability in the ischaemic myocardium and

(a)



(b)

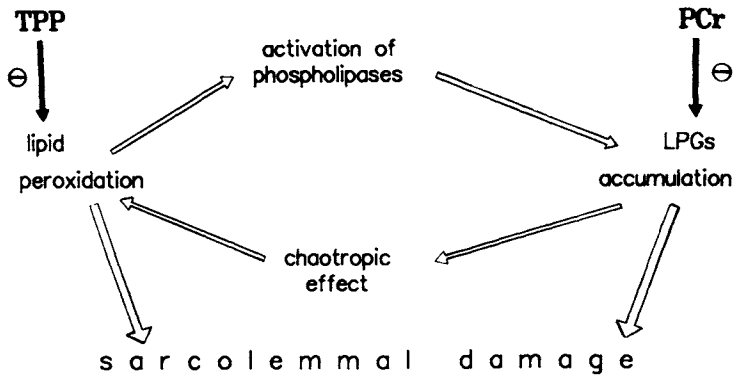


Figure 7.5. (a) Effects of PCr, tocopherol phosphate (TPP), and their combination on the mechanical recovery of the isolated rat heart after normothermic cardioplegic ischaemic arrest. RPP, rate–pressure product (developed pressure × heart rate). Values are means, bars are ± SE. (b) Schematic representation of the possible mechanism of PCr protective effect on the ischaemic myocardium, which is additive to the action of the antioxidant, TPP.

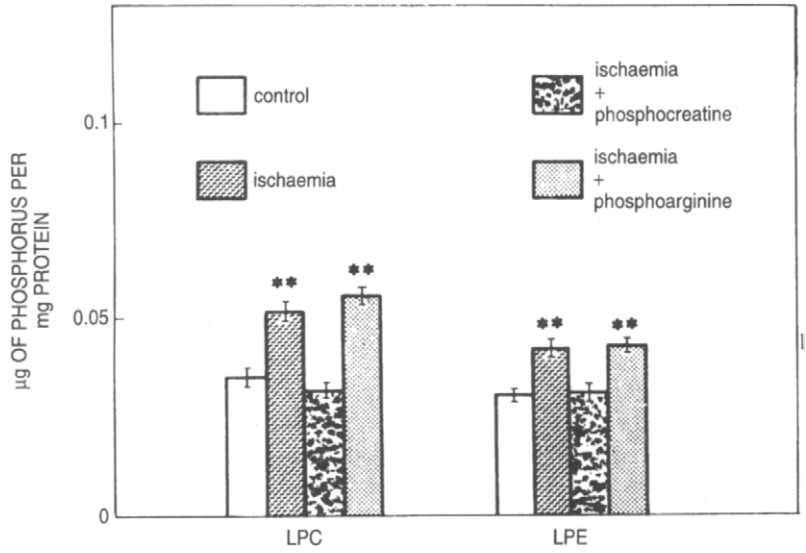


Figure 7.6. Changes in tissue content of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) in the crude sarcolemmal fraction of the heart after 8 min of ischaemia.

to sarcolemmal structure disintegration. The above effects are compounded by the loss of cytosolic components and also by the detergent activities of LPC, whose accumulation further destroys the membranes. Agents which decrease the lysophosphoglyceride accumulation in ischaemic myocardium might also be expected to protect the myocardium from electrophysiological and structural damage. Figure 7.6 demonstrates this activity of PCr. The figure shows that LPC elevation after 8 min of total ischaemia was completely prevented by PCr. It is notable that phosphoarginine did not affect the accumulation of LPG (Fig. 7.6). These observations show that PCr directly affects the metabolism of phospholipids in the membrane and eliminates highly arrhythmogenic intermediates of phospholipid metabolism, the relevance of which is discussed in detail elsewhere (Marshall and Parratt, 1974; Fagbemi *et al.*, 1982; Hearse *et al.*, 1986; Ruda *et al.*, 1988; Afonskaya *et al.*, 1989; Sharov *et al.*, 1989).

6. OTHER BIOCHEMICAL EFFECTS RELATED TO THE UPTAKE OF PCr

Several authors have studied the problem of PCr penetration into the cardiomyocytes through the sarcolemma. Dawn *et al.* (1983), Breccia *et al.* (1985) and Ronca and Ronca-Testoni (1987) found that the administration of ^{32}P-PCr, or

double-labelled PCr during experiments *in vitro*, led to the incorporation of labelled P into ATP and an increased tissue ATP concentration. However, the ATP content rose only by 1–2 μmol/g dry weight over 60–120 min (Dawn *et al.*, 1983; Breccia *et al.*, 1985; Ronca and Ronca-Testoni, 1987), or at a rate of approximately 20 nmol/g dry weight/min. This very low rate of tissue uptake was verified in *in vitro* experiments by Preobrazhensky *et al.* (1986), who found it to be less than 200 nmol/g dry weight/min – a rate which was only increased by a factor of 2 after ischaemia. Such a rate is about 3–4 orders of magnitude lower than the rate of ATP turnover in the working heart (Jacobus, 1985). This indicates that extracellular PCr cannot maintain the intracellular energy metabolism. If PCr penetrates at such a very low rate, it may only be important in maintaining some subsarcolemmal pools of PCr or ATP where it would affect the metabolism of adenine nucleotides. Thus, one may conclude that significant protection of ischaemic myocardium by extracellular PCr may be possible if it is able to stabilize the sarcolemma even without significant penetration into the cells. Its ability to delay enzyme release described above supports this possibility.

7. PHARMACOKINETICS OF PCr UPTAKE BY DIFFERENT TISSUES

Direct determination of the ability of PCr to penetrate from the extracellular space into cardiac cells shows that this process is very slow (Preobrazhensky *et al.*, 1986). The pharmacokinetics of PCr after a single dose injection (Fig. 7.7) is biphasic with clearance half-times of 7 and 50 min (Sharov *et al.*, 1986). Despite an initially rapid clearance phase the urine concentration rises to a plateau at 60 min. Tissue analysis shows that PCr is specifically absorbed into the heart (though transiently) as well as into the brain and skeletal muscle (tissues where its physiological role is important). It is not taken up by the lungs or liver (organs in which it has no known metabolic function) (Wallimann *et al.*, 1989). Such specificity may be directly related to the structural organization of phospholipid membranes. Also, the affinity of such extracellular binding sites for PCr may be very high and regardless of the very rapid clearance, PCr may remain bound to the tissue (Sharov *et al.*, 1986). Thus it can not be ruled out that PCr may be effective even in very low doses though because of rapid clearance rather high doses are needed – in the case of acute myocardial infarction, 5–10 g per day.

8. DIRECT EFFECT OF PCr ON THE CELL MEMBRANE

Sharov *et al.* (1986, 1987) were the first to demonstrate stabilization of the sarcolemma during ischaemia by PCr. When the heart tissue was fixed in the

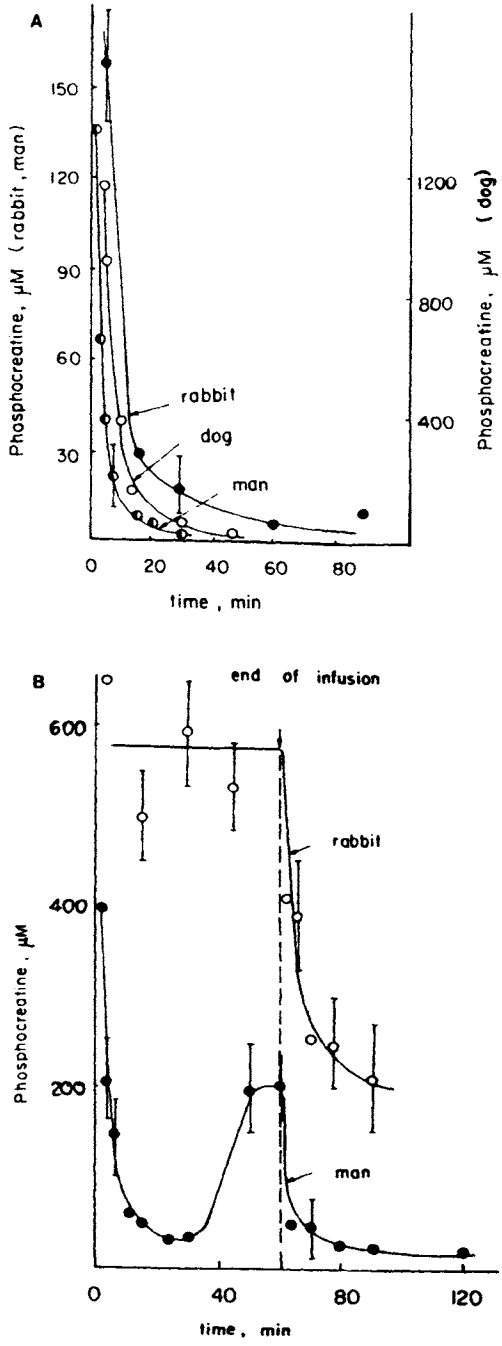


Figure 7.7. Time-dependent distribution of PCr in blood following a single i.v. administration of PCr in doses 1 g/kg.

presence of colloidal lanthanum, the lanthanum particles penetrated the sarcolemma of control re-perfused cardiomyocytes after 30 min of ischaemia. In contrast, lanthanum remained in the extracellular space when PCr was present. Creatine phosphate therefore protected the heart by keeping the lanthanum out of the cell.

Since the sarcolemma is a possible site of action, the effect of PCr on sarcolemmal vesicles was studied with electron spin resonance (ESR) spectroscopy. Sarcolemmal vesicles were isolated from dog hearts (Konorev *et al.*, 1991) and the hydrophobic phospholipid bilayer fluidity of biological membranes examined with spin-labelled probes of fatty acid molecules. The ESR spectrum of the probed membrane reflects the mobility of the probe in the membrane (Konorev *et al.*, 1991). Creatine phosphate (10 mM) increased the fraction of motionally restricted phospholipids at pH 5.5. The fraction of ordered lipids also increased while the pH was elevated up to 8.5. The greater ordering action of PCr was also seen at pH 6.5 and 7.5. From these results the conclusion has been drawn that PCr stabilizes the membranes by decreasing membrane fluidity.

Ca^{2+} (1 mM) also exerted an ordering effect on sarcolemmal vesicles (Konorev *et al.*, 1991), but the pH dependence was different from that of PCr. Thus, Ca^{2+} did not influence the lipid order at pH 5.5 while Ca^{2+} ions considerably increased the fraction of ordered lipids at pH 7.3 and, to a lesser extent, at pH 8.5. Although Ca^{2+} itself did not arrange membrane lipids in a more ordered manner, it potentiated the effect of PCr on lipid order, expressed as an increase of the fraction of motionally restricted lipids (Konorev *et al.*, 1991). This is evidence confirming the synergistic action of Ca^{2+} and PCr on protecting the ischaemic rat myocardium.

9. THE ZWITTERIONIC MECHANISM OF ACTION OF PCr

The explanation of the above effects is that PCr turns the membrane into a more ordered state. Such a property depends upon pH, i.e. it is observed at mildly acidic and neutral pH, but not at high pH. The sarcolemmal membrane contains approximately 25% phosphatidylethanolamine and 5% phosphatidylserine, and both possess amino groups and phosphatidylcholine (52%) with charged groups (Post *et al.*, 1988; Tocanne and Teissie, 1990). Upon deprotonation of NH_3 groups, the membrane surface charge decreases and the orientation of the polar heads is changed (Tocanne and Teissie, 1990). Creatine phosphate presumably binds to NH_3^+ groups of membrane phospholipids through its phosphate group (see Fig. 7.8A). A decrease in membrane surface charges (tighter packing of membrane lipids) may occur due to this binding. The number of protonated amino groups is reduced with increasing pH, and the opportunity of PCr to order the sarcolemmal membrane diminishes concomitantly. Conversely, the negatively charged carboxyl group of PCr may have an electrostatic interaction with the positively charged amino groups

of adjacent phospholipid molecules, thereby enhancing their packing into an ordered domain. This zwitterionic mechanism of interaction between PCr and cellular membrane phospholipids is shown in Fig. 7.8A. The details of electrostatic interaction between the PCr molecule (which has three negative charges and one positive charge), and the neighbouring phospholipids are also shown. Figure 7.8B shows the resulting effect – transition of mobile domain (fluid phase) into structured domain (gel phase) of the sarcolemma in the presence of PCr. Such a mechanism is known for several membrane-stabilizing drugs (Herbette, 1986) and would explain the molecular mechanism whereby PCr protects the ischaemic myocardium.

Ca^{2+} appears to be necessary for PCr interaction with cellular membranes in rendering them more resistant to ischaemic injury. This ion binds directly to anionic phospholipid head groups (Seimiya and Ohki, 1973). The combined action of PCr and Ca^{2+} leads to potentiation of the ordering effect on sarcolemmal vesicles. Involvement of Ca^{2+} in the protective effect of PCr may occur in two ways: (1) Ca^{2+} may be necessary for binding and (or) holding PCr onto the membrane surface, and (2) Ca^{2+} itself may induce similar beneficial changes at the membrane surface (Fig. 7.8A).

As was shown by Weglicki *et al.* (1984), peroxidized phospholipids alter the physical characteristics of adjacent membrane structures and produce a less ordered sarcolemmal state. The phospholipids in the micro-environment of the initial peroxide product would thereby become more available to phospholipase attack. If PCr introduces tight ordering of the phospholipid bilayer, it should also decrease phospholipid degradation by phospholipases and decrease lysophosphoglycerides in ischaemic myocardium.

10. ATTENUATION OF ADRIAMYCIN CARDIOTOXICITY BY EXOGENOUS PCr

The anthracycline antibiotic Adriamycin (doxorubicin) is a widely used chemotherapeutic agent. Regular administration can lead to the development of a cardiomyopathy (Kharasch and Novak, 1981; Goormaghtigh *et al.*, 1982; Muchammed *et al.*, 1982; Taylor and Bulkley, 1982; Cheneval *et al.*, 1983; Ng *et al.*, 1983; Wikman-Coffelt *et al.*, 1983; Doroshow *et al.*, 1985; Pelikan *et al.*, 1986; Singal and Deally, 1987; Chatham *et al.*, 1990; Kester and Sokolove, 1990; Olson and Mushlin, 1990; Doroshow, 1991). The mechanism of this phenomenon is thought to involve multiple factors, such as free radical generation, interaction with nuclear and mitochondrial DNA, changes in mitochondrial function and altered calcium exchange and cellular energetics (Kharasch and Novak, 1981; Goormaghtigh *et al.*, 1982; Muchammed *et al.*, 1982; Taylor and Bulkley, 1982; Cheneval *et al.*, 1983; Ng *et al.*, 1983; Wikman-Coffelt *et al.*, 1983; Doroshow *et al.*, 1985; Pelikan *et al.*, 1986; Chatham *et al.*, 1990; Kester and Sokolove, 1990; Doroshow, 1991). The relative role of any of these may differ depending on the chronic or acute

(A)

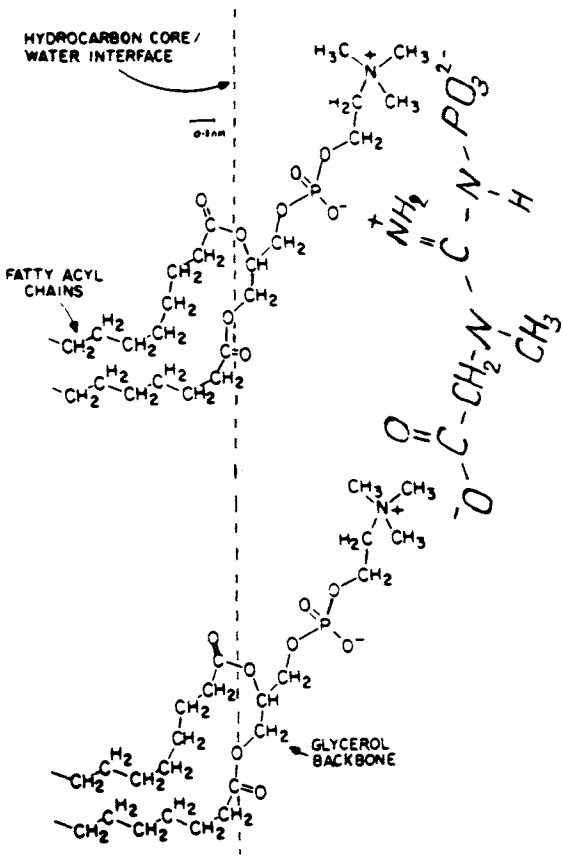


Figure 7.8. (A) Zwitterionic interaction of PCr with the bipolar heads of phospholipid molecules in the membrane surface interface.

cardiovascular effects of Adriamycin. However, in all major cases, the cytotoxic mechanism involves the interaction of the drug with cell surfaces. Numerous investigations have shown that the Adriamycin molecule binds to acidic phospholipids and to some extent to zwitterionic phospholipids (Goormaghtigh and Ruysschaert, 1984; Fiallo and Carnier-Suillerot, 1985; Nicolay *et al.*, 1985; Dupou-Cezanne *et al.*, 1989; Mustonen and Kinnunen, 1991; Wolf *et al.*, 1991a,b).

Thus, Adriamycin and exogenous PCr both interact with the polar heads of phospholipids. This would appear to imply that since the sites of interaction are similar, Adriamycin and PCr may mutually exclude the binding of each other. Such a mechanism might explain how exogenous PCr could prevent the development of cardiomyopathy due to Adriamycin cardiotoxicity and this hypothesis has recently been examined and is discussed below as well as in Chapter 8.

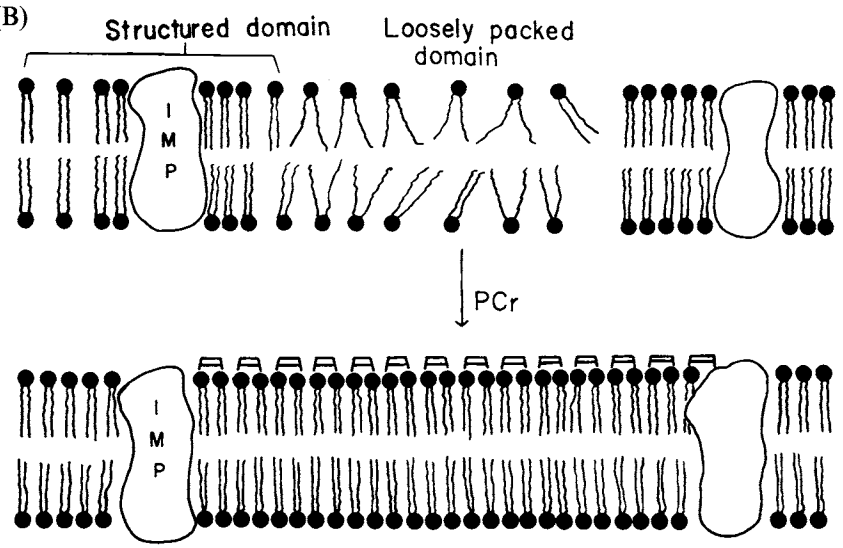


Figure 7.8. (continued) (B) Shift of the loosely packed (mobile phase) phospholipid domain to structured (gel phase) domain induced by PCr.

In experiments using the isolated perfused working heart, Saks *et al.* (1995), the aortic output was maintained constant during the perfusion at any workload. After the addition of 4 μM Adriamycin (2.32 mg/l) to the perfusate, acute cardiac insufficiency developed (Fig. 7.9). Also, as seen in Fig. 7.9B, the effects of Adriamycin are workload-dependent. Figure 7.9 demonstrates the recovery of heart function in the working model. At low aortic pressure (low cardiac work), Adriamycin induced a fall in function by 50% and this effect increased with time. At high workloads (filling pressure 15 and aortic pressure 100 and 130 cm of water), the "myopathic" hearts lost their ability to pump.

In experiments where PCr was present during treatment of the heart with Adriamycin, the cardiac function remained greater than 80–90% of the initial value ($P<0.001$) (Fig. 7.9). Coronary flow was not changed after PCr addition, indicating that oxygen and substrate delivery were not limiting. Therefore, the effect of PCr is not related to possible changes in coronary circulation or to some anti-ischaemic property.

These results confirm the suggestion made *a priori* on the basis of information on the mechanism of both Adriamycin and exogenous PCr interaction with the phospholipid bilayer of cellular membranes. Comparison of model and natural membranes has led to a conclusion that Adriamycin penetration into the lipid bilayer depends on the molecular packing of the lipid where the packing prevents Adriamycin penetration (Dupou-Cezanne *et al.*, 1989). The interaction of PCr with the membrane phospholipids is thought to be zwitterionic in nature (see Fig. 7.8 and 7.10). Shielding of the electrostatic charges

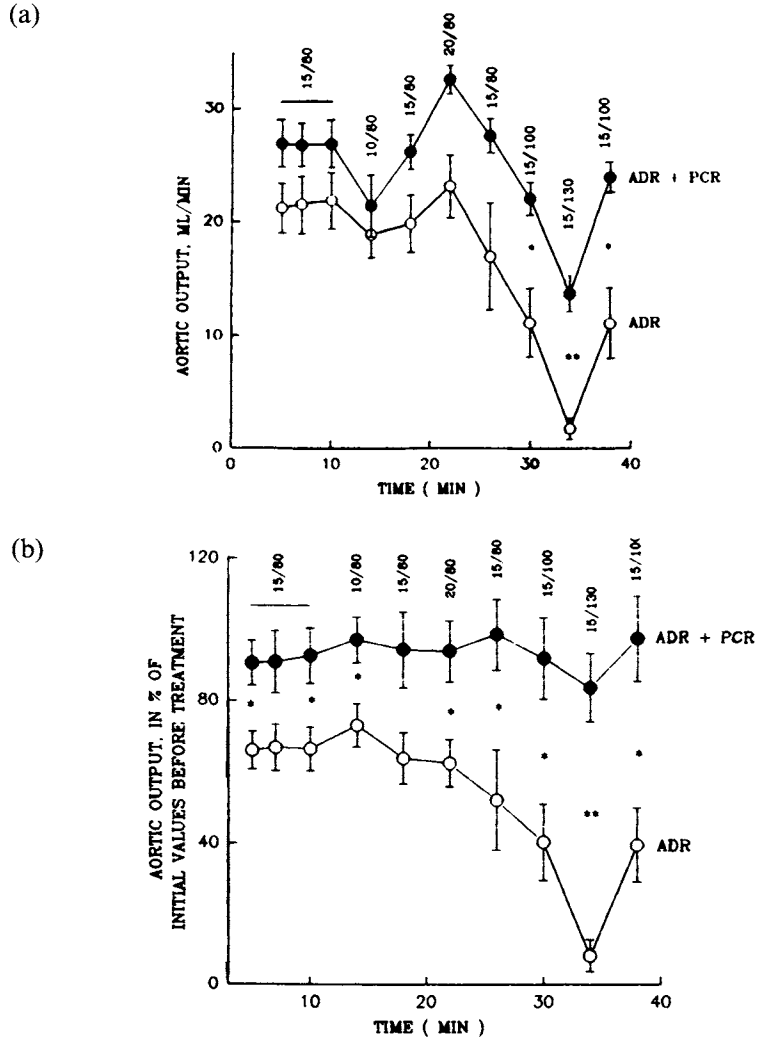


Figure 7.9. Changes in aortic output from an isolated perfused heart exposed to 4 μM of Adriamycin in the absence (ADR) and the presence of 10 mM PCr (ADR + PCr). The numbers show the filling and aortic pressures. (A) Absolute values; (B) relative values (ratios of aortic pressure after ADR addition to that initially under the same conditions). * $p<0.05$; ** $p<0.001$.

and increased lipid packing in the membrane by PCr (in the presence of calcium) may remove Adriamycin from the membrane and prevent penetration into the bilayer. Therefore, the attentuation of Adriamycin cardiotoxicity by PCr supports the proposal that a zwitterionic interaction occurs between PCr and membrane phospholipids.

The efficacy of PCr in preventing Adriamycin toxicity is of considerable

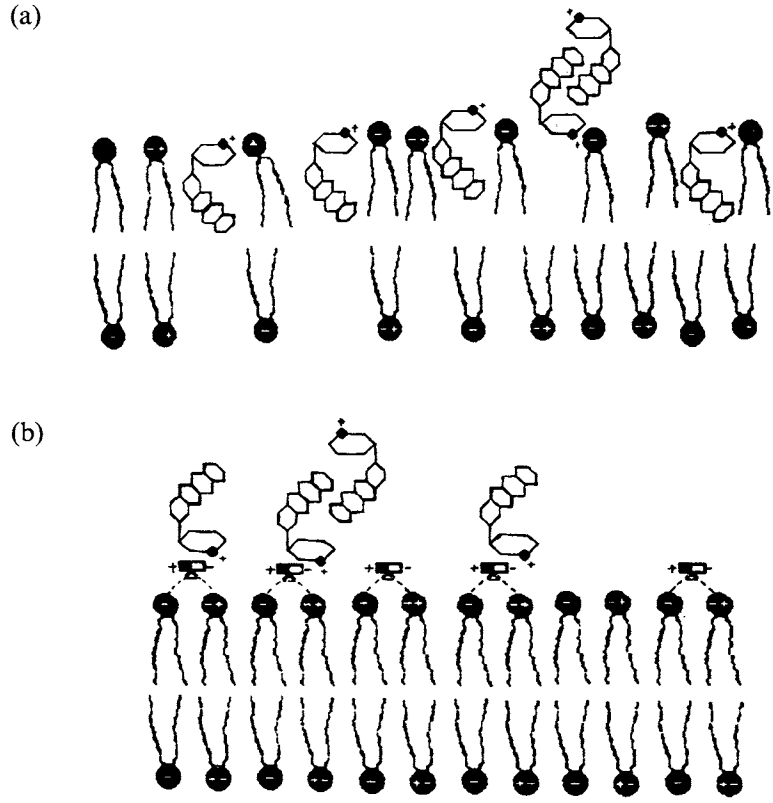


Figure 7.10. Schematic representation of the possible mechanism of the attenuation by PCr of acute cardiotoxicity caused by Adriamycin. (a) Arrangement of Adriamycin molecules in the membrane interface as suggested by Wolf *et al.* (1991a,b). The positively charged amino group of Adriamycin molecules (shown as black dots) are electrostatically bound to the phospholipid head groups and the anthracycline moieties are between the lipid molecules creating small focal defects. (b) In the presence of PCr and calcium, the zwitterionic interaction between this complex and the polar heads of the phospholipids results in tight packing of phospholipids in the membrane and shielding of their polar heads from the Adriamycin molecules, which will remain outside of the membrane interface.

interest. Studies are required to determine whether or not the PCr is equally effective if used for the prevention of chronic Adriamycin cardiotoxicity – both in experimental preparations and in patients. Since rapidly growing cancer cells have a significantly altered cell structure and metabolism, including excessive plasma membrane blebbing, enhanced metabolite transport and increased rates of glycolysis (Petersen and Bustamante, 1980; Alberts *et al.*, 1983), it is possible that PCr will not alter the effect of Adriamycin on the mitotic cells. If this is the case, then the protective effect of PCr may be very selective to the heart and so prevent the development of cardiomyopathy.

11. EXTRACELLULAR EFFECTS OF PCr

An important mechanism for the action of extracellular PCr in pathological tissues may be the ability to inhibit ADP-induced platelet aggregation (Neely *et al.*, 1973). This is most likely to occur due to the CK reaction. Administration of exogenous PCr activates the CK which is released into blood during ischaemia and thus eliminates ADP by rephosphorylation via PCr and CK to ATP. However, a direct effect of PCr on the platelets and erythrocytes cannot be excluded (Gard *et al.*, 1985). These effects may lead to significantly improved microcirculatory perfusion and elevated coronary flow into the ischaemic zone (Kryzhanovsky *et al.*, 1987). Moibenko *et al.* (1992) have found that exogenous PCr has a protective effect on the structure and function of coronary vessels during immune (cytotoxic) damage to the heart. This may relate to possible stabilization and preparation of endothelial cell membranes (Moibenko *et al.*, 1992). Accordingly, it preserves the endothelium-dependent vasodilatory effects of humoral agents (Moibenko *et al.*, 1992). Also, Rodriguez *et al.* (1991) have recently shown that exogenous PCr preserves renal function in the *in vitro* model of renal ischaemia. Indirect clinical data demonstrate a protective effect when cerebral function is assessed in patients with ischaemic disease and in those after surgery (Breccia *et al.*, 1985). Thus, we may suppose that the membrane protective effect of PCr is a more general phenomenon which is not confined to ischaemic heart muscle alone.

12. SUMMARY

In summary, studies on the biochemical basis for a therapeutic effect of PCr demonstrate a role in preserving contractile function, maintaining intracellular ATP and PCr and reducing CK loss. The anti-ischaemic effect is Ca^{2+}-dependent and an antioxidant property has been confirmed. The mechanism of action is thought to relate to the preservation of sarcolemmal membranes. While reservations about membrane penetration of the molecule are expressed, pharmacokinetic data have shown the uptake of exogenous PCr by heart, skeletal muscle, brain and to some extent by kidney, but not by lung or liver tissues (Breccia *et al.*, 1985, and discussed above). Thus, the protective effect of PCr is directly related to the tissue uptake due to some specific phospholipid composition of the cell membranes, and in this respect, it would be interesting to analyse the available data for all tissues studied. A mechanism of protection related to stabilization of the sarcolemma without significant penetration into the cells can also be envisaged.

The results of the various studies on PCr present some important points for consideration. Since charged phospholipids are located at both sides of the sarcolemma (Post *et al.*, 1988), exogenous and endogenous PCr may be

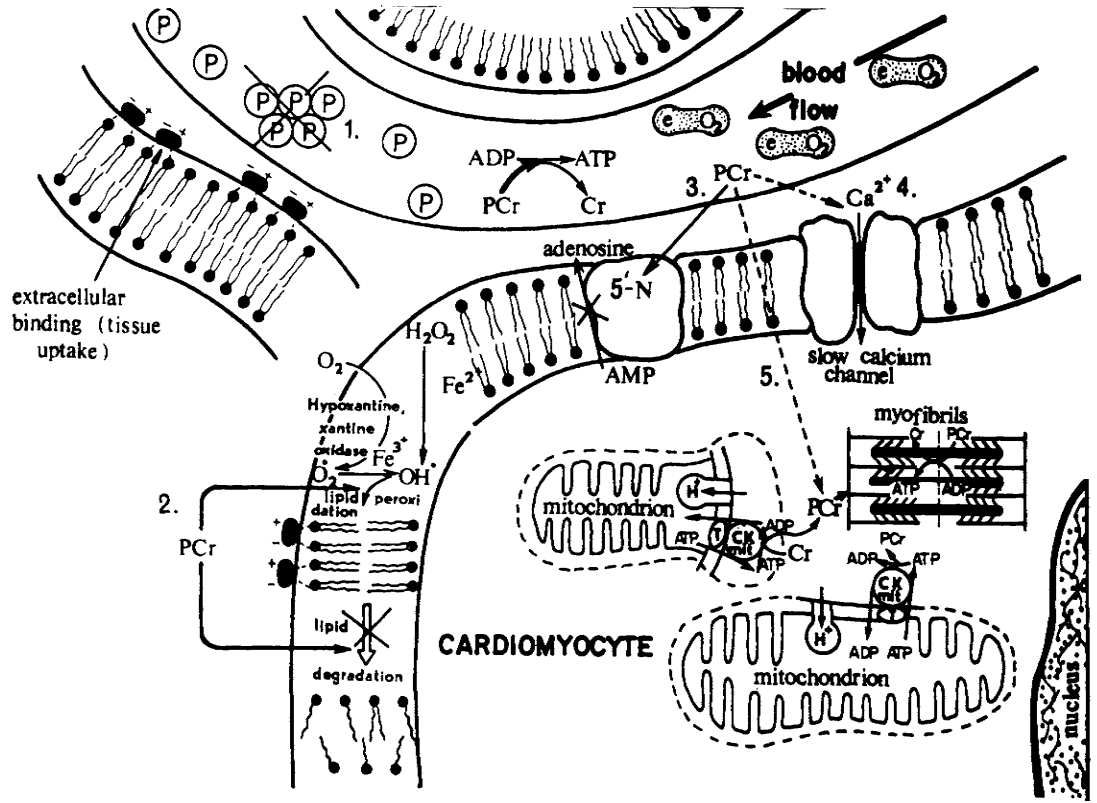


Figure 7.11. General presentation for the mechanism of the cardioprotective action of PCr (according to Kryzhanovsky, 1993). Abbreviations: e, erythrocyte and ±, zwitterionic action of PCr. The following components of the protective mechanism are shown: (1) inhibition of platelet aggregation by removal of ADP; (2) the rate of lipid peroxidation and degradation is decreased because of the increased structural ordering of phospholipid bilayer; (3) inhibition of 5′-*N* results in preservation of the cellular adenine nucleotide pool; (4) very slow penetration into the ischaemic cells; (5) effect on the inward Ca^{2+} flux in the hypodynamic (~hypokinetic) state, which is absent in normal myocardium.

equally important for sarcolemmal stability. Rapid intracellular PCr depletion in ischaemic heart tissue could be one of the factors relevant to membrane destabilization and the increased rate of breakdown (Ronca *et al.*, 1989; Zucchi *et al.*, 1989). Exogenous PCr may stabilize the membrane after binding to its external surface without penetration into the cells. Such a membrane-stabilizing effect might also be the basis for the ability of the molecule to protect the heart against other types of damage such as that associated with oxidative injury. The proposed protective action of exogenous PCr on ischaemic cardiac (and skeletal muscle, brain and kidney) cells is illustrated in Fig. 7.11.

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8

Effects of Creatine Phosphate on Cultured Cardiac Cells

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Key words: creatine phosphate, muscle, cardiac, hypoxia, arrhythmia, adriamycin, phosphocreatine, phosphoarginine, cardiotoxicity

1. INTRODUCTION

Cardiac muscle cells are unique in that they maintain their ability to beat in culture for prolonged periods. The beating of the cells is both characteristic of and dependent upon their environment, which can be rigorously controlled by the culture medium. Thus, if the medium is made hypoxic, the cells stop beating. The response to such an intervention is used as a model of the whole organ under similar conditions. Therefore we have used cardiac muscle cells to examine how the heart may respond when exposed to certain conditions and appropriate treatments.

In order to utilize the beating as a measure of physiological function, we have constructed a closed microscopic stage system that controls temperature and pH, two variables to which cardiac cell beating is particularly sensitive. We have explored a variety of techniques to characterize heart cell beating ranging from direct visual observation with a stopwatch to a video-enhanced computer-based image processing system.

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The system consists of a video camera (RCA Model TC 1005) which transmits the image of the cardiac cells onto a television monitor (Sanyo Model VM 4155). A photoresistor (which can be manoeuvred to any point on the screen) detects changes in light intensity resulting from pulsations (beats) of the cells. Each beat is permanently recorded on paper and computer. The system allows for the measurement and computation of the interval of time (to the ms) between beats (which is instantaneously converted to beats/min) and standard deviations. Thus a precise measurement of chronotropic changes in contraction is made.

Although the polygraph recordings document obvious arrhythmias, quantitative measurement of these perturbations can be made by the microcomputer. Under the conditions developed for growing healthy cardiac cells, maintaining a steady temperature and controlling pH, these cells beat in a regular fashion (Lampidis, 1983). The spontaneous contractions of these cells are rhythmic and remain so for prolonged periods (>10 days) in culture (Lampidis *et al.*, 1980). Therefore, studies at the cellular and subcellular level on the genesis of arrhythmias as well as on the potencies of anti-arrhythmic drugs can be addressed using this system.

With the ability to maintain functionally active cardiac cells in culture for prolonged periods, we began to develop this system as an *in vitro* model to study cardiopathogenesis produced in patients treated with the potent antitumour agent Adriamycin (ADM). ADM-induced cardiomyopathy is a cumulative dose-limiting toxicity which prevents a more efficacious use of this drug as an antitumour agent. We have previously shown that ADM and a number of other anthracycline analogues which are positively charged (at physiological pH) are accumulated preferentially in cardiac muscle cells as opposed to co-cultured cardiac fibroblasts (Lampidis *et al.*, 1981). It has also been observed with compounds other than the anthracyclines (xanthene dyes) that charge determines preferential accumulation in cardiac muscle rather than in non-muscle cells (Lampidis, 1983). Therefore we have been able to demonstrate that an important consideration for whether an anthracycline will preferentially accumulate in cardiac muscle cells or not (and thus increase its specific toxicity toward these cells) is its charge (Lampidis *et al.*, 1984). The most likely explanation for this phenomenon is the high electronegative charge and the abundance of negatively charged molecules in cardiac muscle cells (Lampidis *et al.*, 1989; Van Priebe *et al.*, 1993). The nature and function of the interaction of creatine phosphate (PCr) and the cardiac membrane is discussed by Saks in Chapter 5.

ADM-induced cardiotoxicity has been reported to resemble cardiac ischaemia (Seraydarian and Naginieni, 1987) and lowered PCr levels is one of the effects found in many cardiopathologies (Chapters 3 and 9). Here we discuss the effects of exogenous PCr on cardiac cells treated with ADM.

2. PROTECTION BY CREATINE PHOSPHATE AGAINST ADRIAMYCIN

When normal cardiac cell cultures were exposed to 20 μg/ml of PCr, no effects on contractility were observed (Fig. 8.1). This observation would coincide with an interpretation that PCr levels in intact cardiac cells are either not affected by exogenous PCr or that higher intracellular levels do not affect contractility (Table 8.1). As discussed in Chapter 7, the uptake of PCr into heart cells is very slow where creatine is taken into the cells, leading to a substantial accumulation of creatine and PCr (Chapters 3 and 13).

In an attempt to mimic anoxia and to determine whether addition of exogenous PCr could intervene, we exposed the cardiac cells to 100% CO_2 in the presence and absence of PCr (Table 8.1). When cells are exposed to 100% CO_2 for 30 or 60 min they stop beating within 6–7.5 min. and become arrhythmic shortly before arresting. Upon reperfusion with normal CO_2 levels and air, cells begin beating within 12–15 min and are arrhythmic for a short period before resuming a normal rhythmic rate. Creatine phosphate at concentrations of 20 μg/ml does not alter the arrest or recovery time and has no effect on the arrhythmias. Since myocytes regain their normal beating patterns after exposure to 30, 60 min or longer of CO_2 it appears that they are able to withstand anoxia without apparent damage. This observation agrees with a recent report which indicates that activation of lipases is an important mechanism leading to toxicity when cardiac cells are placed in anoxic conditions at normal or elevated pH (Lemasters, 1993). Moreover it was shown that lipase activity is inhibited at lowered pH, which clearly occurs when cells are exposed for prolonged periods to 100% CO_2, as was the case in our experiments.

When cells are treated with 0.1 μg/ml of ADM for 1–2 days and placed in serum-free medium for 1 h or more, they stop beating. Interestingly, 4 min after adding 20 μg/ml of PCr, the cells resume beating and continue beating for at least 48 h (Fig. 8.2). When PCr-stimulated cells are rinsed in serum-free medium they stop beating again and can be re-stimulated by re-addition of 20 μg/ml of PCr. Although we have repeated this, the quantitative responses in culture are varied.

In contrast to our results with PCr, phosphocreatinine did not stimulate beating in ADM-arrested cells (Fig. 8.3). These experiments were carried out under the same conditions as those above. In cultures that did not respond to phosphocreatinine, it was found that they were still able to respond to treatment with PCr. These results are reminiscent of those discussed in Chapter 7, where it was found that phosphoarginine (another PCr analogue) offered no cardioprotection or membrane stabilization where PCr had a striking effect.

When cardiac cells were treated with lower doses of ADM (0.05 μg/ml), which produced arrhythmias, PCr had no effect on beating patterns (Fig. 8.4). This is in contrast to the reversibility of ADM-induced arrhythmias with verapamil or tubulin-binding agents (Lampidis *et al.*, 1992). Whether, at these

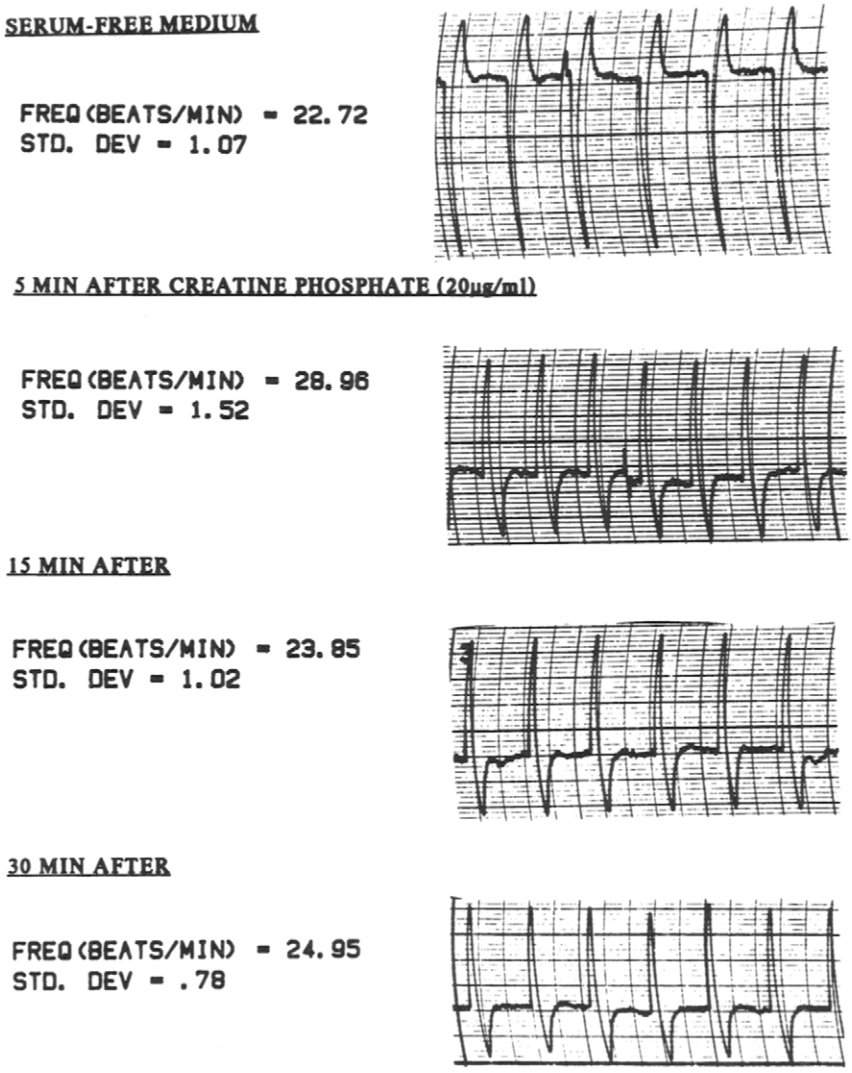

Figure 8.1. Non-stimulatory effect of PCr on intact normal cardiac cells. At 20 μg/ml. PCr does not significantly change the rate (average of >100 beats±SD) of synchronously beating cardiac cell cultures during 30 min of continuous treatment. Polygraph recordings (15 s sampling at 5 mm/s) are representative beating patterns of the culture at each time point indicated.

Table 8.1. Cardiac cell response to 100% CO_2 (30 min) + or − PCr

Culture number	Time to arrest (min)	CO_2 time (min)	Recovery time (min)	PCr
1	7.5	30	14	−
2	7.5	30	14.5	−
3	7.5	30	14.5	−
4	7	30	13.5	+
5	7	30	14.5	+
6	6	30	15	+
1	6.5	60	15	−
2	7	60	12	−
3	7.5	60	14	+
4	7.5	60	12	+

lower ADM doses, PCr crosses the cell membrane or acts at ion channels in some unique way, with or because of ADM, remains to be determined. One must remember that PCr normally does not cross the membrane to any significant extent (Chapter 7), so more work needs to be done to determine the mechanism of action.

3. SUMMARY

We report that cardiac cells growing in culture are not irreversibly damaged by prolonged exposure to 100% CO_2. The addition of exogenous PCr does not alter the arrhythmic effect produced by 100% CO_2 treatment nor the arrest or recovery times. However, when cells are treated with ADM and cardiac cell beating is arrested in serum-free medium, PCr (but not phosphocreatinine) is able to restore beating. Since intracellular PCr is known to be lowered in ADM-treated hearts, as well as other pathological muscle tissues (Chapters 3 and 9), it is possible that addition of this compound exogenously restores some minimal level necessary for beating. It will be interesting to see whether PCr may have clinical application in patients treated with ADM.

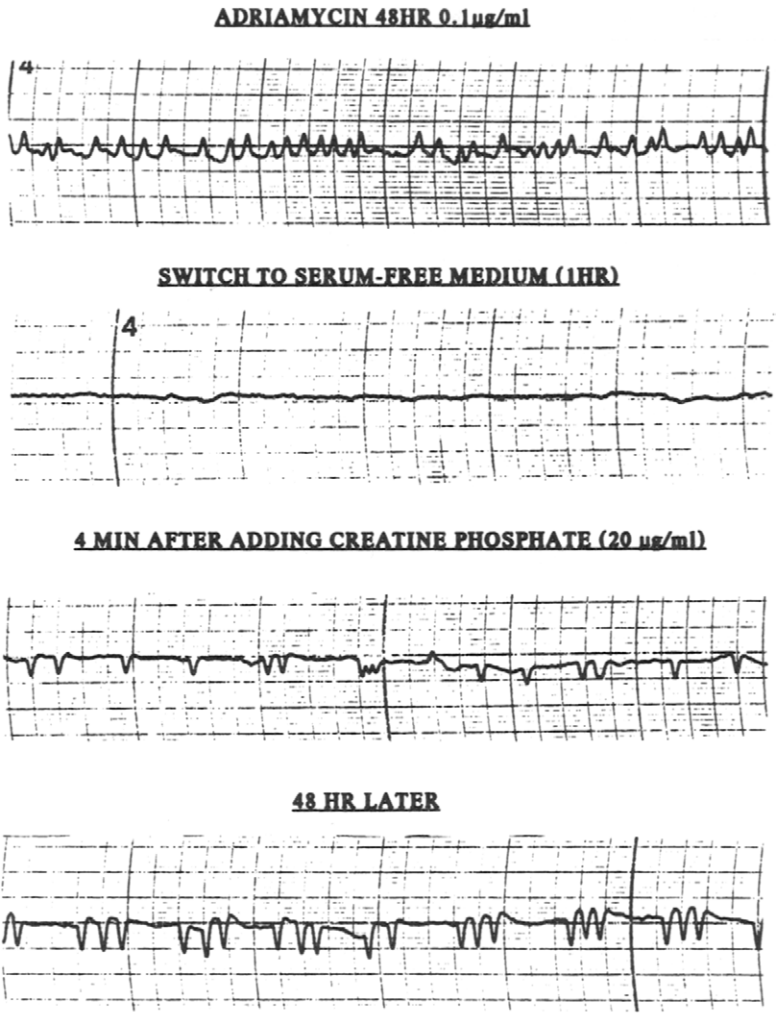

Figure 8.2. Stimulation of ADM-arrested cardiac cells by PCr. Cardiac cultures treated with ADM (0.1 μg/ml) for 48 h and then rinsed in serum-free medium for 60–90 min stop beating. Four minutes after addition of 20 μg/ml PCr cells resume beating and continue beating for at least 48 h in serum-free medium. Polygraph recordings (30 s sampling at 5 mm/s) are representative beating patterns of the culture at each time point indicated.

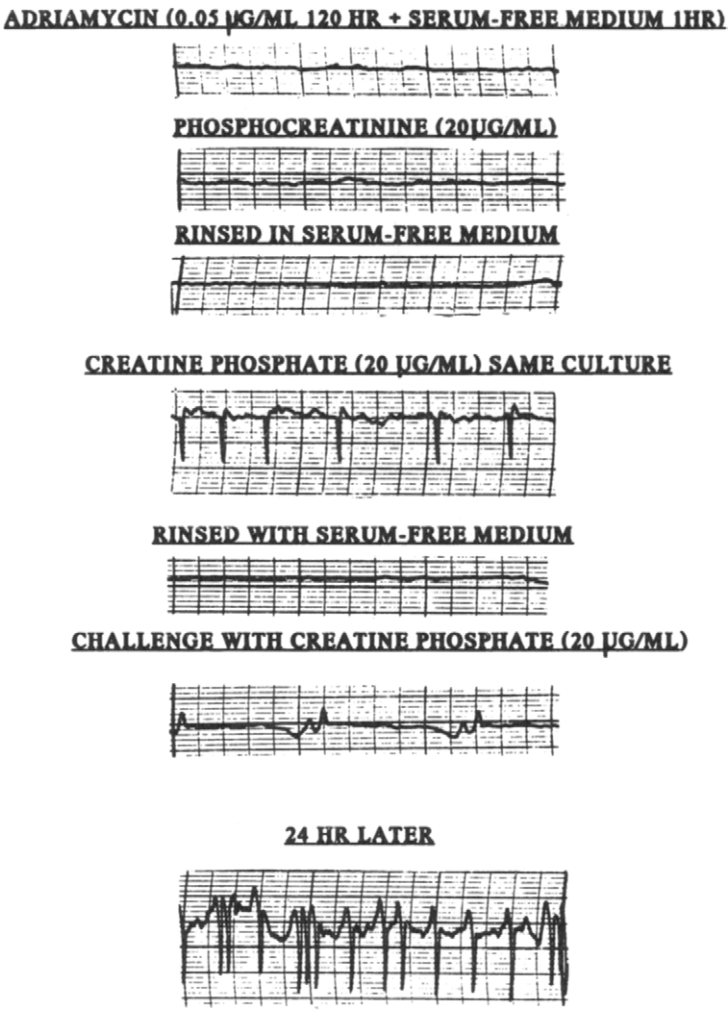

Figure 8.3. Phosphocreatinine does not stimulate ADM-arrested cardiac cells. Cardiac cultures treated with ADM (0.5 μg/ml) for 120 h and then rinsed in serum-free medium for 60–90 min stop beating. After addition of 20 μg/ml phosphocreatinine the cells remain arrested. They resume beating again when rinsed and treated with PCr (20 μg/ml) and continue beating for at least 48 h in serum-free medium. Polygraph recordings (15 s sampling at 5 mm/s) are representative beating patterns of the culture at each time point indicated.

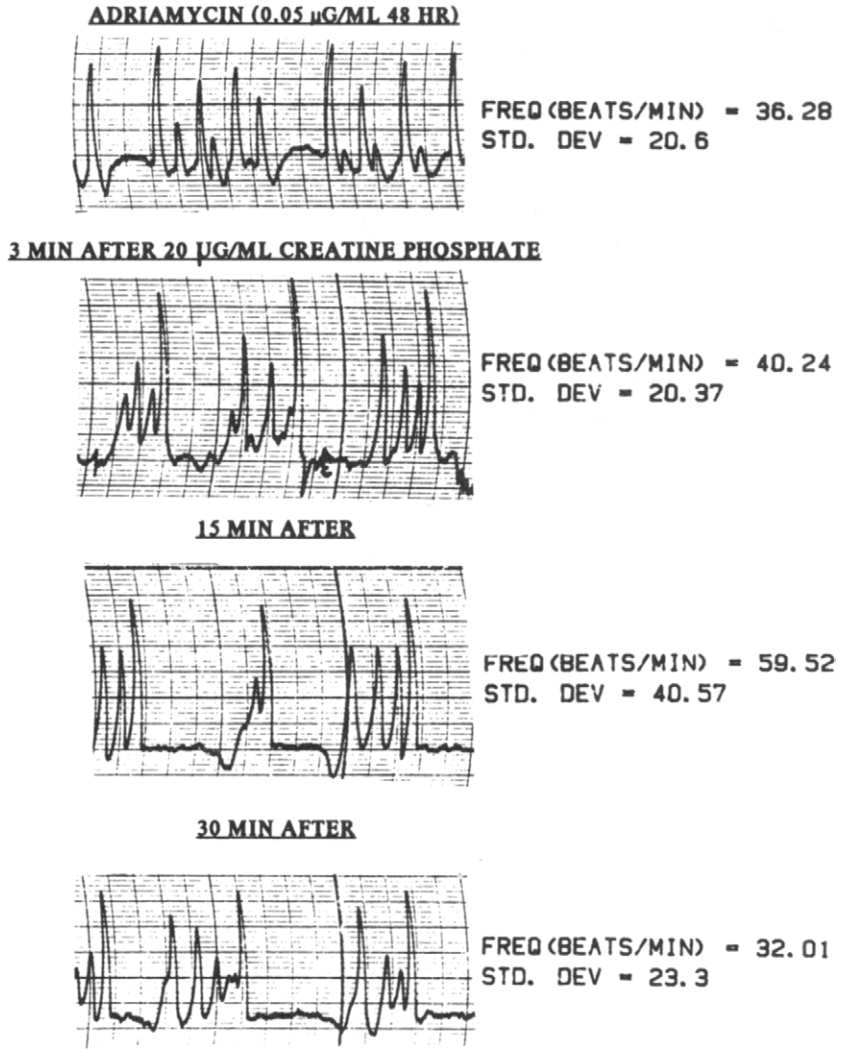

Figure 8.4. Absence of anti-arrhythmic activity of PCr. Cardiac cultures treated with ADM (0.05 μg/ml) for 48 h and then rinsed in serum-free medium (5 min) beat arrhythmically. Thirty minutes after addition of 20 μg/ml PCr, cells continue beating arrhythmically. Although beating rates (average of >100 beats) change during 30 min of treatment, PCr does not reverse arrhythmic beating. Polygraph recordings (15 s sampling at 5 mm/s) are representative beating patterns of the culture at each time point indicated.

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9

Creatine Phosphate: *in vivo* Human Cardiac Metabolism Studied by Magnetic Resonance Spectroscopy

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Key words: creatine phosphate, magnetic resonance spectroscopy, myocardium aortic stenosis, aortic incompetence, hypertrophy, heart failure

1. INTRODUCTION

Creatine phosphate (PCr) is recognized as a key metabolite in the biochemical pathways that supply high-energy phosphates for muscle contraction and other energy-requiring processes. The intracellular concentration can be measured non-invasively using ^{31}P magnetic resonance spectroscopy (MRS) and

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over the years numerous studies have been performed in animal models to characterize cardiac spectra and describe the reproducible changes which occur following various interventions designed to increase workload, decrease substrate supply, etc. (Garlick *et al.*, 1977; Jacobus *et al.*, 1977; Radda, 1983, 1986). The potential of such a non-invasive technique for the assessment of biochemistry in man is clear and already several groups have examined control subjects and patients with cardiac and other disease.

Changes in myocardial PCr levels are closely related to ATP consumption through the creatine kinase reaction as described earlier (see Section I). The relationship is represented by the PCr/ATP ratio and in animal models of acute intervention, increased ATP consumption is associated with a decrease in PCr. Exercise of normal human skeletal muscle produces a similar change as shown by the lower PCr/(PCr+P_i) ratio (Taylor *et al.*, 1983; Wilson *et al.*, 1985; Massie *et al.*, 1987).

2. THE NORMAL HEART: RESTING METABOLISM

The relative concentrations of PCr and ATP in the human heart *in vivo* are measured using surface coils (Ackerman *et al.*, 1980) as outlined elsewhere, e.g. Andrew *et al.* (1990). A typical cardiac spectrum from a normal control collected using one reported technique – phase-modulated rotating frame imaging (PMRFI: Blackledge *et al.*, 1988) – is shown in Fig. 9.1. Spectra are localized to the myocardium by stacked plots in PMRFI and by magnetic resonance imaging (MRI), and other imaging methods such as image selected *in vivo* spectroscopy (ISIS: Ordidge *et al.*, 1986) and one-dimensional chemical shift imaging (1D CSI: Bottomley *et al.*, 1990). Values for PCr/ATP in a selection of reported studies are in Table 9.1.

Human and animal cardiac spectra are composed of peaks derived from phosphates at different chemical shifts. On reading the spectrum from left to right, 2,3 diphosphoglycerate (2,3 DPG), inorganic phosphate (P_i), phosphodiester (PDE), creatine phosphate (PCr) and three peaks for ATP can be identified. Blood, heart and skeletal muscle contain P_i, PDE and ATP. Creatine phosphate is present in skeletal and cardiac muscle and absent from blood. The presence of a prominent 2,3 DPG peak indicates that the sample volume has a significant amount of blood present.

In most reports the spectra are analysed by comparing the PCr peak to βATP (Hardy *et al.*, 1991). Some investigators use γATP (Blackledge *et al.*, 1987; Conway *et al.*, 1991 a & b) because of potential βATP peak distortion associated with off-resonance effects. Ratios calculated using different ATP peaks may be at variance in different studies since the amplitude and area of individual phosphate peaks may vary. However, if the same ATP peak is used for patients and controls within a study, the comparison should be valid.

The normoxaemic blood which contaminates the cardiac spectrum has a

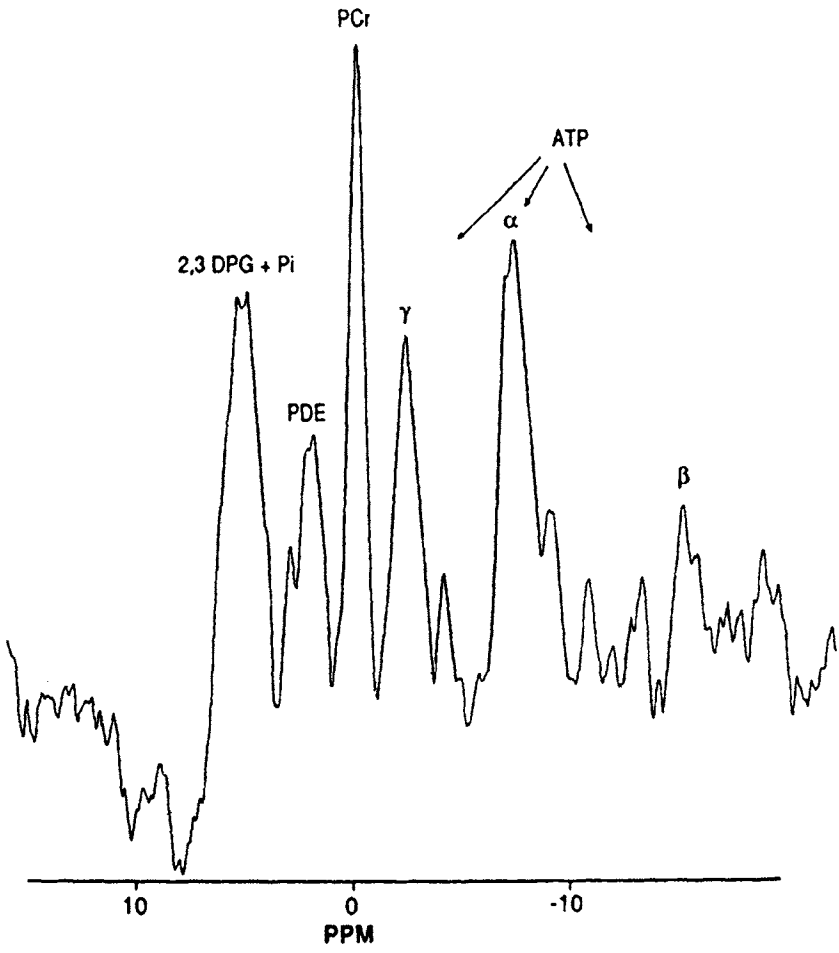

Figure 9.1. Cardiac spectrum from a control acquired using the phase-modulated rotating frame imaging technique (PMRFI) (2,3 DPG+P_i, 2,3 diphosphoglycerate and inorganic phosphate; PDE, phosphodiester; PCr, creatine phosphate; ATP, adenosine triphosphate, γ, α and β phosphates; ppm, frequency in parts per million).

pattern similar to that shown in Fig. 9.2. There is no peak for PCr at 0 ppm. The amplitude of the PDE signal generally exceeds that of the ATP peaks. The ratio of the 2,3 DPG peak (both phosphate peaks combined) to γATP is approximately 6:1, based on ^{31}P MRS measurements on *ex vivo* venous blood (Conway *et al.*, 1991a). The cardiac spectrum which is contaminated by signal from cavity blood will contain extra ATP and the area of the ATP peak from blood will be approximately one-sixth that of the area of the 2,3 DPG signal in the cardiac spectrum. Thus, in order to "purify" the cardiac spectrum the ATP peak area should be reduced according to this ratio. Hardy *et al.* (1991)

Table 9.1. PCr/ATP ratios determined by ^{31}P MRS at 1.5–2.0 T in uncorrected[a] and saturation- and/or blood-corrected spectra.

Authors	PCr/ATP ratio	Year
Bottomley[a]	1.30±0.40	1985
Blackledge *et al.*[a]	1.53±0.25	1987
Schaefer *et al.*[a]	1.23±0.27	1988
Conway *et al.*[a]	1.50±0.20	1988
Luyten *et al.*[a]	1.37±0.09	1989
Weiss *et al.*	1.72±0.15	1990
Aufferman *et al.*	1.59±0.11	1991
Hardy *et al.*	1.80±0.21	1991
Bottomley *et al.*	1.93±0.21	1991
de Roos *et al.*	1.65±0.26	1992
Neubauer *et al.*	1.95±0.45	1992
Yabe *et al.*	1.85±0.2	1994

subtracted an area of βATP that was 15% of the integrated signal of the two DPG peaks. This was based on literature reports indicating that ATP is 30±2% of the 2,3 DPG in blood.

The signal from 2,3 DPG is contaminated by signal from P_i since the frequency shift of one of the phosphates in 2,3 DPG coincides. The blood P_i concentration is low and de Roos *et al.* (1992), using proton decoupling, detected negligible P_i in the normal heart. Thus, it is generally accepted that the P_i signal is not significant compared with background noise in the normal heart and so it is unlikely to be a major source of error when adjusting the ATP for blood contamination.

While most of the currently reported PCr/ATP values are within the range 1.5–2.0, some have reported much lower ratios (e.g. Bottomley 1985; Schaefer *et al.*, 1988). In the earlier studies this may relate to the failure to allow for blood contamination and saturation correction and the more recent higher ratios are largely related to these factors. However, other anatomical and structural features may also explain the differences in reported values for PCr/ATP ratios. Since the spectroscopy techniques measure signal from the nuclei of atoms which are common to skeletal muscle, heart and blood, contamination of the myocardial PCr and myocardial ATP by signal from contiguous tissues may occur. Differences in localization from individual to individual could be a source of variability. The spectroscopy technique must measure accurately from a relatively deep, thin and moving organ and provide a localized signal in which the cardiac PCr must be separate from that of the chest wall muscle and the ATP signal must be separated in blood, heart and skeletal muscle. Bottomley (1994) recently examined this problem and identified differences in nuclear relaxation times as an important cause of potential variability in the measurements reported by different investigators.

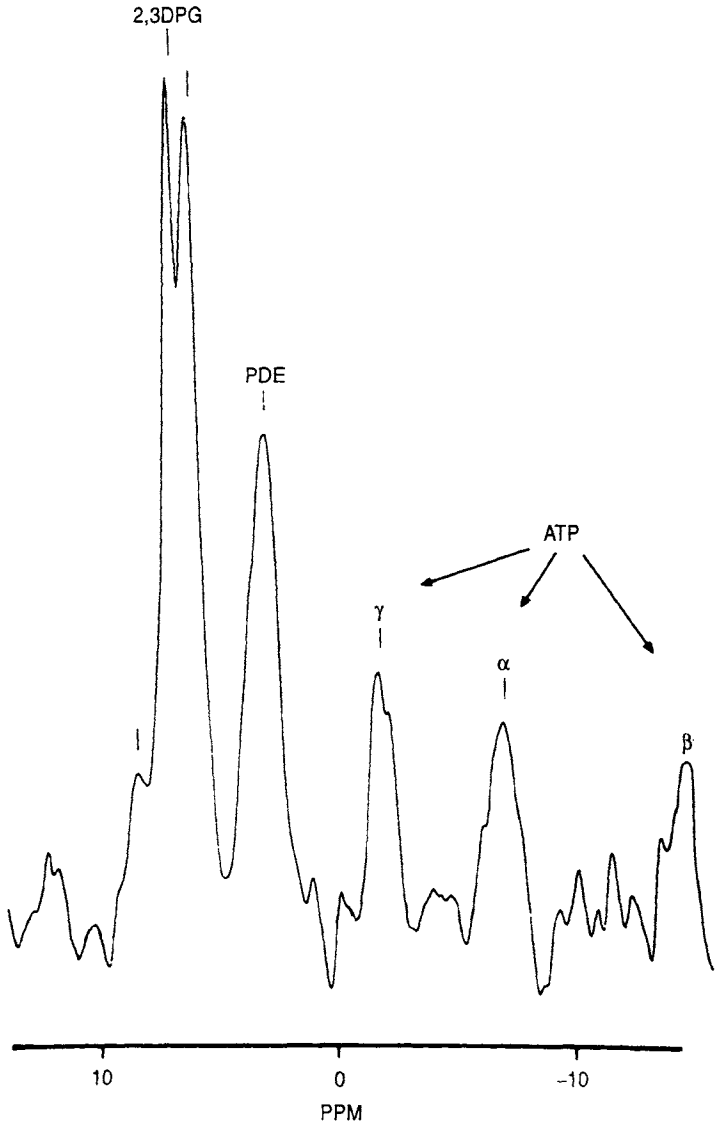

Figure 9.2. Spectrum of *ex vivo* human blood (PDE, ATP and ppm: as in Fig. 9.1). The main peak to the left of the PDE is the 2 phosphates of 2,3 DPG.

3. THE NORMAL HEART: ISOTONIC AND ISOMETRIC EXERCISE

Little is known about the effects of dynamic exercise on *in vivo* high energy phosphorus metabolism and the ratio of PCr to ATP in the normal human heart. Characterization of normal subjects is important for the purposes of

comparison with patient metabolism. As with the resting ECG, resting ^{31}P spectra may have a limited role since most currently available cardiac measurements often fail to demonstrate abnormalities without stress testing. Methods for performing exercise while acquiring ^{31}P spectra are therefore required and a number of these have been described.

Exercising in the confined space of a magnet poses unique problems. Standard supine exercise is limited because the heart lies furthest away from the coil in this position and there is free respiratory movement of the chest wall. The prone position is more suited for cardiac spectroscopy and hence a method of exercising in the prone position – where the body is immobilized and foreleg movement alone allowed (Pronex) – has been reported (Conway *et al.*, 1988). The physiological response to this form of exercise has been characterized using blood pressure (BP), heart rate (HR) and total body oxygen consumption ($\dot{V}O2$) measurements.

The exercise equipment for studying the physiological effects of prone exercise is shown in Fig. 9.3. Cylinders are used for weights and the load increased by adding water from reservoirs. The subject lies prone on the couch with each foot suspended on a cable. This position corresponds to that in the bore of the magnet and provides ideal stability of the thorax. Expired air is measured for volume and analysed for oxygen, nitrogen and carbon dioxide using a mass spectrometer.

In the study protocol reported (Conway *et al.*, 1988) exercise is performed by lifting weights approximately 25 cm with each foreleg extension 50 times per minute. Expired air is collected during the 3rd, 11th and 19th minutes of exercise. A volume of water equivalent to 2.5 kg is lifted during the first 3 min (Pronex, stage 1). The weight is increased by 1.25 kg during the 4th, 8th, 12th and 16th minutes of exercise such that during collection of the second and third samples of expired air the workloads are 5 kg (Pronex, stage 2) and 7.5 kg (Pronex, stage 3), respectively.

The measurements obtained during prone exercise were compared with those obtained from the same subjects during treadmill exercise using the Bruce protocol (Bruce, 1971). Blood pressure and HR were recorded every minute and the $\dot{V}O2$ was measured using the above procedure from 10 ml expirates collected during the third minute of each of Bruce protocol stages 1 to 4. The measurements performed during incremental prone exercise are compared with those performed during Bruce protocol treadmill exercise in Figs 9.4–9.6 (Mean ± S.D.).

3.1. Physiological changes during prone and treadmill exercise

The mean value for the heart rate at stage III of the prone exercise is between the rates at stages 2 and 3 of the Bruce protocol. The mean systolic blood pressures at each stage closely matches that during treadmill exercise. The diastolic

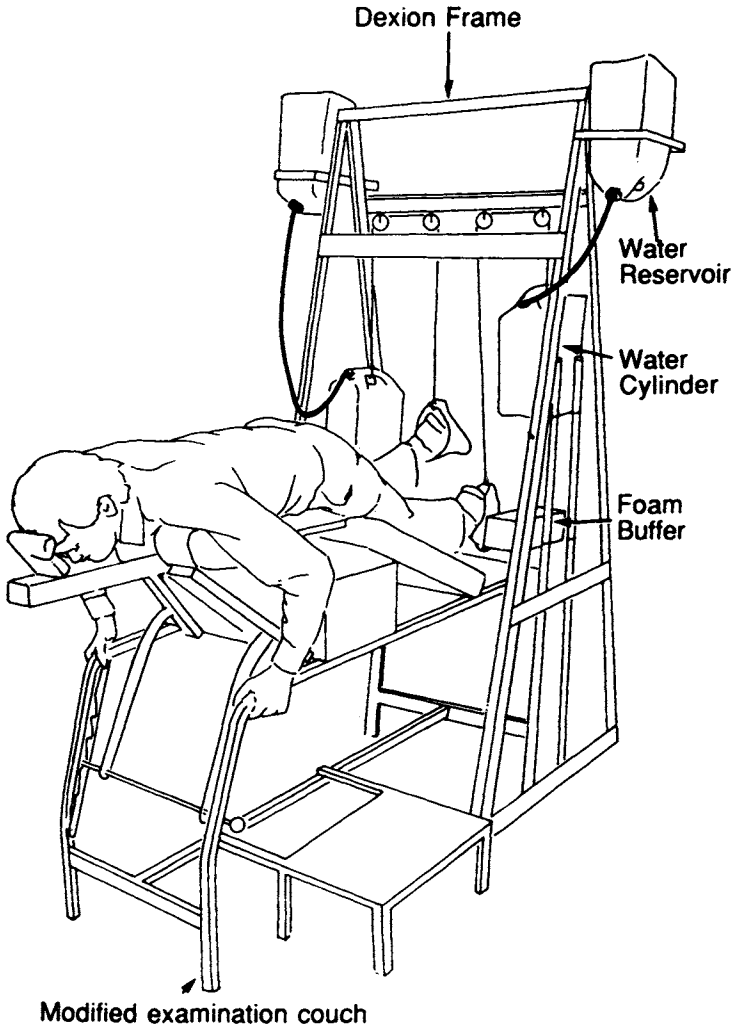

Figure 9.3. Ergometer for the performance of prone exercise. The feet are attached to the cylinders via cables.

blood pressure (DBP) response differs in the two techniques since it falls during treadmill exercise and the higher resting prone diastolic blood pressure rises as the workload is increased during prone exercise.

At rest, the oxygen consumption is equivalent before both types of exercise and is similar to the normal values reported by other investigators. The prone exercise oxygen consumption (stage III) compares favourably with that at stage 1 of the Bruce protocol.

In Fig. 9.6 the rate–pressure products of the two types of exercise are shown.

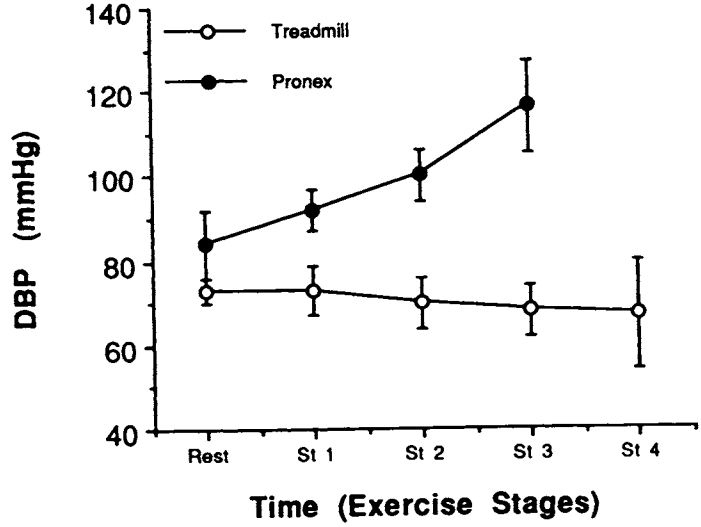

Figure 9.4. Diastolic blood pressure (DBP) at rest and during prone (Pronex) and upright exercise (Treadmill).

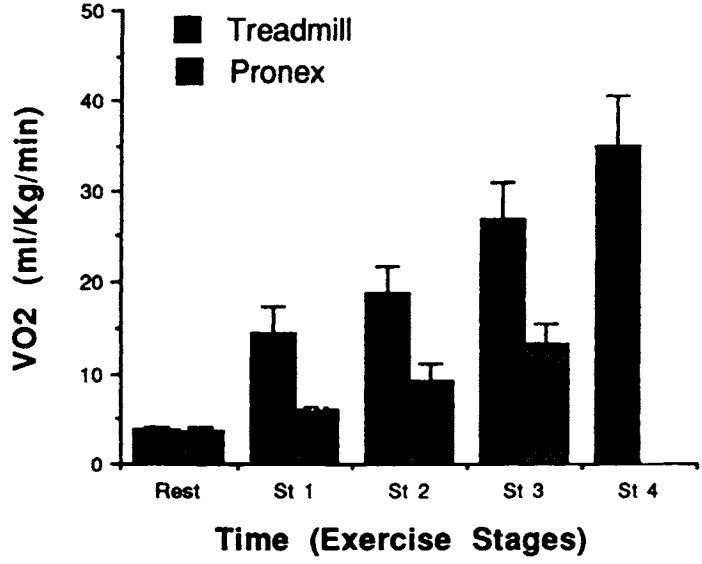

Figure 9.5. Total body oxygen consumption ($\dot{V}O2$) at rest and during prone and upright exercise.

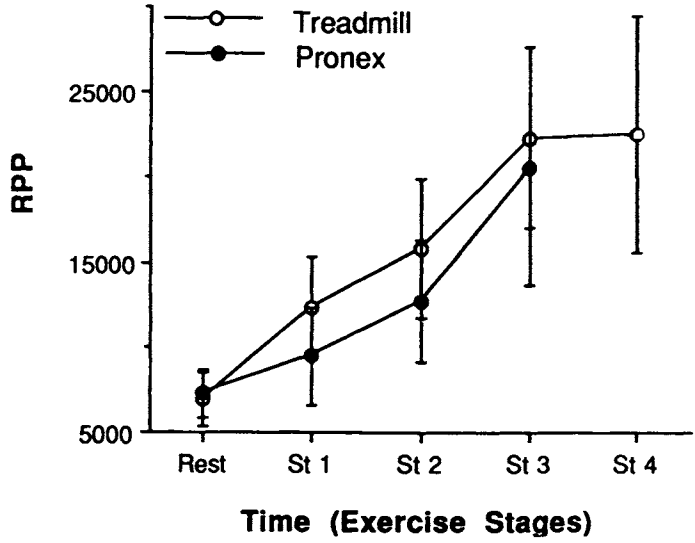

Figure 9.6. Rate–pressure product at rest and during prone and upright exercise.

High rate–pressure products occur during prone exercise and indeed during the final stage of the prone exercise protocol the rate–pressure products are over 20000 and close to those found during stages 3 and 4 of the Bruce protocol.

3.2. Physiological changes during isometric exercise

Sustained handgrip or isometric exercise is another method which can be used for studying the effects of myocardial stress on the intracellular PCr concentration. The effects of this type of exercise on the circulation are well documented (Lind *et al.*, 1964; Donald *et al.*, 1967). There is a rapid elevation in systolic and diastolic blood pressure which leads to significantly increased afterload. The effects are shown to be transient and generally reverse within 1 min of recovery.

3.3. Cardiac high energy phosphate metabolism during isotonic and isometric exercise

High-energy phosphate measurements during dynamic exercise are shown in Fig. 9.7 for a subject undergoing prone exercise in a 1.9 T whole-body magnet. The exercise was performed using the 5.0 kg workload which was evaluated during the physiological studies. Thus, at a level of exercise which is close to that at stage 1–2 of the Bruce protocol, when the oxygen consumption is 9.2 ± 2.0 vs 14.5 ± 2.9 ml/kg/min (Bruce stage 1) and the rate–pressure product

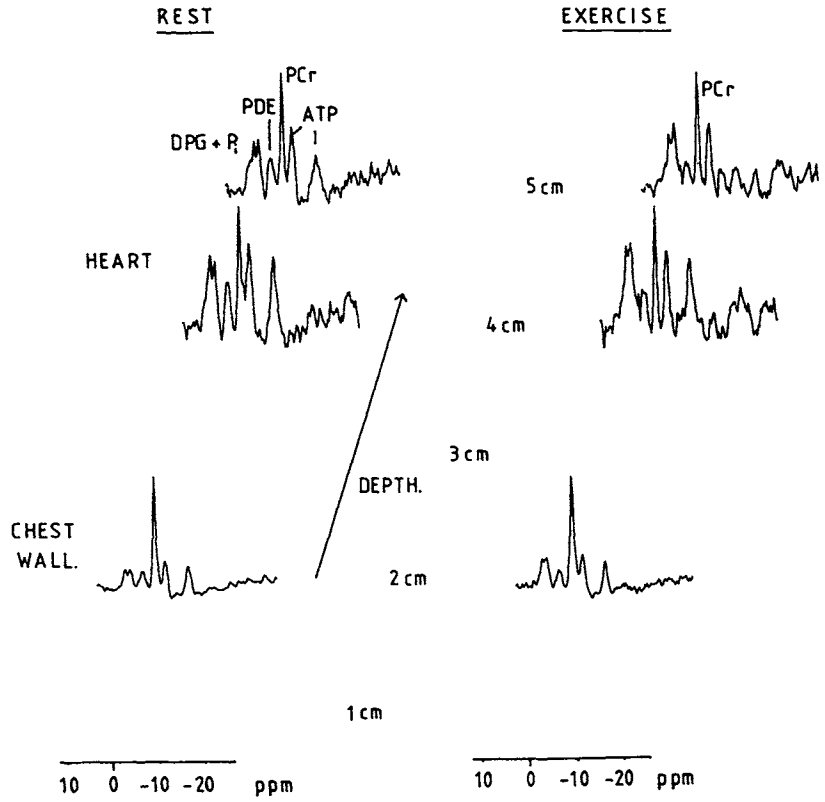

Figure 9.7. Human cardiac spectra acquired during dynamic prone exercise using ^{31}P MRS (abbreviations as in Fig. 9.1). Reproduced with permission from Conway *et al.* (1991b) BMJ Publications.

is 12670±3580 vs 12290±3028, no change develops in the PCr/ATP ratio (rest, 1.56±0.2; exercise, 1.58±0.14).

Similar observations have been made by Weiss *et al.* (1990) using an isometric exercise for 7–8 min at 30% of maximal voluntary contraction. Two minutes were allowed to achieve steady state before signal acquisition. Spectra from normal controls which do not alter during isometric exercise have also been acquired by Yabe *et al.* (1994).

These findings demonstrate that during both isotonic and isometric exercise the PCr/ATP is unchanged in normal subjects. Thus, despite an increase in workload associated with measurable significant haemodynamic changes, the cellular PCr content is unaltered.

Several questions about normal cardiac metabolism still remain to be answered, e.g. can MRS be used to demonstrate the absence of change in the PCr level with each contraction in the normal heart, as has been shown by

Table 9.2. Examples of direct measurements of high-energy phosphates in models of ventricular hypertrophy and heart failure.

Publication	Preparation and experimental detail		Cr[a]	PCr[a]	ATP[a]
Hochrein and	GP, *in situ*	N		7.2	3.5
Doring (1958)	Vol. load++	CHF		–32%	–11%)
Feinstein	GP, *in situ*	N		8.4	5.6
(1960)	(Aortic constriction)	CHF		–54%	–24%
Pool *et al.*	Cats, RVH				
(1967)	Right ventricle:	N	15.5±0.6	7.2±0.5	5.1±0.2
		RVH	12.5±0.9	5.4±0.5	5.1±0.4
		CHF	9.70±0.6	4.1±0.4	5.1±0.6
	Left ventricle:	N	17.4±0.5	7.9±0.7	5.8±0.3
		LVH	17.0±1.3	8.8±1.0	6.3±0.6
		CHF	12.2±0.4	5.9±0.8	6.0±0.6
Coleman *et al.*	Dogs, Sham op. (n=8)		21.3±0.8	9.0±0.6	6.4±0.3
(1971)	Ch Tachy CHF (n=6)		13.8±0.6	7.1±0.6	5.4±0.7
Attarian *et al.*	Dogs, Subendo	Con		42.1±0.9	23.8±0.7
(1981)		LVH		27.1±3.6	18.2±1.7
	Dogs, Subepi	Con		45.6±1.9	23.5±1.0
		LVH		41.2±2.5	22.4±0.7

[a] Values are μmol/g dry tissue.

Creatine (Cr); phosphocreatine (PCr) adenosinetriphosphate (ATP); congestive heart failure (CHF); left-ventricular hypertrophy (LVH); guinea pig (GP); control (CON); normal (N); tachycardia (Tachy); subendocardial (Subendo); subepicardial (Subepi).

Wikman-Coffelt *et al.* (1983)? Can ^{31}P MRS reliably demonstrate different metabolic concentrations in the epicardial compared with the endocardial segments? Such questions represent significant technical challenges at present but are an important part of future strategy since PCr/ATP ratios measured during diastole or systole alone or as a mean measurement across the myocardium may fail to differentiate clearly the demands on PCr metabolism – both in controls and in patients with ischaemia and other cardiac disease.

4. LEFT VENTRICULAR HYPERTROPHY

The relationship between the concentration of PCr and ATP in the hypertrophied heart has been the subject of many studies using both wet biochemical methods and non-invasive cardiac spectroscopy. Changes in the concentration of PCr and in the ratio of the PCr to ATP have been identified in animal models of hypertrophy associated with heart failure due to hypertension and valvular heart disease and the early work was reviewed by Furchgott and Lee (1961). A summary of the findings in a number of the original studies is presented in Table 9.2 (see also Chapter 3). Using MRS, Clarke *et al.* (1989) observed that the total PCr content was decreased in triiodothyronine-treated

hypertrophied hearts. Recent studies on the dog model of left ventricular hypertrophy (LVH) produced by banding the aorta suggest that a decrease in the ratio of PCr to ATP occurs in significant myocardial hypertrophy (Zhiang *et al.*, 1993) compared with moderate hypertrophy (Aufferman *et al.*, 1990). The ratio of these metabolites decreases markedly with pacing-induced tachycardia in the hearts whose mass is doubled compared with control (Zhiang *et al.*, 1993). Hence, a number of classical and ^{31}P MRS studies indicate that lower PCr/ATP ratios can occur in heart failure associated with LVH. Creatine phosphate is depleted in biopsy specimens on patients suffering from both aortic stenosis and coronary artery disease (Swain *et al.*, 1982), whilst Ingwall *et al.* (1985) found cardiomyopathy biopsies with low creatine concentrations. The hypertensive rat in transition to heart failure has similarly low PCr (Ingwall *et al.*, 1990). However, reduced PCr may not be a universal finding since Chidsey *et al.* (1966) found no reduction in the concentration of high-energy phosphates in papillary muscle preparations of surgical patients with heart failure associated with mitral and other valve disease, and Pool *et al.* (1967) detected normal PCr and ATP concentrations in *in vitro* isolated papillary muscles, even though they demonstrated intrinsically depressed contractility.

These observations suggest that altered PCr to ATP ratios can be expected in some patients with LVH due to valve disease, hypertension, congenital heart disease and cardiomyopathies. Over the years, the pathology, electrophysiology, haemodynamics and other pathophysiological aspects of LVH have been examined (Swynghedauw, 1990) but much is yet to be learned about the cellular biochemistry of the human hypertrophied heart. Furthermore, myocardial hypertrophy is associated with the development of heart failure and characterization of the biochemical features of the failing human heart represents an important objective *per se*.

4.1. Valvular heart disease

In order to examine concentric compared with eccentric hypertrophy in the presence and absence of heart failure, Conway *et al.* (1991a, 1993) performed studies on a group of patients with aortic stenosis and a group with aortic incompetence.

During the initial studies using the PMRFI technique (1.9 T magnet and a 6.5 cm surface coil) 14 patients were examined (Conway *et al.*, 1991, 1993), of whom six had aortic stenosis and eight aortic incompetence. The PCr/ATP ratios were compared with 13 healthy controls (PCr/ATP: 1.5 ± 0.2). Six patients were on treatment for heart failure with diuretics and/or vasodilators. The mean aortic valve gradient was 59 ± 34 mmHg and the severity of aortic incompetence was grade 3–4. The left ventricular end diastolic pressure was 16 ± 7.8 mmHg in 10 of the patients.

Examples of spectra are shown in Fig. 9.8. These are taken from stacked plots such as those shown in Fig. 9.9. The low amplitude of the signal from 2,3 DPG suggests that the sample volumes predominantly contain myocardium.

The ratio of PCr to γATP between eccentric versus concentric LVH was not significantly different (n=6: 1.45±0.32 vs n=8: 1.23±0.3). On examination of the PCr/ATP ratio according to symptoms [New York Heart Association (NYHA) status] and treatment with diuretics, low PCr/ATP ratio was found in those with symptoms of breathlessness requiring therapy (1.1±0.32) compared with normal ratios in the asymptomatic group (1.5±0.15; $p<0.004$). This difference did not relate to structural factors such as the posterior wall thickness or left ventricular mass.

These findings suggest that in patients with failing hearts a decreased PCr to ATP ratio develops. The mechanism of such changes in the PCr/ ATP ratio is unknown. Altered steady state PCr concentrations may reflect altered creatine metabolism in failing hypertrophied tissue. The importance of these observations lies in the demonstration of abnormalities which may respond to therapeutic intervention and the possible role of administered creatine and PCr is described below.

Much work has yet to be done to examine metabolism in hypertrophied tissue associated with heart failure. In contrast to the animal model, patients with myocardial hypertrophy may have disease complicated not only by therapy for fluid retention, but also by perfusion abnormalities associated with coronary artery disease. Thus, human LVH is likely to be more complicated and require careful case selection for study. Attention to such factors, the application of better localizing techniques and the introduction of methods such as magnetization transfer (Forsen and Hoffman, 1963; Bittl and Ingwall, 1987) are required so as to interrogate further the dynamics of high-energy metabolism.

4.2. Hypertension

Hypertension has been identified as one of the most important causes of heart failure in man (Kannell *et al.*, 1991). Those patients with hypertension who develop LVH are at greatest risk – three-fold excess risk compared to patients with hypertension alone (Kannell, 1983). The increased LV mass is also associated with increased mortality and in the original cohort studied at Framingham (Kannell, 1983) electrocardiographic LVH was associated with an eight-fold increase in mortality. Dunn *et al.* (1990) report a doubling of mortality amongst patients with ECG evidence of LVH and strain. Heart failure frequently develops insidiously in such patients and the deterioration in pump function can occur without overt symptoms attributable to coronary artery disease. The mechanism of impaired left ventricular function in

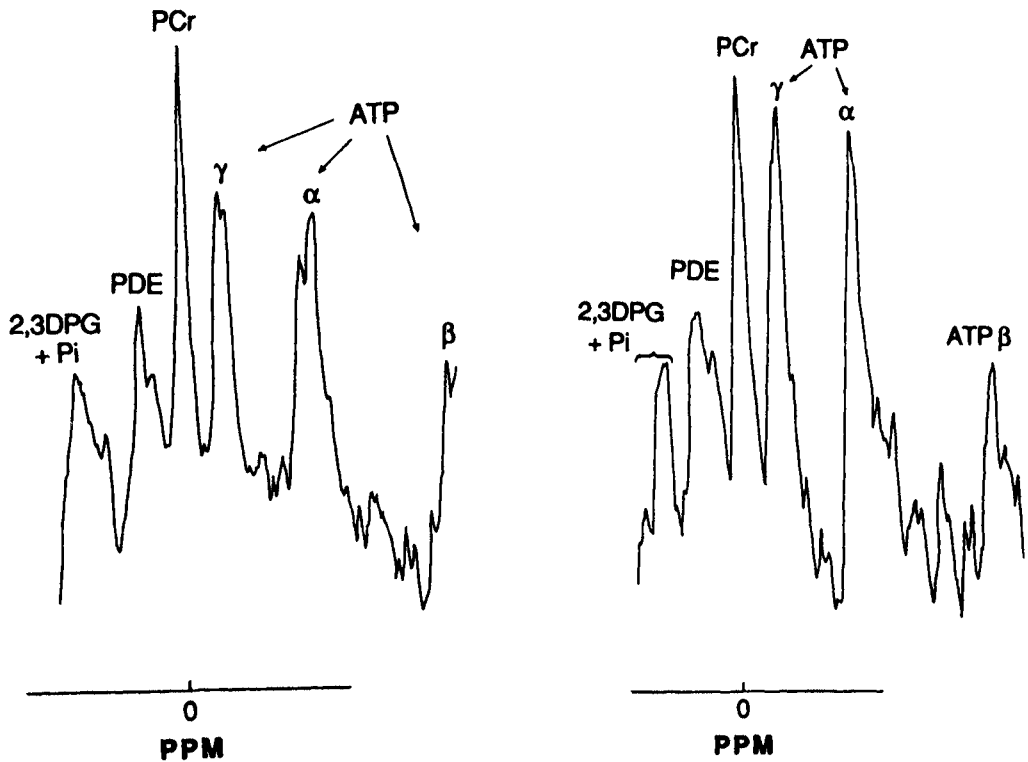

Figure 9.8. Individual spectra from patients with mild (left) and severe (right) myocardial hypertrophy (abbreviations as in Fig. 9.1).

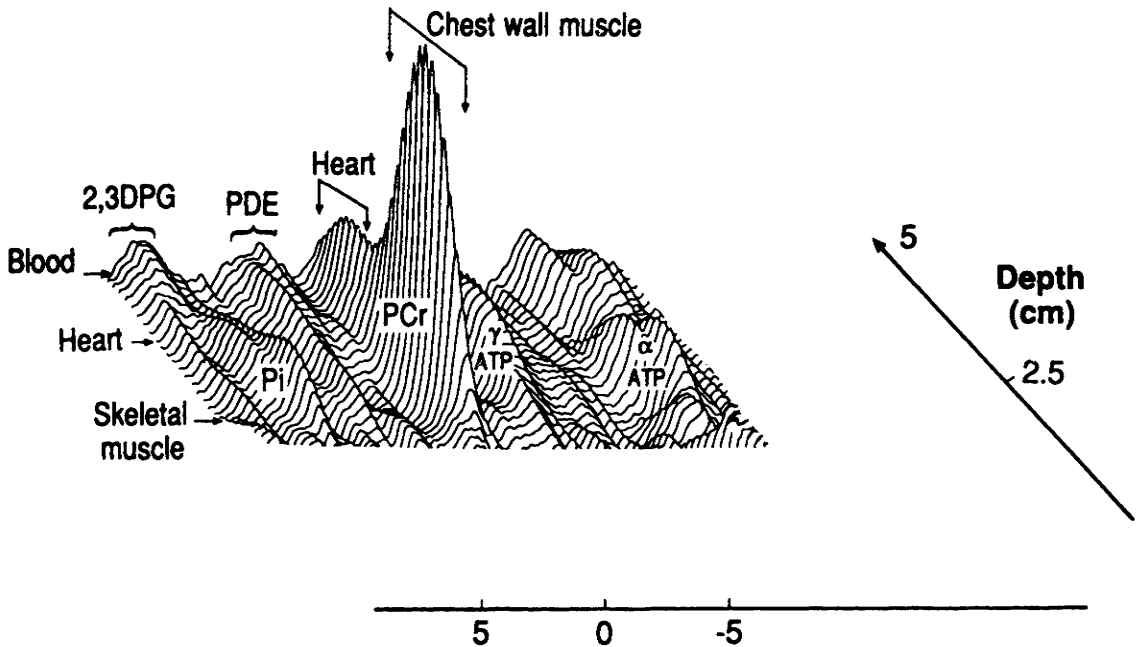

Figure 9.9. Stacked plot of data sampled using a 6.5 cm surface coil. The stacked plot is constructed of adjacent individual spectra with the spectra from near the surface at the front. The creatine phosphate (PCr), the amplitude of which is greatest in chest wall muscle, steps down to a plateau in the region of the heart. The inorganic phosphate (P_i) peak in skeletal muscle is clear. The γ and αATP peaks are shown. Modified and reproduced with permission (Conway *et al.*, 1991a; Lancet Publications).

hypertension is poorly understood and both systolic and diastolic dysfunction have been recognized (Shepherd *et al.*, 1989). Metabolic abnormalities may be central to failure and the identification of such changes would be valuable for more careful management and a better prediction of prognosis.

Heart failure is likely to develop in those with hypertension who develop myocardial hypertrophy with or without associated coronary artery disease. Electrocardiographic LVH is present in 34.5% of men and 12.8% of women with hypertension (Dunn *et al.*, 1990) and this group are an important population in whom metabolism must be characterized.

Messerli and Devereux (1983) proposed that the development of myocardial disease is likely to occur once the hypertrophy has developed to a significant degree and has passed through a "critical mass". Thus, in the early stages of the disease compensatory hypertrophy may develop which is similar to that found in athletes (Pelliccia *et al.*, 1991) and metabolic measurements will be unchanged from normal. Evidence for this has been found among hypertensive patients without heart failure where the PCr/ATP ratio was normal (Conway *et al.*, 1992), supporting the observations of Schaefer *et al.* (1990). Patients with increased left ventricular mass due to a combination of concentric hypertrophy and cardiac dilatation risk developing systolic failure, while those with pure concentric hypertrophy are likely to present with diastolic failure. Determining whether or not there is a metabolic difference between systolic and diastolic heart failure is central to the full understanding of hypertensive heart disease.

4.3. Hypertrophic cardiomyopathy

Segmental septal or apical hypertrophy is a genetic abnormality (Clark *et al.*, 1973; Maron *et al.*, 1986) which appears to develop independently of increased pressure or volume overloads. In contrast to concentric hypertrophy, where the new fibres are laid down perpendicular to the normal fibre orientation (Perennec and Hatt, 1990), in asymmetric septal hypertrophy fibre disarray is seen (Maron *et al.*, 1987). This structural change is associated with premature death which often results from ventricular arrhythmias. These patients are also prone to develop heart failure, particularly when there is significant obstruction to cardiac emptying due to hypertrophic obstructive cardiomyopathy (HOCM). The mechanism of pump failure in such cases may be similar to aortic stenosis or hypertension since the hypertrophied free wall or other segments – not inherently affected by the gene defect – are at risk of deteriorating independently.

Few ^{31}P MRS studies have been performed to examine metabolism in tissue with fibre disarray since the base of the septum is very deep relative to the surface coil. Rajagopalan *et al.* (1987), de Roos *et al.* (1992), Masuda *et al.* (1992) and Sakuma *et al.* (1993) have reported a low PCr/ATP ratio relative to

their normal controls. These findings raise the possibility that HOCM leads to abnormal metabolism at particular stages of the illness and ^{31}P MRS provides the opportunity to characterize patients metabolically and thereby determine whether procedures such as myomectomy or embolization are required to prevent myocardial deterioration.

5. ISCHAEMIC HEART DISEASE

The earliest studies on phosphate metabolism using MRS in the isolated perfused rat heart demonstrated that high-energy phosphates and pH changes resulting from ischaemia are elegantly reflected by alterations in the amplitude and area of PCr and P_i and by the frequency shift of P_i relative to PCr (Jacobus *et al.*, 1977; Garlick *et al.*, 1977, 1979). Studies on both localized (Stein *et al.*, 1986; Bottomley *et al.*, 1987b; Guth *et al.*, 1987; Rajagopalan *et al.*, 1989) and global ischaemia (Garlick *et al.*, 1979; Schwartz *et al.*, 1990) show decreased PCr/ATP ratios and a fall in pH. Such changes are largely determined by the degree of tissue perfusion and the altered concentration of individual metabolites reflects the extent of tissue hypoperfusion. This marker of flow has been found to be very sensitive and even with a 20% reduction in endocardial blood flow by 20% the ratio of the PCr to P_i falls (Schaefer *et al.*, 1990), reflecting greater PCr depletion and accumulation of P_i.

Many investigators have explored ^{31}P MRS in patients with coronary disease and, in contrast to the well-controlled animal model, the ischaemic human heart is subject to more variable and complex baseline perfusion. Thus, some patients may have co-existent segmental intermittent flow and persistently reduced flow and the myocardium may demonstrate dysfunction upon restoration of blood flow following ischaemia (the stunned myocardium). The high-energy phosphates in hibernating myocardium associated with chronically reduced blood flow may recover fully if the degree of ischaemia is moderate (Schaefer *et al.*, 1992).

Almost all currently available investigations for evaluating and diagnosing ischaemia require the application of physiological or pharmaceutical stress. The methods for performing exercise which are available for stress testing during ^{31}P magnetic resonance spectroscopy have been outlined above. Dobutamine stress testing has also been examined (Schaefer, 1993b). In this technique the infusion was commenced at a rate of 2 μg/kg/min and increased incrementally thereafter at a rate of 2 μg/kg/min every 4 min. By monitoring the ECG, haemodynamics and patient symptoms, the dose of dobutamine required to produce a significant increase in myocardial metabolic demand can be determined and this dose used during the performance of spectroscopy. In a case reported, dobutamine produced a clear reduction in PCr (Schaefer, 1993a).

Measurement of the PCr/ATP ratio during dynamic exercise has been performed by Conway *et al.* (1988) using the "Pronex" technique. At a level of

exercise which produced changes in the rate–pressure product equivalent to stage 2 of the Bruce protocol in controls, the PCr/ATP decreased in a patient with three-vessel coronary disease (Fig. 9.10). This patient did not suffer chest pain during the study despite a blocked left anterior descending coronary artery and severe circumflex and right coronary artery stenoses.

Two studies are reported where the PCr/ATP ratio was measured using isometric exercise in patients with severe coronary artery disease (Weiss *et al.*, 1990; Yabe *et al.*, 1994). This exercise technique was used because it avoids the problems of movement and it has been fully characterized in patients with coronary artery disease undergoing cardiac catheterization. Kivowitz *et al.* (1971) found that isometric exercise raises the mean arterial pressure from 84 mmHg to 104 mmHg. This was associated with a 45% increase in coronary sinus flow and myocardial oxygen consumption and an increase in systemic vascular resistance. Those patients with poor coronary reserve were found to develop a rise in left ventricular end diastolic pressure (LVEDP) in association with the increase in stroke work. These observations were supported by the findings of Helfant *et al.* (1971). However, this group noted that the increase in cardiac output was confined to patients without catheter evidence of heart disease. Indeed, isometric exercise reduced the stroke index in patients with coronary disease. The mechanism of these effects has been studied by Mitamura *et al.* (1984) using echocardiography. The regional wall motion abnormalities that they observed appear to relate to a reduction in the calibre of the vessel lumen (35%) as demonstrated by Brown *et al.* (1984). Thus, isometric exercise produces effects on the ischaemic left ventricle, not primarily through higher rate–pressure products, but by coronary vasoconstriction.

Weiss *et al.* (1990) studied 16 patients with severe left anterior descending or left main disease where the average severity of stenosis was $87 \pm 11\%$. Nine patients also had circumflex disease and in 11 cases the right coronary artery was also severely stenosed. Using isometric handgrip exercise at 30% of maximum capacity, clear reductions in the amplitude of the PCr were identified during exercise. Compared with normal PCr/ATP ratios of 1.72 ± 0.15, 1.74 ± 0.17 and 1.77 ± 0.16 before, during and after exercise, patients with coronary artery disease had ratios of 1.4 ± 0.31, 0.91 ± 0.24 and 1.27 ± 0.38 at equivalent times.

More recently these findings have been examined by a Japanese group (Yabe *et al.*, 1994) using depth resolved surface coil spectroscopy (DRESS) spectroscopy (Bottomley *et al.*, 1984). Twenty-seven patients were examined in whom severe stenosis of the left anterior descending (LAD) coronary artery ($>75\%$) was present. The findings were compared with thallium scintigraphic measurements and the patient group was divided according to whether or not a fixed thallium defect was present. Significant haemodynamic changes occurred in the heart rate, blood pressure and rate–pressure product with isometric exercise.

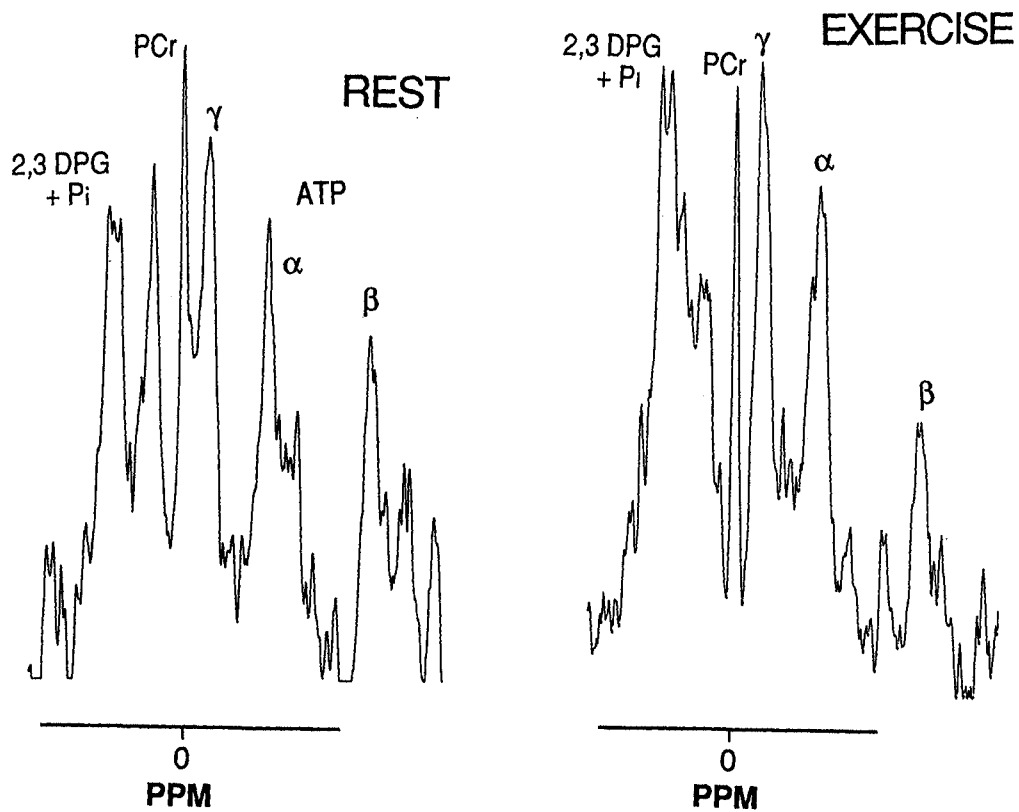

Figure 9.10. The cardiac spectra at rest and during dynamic foreleg exercise in a patient with severe three-vessel coronary artery disease (abbreviations as in Fig. 9.1).

In support of the observations of Weiss *et al.* (1990), Yabe *et al.* (1994) reported that in those with a reversible thallium defect, the PCr/ATP fell from 1.60±0.19 to 0.96±0.28 ($p<0.001$) with isometric handgrip exercise. This compared with an unchanged control ratio (rest 1.85±0.28 vs exercise 1.90±0.23). The patients with a fixed thallium defect had low PCr/ATP ratios at rest which was unchanged with exercise.

The potential role of ^{31}P MRS is as a non-invasive test for myocardial ischaemia. Such a role would be particularly valuable for characterizing the patient who presents with ischaemia and for monitoring the result of coronary revascularization. When fully assessed as a clinical investigation, MRS will likely be valuable in the screening of patients at high risk of developing ischaemia and will reduce the need for invasive angiography in patients presenting with atypical chest pain and conditions such as Syndrome X.

5.1. Acute myocardial infarction

Total myocardial ischaemia is associated with well-described changes in high-energy phosphate concentrations (Bing, 1965). In studies performed by Wollenberger and Krause in Berlin during the early 1960s, the elevated tissue orthophosphate and fall in phosphocreatine concentration was already recognized. Up to 80% of the phosphocreatine is depleted within 1 min of the onset of ischaemia in the dog heart (Braasch *et al.*, 1968) and the ATP in *in vivo* models decreases by 65% within 15 min (Jennings *et al.*, 1978). Ischaemia is characterized by an initial period of rapid metabolism of high-energy phosphates followed by a more linear period while anaerobic glycolysis is supplying the energy. By 120 minutes, the tissue high-energy phosphates will have decreased by over 96%. The cessation of glycolysis prevents further ATP utilization (Jennings *et al.*, 1981). Neely *et al.* (1973) suggest that 25% of the total ATP depleted in the first 2 min of ischaemia is used to support mechanical function and its concentration thereafter does not relate to mechanical performance because the latter falls from 5 min onwards even though the ATP may be at 75% of baseline levels.

These changes have been elegantly studied non-invasively using ^{31}P MRS. The phosphocreatine measurements are likely to be more accurate using this technique because, being a non-destructive *in vivo* method, risk of rapid breakdown of metabolites in *ex vivo* samples is avoided.

Localized ischaemia reflects the myocardial changes in the early phases of acute myocardial infarction (AMI) and Stein *et al.* (1986), amongst others, demonstrated the low PCr/ATP ratio and the fall in pH. Also, the non-infarcted tissue in the rat AMI model contains decreased phosphocreatine and creatine levels (Leonard Arnolda, *et al.*, personal communication).

Measurements on animal models of AMI using MRS demonstrate, as might be expected, that the overall concentration of high-energy phosphates is

decreased. Some experiments (Bottomley *et al.*, 1987b) appear to indicate that the ratio of phosphocreatine to ATP may be similar to normal in the infarcted tissue for hours and days after the acute event. These observations (Paul A. Bottomley, personal communication) might indicate that the ATP may be depleted independently of phosphocreatine.

Measurements on the human heart within days of acute myocardial infarction have been performed by Bottomley *et al.* (1987b) and Conway *et al.* (1992a). Others have studied patients with older myocardial infarctions (i.e. more than 2 weeks after the acute event) (Mitsunami *et al.*, 1992; Neubauer *et al.*, 1992; Luney *et al.*, 1993). In the four patients studied 5–9 days after AMI, Bottomley found no difference in the PCr/ATP ratio. This finding was similar to that reported by Mitsunami *et al.* and Neubauer *et al.* but contrasted with the low PCr/ATP in the five patients studied by Luney *et al.* at a time 3 months after infarction.

In a study performed within 4 days of a large anterior myocardial infarction, Conway *et al.* (1992) demonstrated a low level of signal from the region of the heart consistent with the extensive degree of damage associated with early mortality. Reliable PCr/ATP measurement in such infarctions is difficult because of the low signal-to-noise ratio and this may be a factor in the discrepency amongst findings reported so far. Another reason for the differences is that some of the tissue in the sample volume may be failing – especially if it is poorly perfused in the peri-infarct zone.

Careful studies of similar types of myocardial infarction, e.g. anterior versus inferoposterior, early compared with late, and in patients who recover compared with those who develop heart failure, are required in order to fully characterize myocardial infarction. Also, the size of the infarcted area compared to the area of the sample volume will need to be accurately determined using techniques such as magnetic resonance imaging (de Roos *et al.*, 1990) so that the sampling of normal and hypoperfused peri-infarct tissue can be reduced to a minimum.

The pattern of both energetic and functional recovery following a period of ischaemia is heavily dependent on the duration of the insult, an observation especially relevant to modern thrombolytic therapy. Lethal myocardial injury will occur if the duration of total ischaemia extends beyond 1 h in the dog model (Jennings and Reimer, 1981). This may not apply directly to the human heart where collaterals and dual circulation are well described, but the process may well be similar. If oxygen and key substrates can be supplied to the cells early enough then recovery will occur. After a critical period, cellular function may not recover and on reperfusion massive swelling, contraction bands, the accumulation of calcium phosphate and disruption of the cell membrane is seen. Jennings and Reimer (1981) contend that cell death occurs when high-energy phosphate, principally ATP, drops to levels insufficient to maintain the cells' structural integrity.

Consequently, study of the very early hours after acute coronary occlusion

should be the goal since successful thrombolysis may be demonstrable non-invasively as a restoration of the PCr/ATP ratio. Failure of the high-energy phosphates to recover within a few hours (particularly in the very young patient) might then be an indication to proceed to cardiac catheterization and angioplasty. Future studies must be directed at measurement of tissue metabolite concentrations since extensive and completed infarction will lead to gross depletion of active metabolites.

Some animal and human studies indicate that the P_i rises following myocardial infarction. While this is difficult to measure in cardiac spectra contaminated by blood (a feature that is likely to be commonly seen in the dilating infarcted heart), it does provide a potential index of infarction which may be relevant in diagnosis. Thus, if the P_i rises early, the change may precede ECG and enzyme evidence of infarction and lead to earlier thrombolytic therapy.

6. MYOCARDITIS AND DILATED CARDIOMYOPATHY

Inflammatory conditions such a myocarditis cause acute deterioration in cardiac function and patients often present with pulmonary oedema or congestive cardiac failure. The changes in high-energy phosphorus metabolism associated with such damage to the myocardium in man are largely unknown. Inflammation usually leads to irreversible damage and extensive myocardial fibrosis. Once this stage is reached, chronic heart failure develops and patient survival may depend on the integrity of areas or segments of the myocardium which recovered from the initial inflammation. Animal models of myocarditis secondary to Adriamycin (Keller *et al.*, 1986) and alcohol have been studied and while no measurable metabolic changes were seen following the cytotoxic drug, a reversible decrease in PCr/ATP was detectable in animals fed a high alcohol diet (Auffermann *et al.*, 1988,1989).

A number of groups have studied dilated cardiomyopathy in man using ^{31}P MRS (Schaefer *et al.*, 1990; Aufferman *et al.*, 1991; Hardy *et al.*, 1991; de Roos *et al.*, 1992; Masuda *et al.*, 1992; Neubauer *et al.*, 1992). Studies on over 70 patients are documented. Coronary artery disease was absent in the majority although nine of the 20 patients studied by Hardy *et al.* (1991) had documented ischaemia. Dilated cardiomyopathy patients were generally classified as symptomatic of heart failure and were on therapy for fluid retention. The ejection fraction varied from study to study [as low as 9% in one study (de Roos *et al.*, 1992) and up to $33 \pm 14\%$ in the group studied by Neubauer *et al.* 1992)]. The NYHA classification also indicated some heterogeneity with some patients being asymptomatic in NYHA class I while others were significantly dyspnoeic at rest (NYHA class IV).

The PCr/ATP ratio did not appear to be abnormally low in these patients compared with control except in the study of Hardy *et al.* (1991), where the PCr/ATP ratio was decreased by approximately 20% in the diseased

myocardium. This difference did not appear to result from the incorporation of the patients with coronary artery disease since the non-ischaemic patients were also found to have reduced PCr/ATP ratios.

The differences between the groups may relate to the techniques used for spectroscopy and the difficulties of localizing signal in dilated thinned hearts with cardiomyopathy. Indeed, contamination by blood may result in a much greater contribution of blood ATP to the cardiac spectrum and unless this is allowed for any reported measurements of PCr/ATP in such hearts, where adjustment has not been made, must be treated cautiously. Since subtraction of the contaminating ATP will increase the PCr/ATP ratio, the high ratios that may arise after proper adjustment raise the question of significant skeletal muscle contamination.

The above reports are based on patients with chronic disease. Similarly low signal from such hearts have been observed using the PMRFI technique (Conway *et al.*, 1992a), both in early acute myocarditis and in the later chronic phase of the disease. However, as well as low ratios, absent signal from PCr at the depth of the heart is frequently seen. Some subjects who present with acute heart failure due to myocarditis recover with little evidence of chronic congestion apart from a variable degree of cardiac dilatation seen on chest X-ray and by echocardiography. In such cases the heart may have normal myocardial high-energy phosphorus metabolism which is consistent with some reported measurements.

Schaefer *et al.* (1990) suggest that phosphodiesters (PDE) are elevated in dilated cardiomyopathy (Schaefer *et al.*, 1990). Others have indicated that the large PDE signal comes mainly from blood (de Roos *et al.*, 1992). While blood is likely to be the main source of this metabolite, the size of the PDE peak in a number of patients argues against serum as the only source and suggests that the phosphate signal from glycerophosphorylcholine and glycerophosphorylethanolamine is abnormally raised. Lipid deposits and fatty infiltration of the myocardium may be the source of such a signal and the amplitude of the PDE peak may yet have an important role.

7. CARDIAC TRANSPLANTATION

The viability and functioning of the transplanted heart depends on a number of factors. One which is central to management and therapy is the rejection process. At the present time, the most reliable method of accurately determining whether or not there is active rejection is right ventricular endomyocardial biopsy. This poses a large demand on resources and is not without some risk and discomfort for the patient. Methods for non-invasively monitoring the myocardium are required and since metabolic changes result from the rejection process the role of ^{31}P MRS has been studied by a number of groups.

Canby *et al.* (1987) were some of the earliest investigators to examine the technique using an animal model. A non-working rat heart was inserted in the neck of a recipient rodent. The hearts were studied at 4.7 T daily for several days prior to excision for histological study. No immunosuppression therapy was used. The behaviour of metabolites in the isografts and allografts was different and the PCr/ATP was unchanged in the former whereas the latter had a significantly reduced PCr/ATP by day 4. In a similar study on rats, Haug *et al.* (1987) showed that treatment with cyclosporin prevented allograft rejection and the PCr/P_i ratio was normal. The rejecting heart spectrum shown in this report has a large 2,3 DPG+P_i peak indicating that there may have been blood contamination of the sample volume. The non-immunosuppressed beagle heart transplant was studied by Fraser *et al.* (1988) and decreased PCr/ATP was shown to occur within 2–3 days. These metabolic changes preceded histological evidence of rejection which was seen at day 4.

Such observations lead to interest in the application of the technique in patients in the expectation that low PCr/ATP ratios would represent the early stages of the rejection process. However, the animal studies were performed on the non-working heart which was not immunosuppressed with steroids and azathiaprine.

In the animal models of cardiac transplantation unchanged PCr/ATP ratios were seen in the non-rejecting heart and this finding has been confirmed in man (Herfkens *et al.*, 1988; Evanochko *et al.*, 1990). However, the detection of rejection based on the PCr/ATP has not been reliably demonstrated despite studies on 176 patients post-transplantation to date (Herfkens *et al.*, 1988; Evanochko *et al.*, 1990; Bottomley *et al.*, 1991; Wolfe *et al.*, 1991; van Dobbenburgh *et al.*, 1993). Low PCr/ATP was identified in patients with histological evidence of rejection but the changes have failed to correlate adequately with the severity of histological changes associated with the rejection process. At present ^{31}P MRS assessment of rejection cannot be recommended. The reason for the failure of the technique is unclear but may relate to problems such as contamination. Further evaluation of the technique is required with emphasis on P_i measurements and the metabolic balance measurable by magnetization transfer techniques (Forsen and Hoffman, 1963).

8. CREATINE PHOSPHATE CHANGES IN TACHYCARDIA AND RHYTHM DISORDERS

When the heart rate is increased by ventricular pacing to produce heart failure the PCr concentration falls (Coleman *et al.*, 1971). Increasing the heart rate of dogs with severe left ventricular hypertrophy also decreases in the PCr/ATP ratio (Zhiang *et al.*, 1993).

Human rhythm disorders which simulate such tachycardic conditions are ventricular and supraventricular tachycardia and rapid atrial fibrillation (AF).

Patients with controlled AF have been examined during valvular heart disease studies and while many have low PCr/ATP ratios (Conway *et al.*, 1994), the cause of the decreased PCr concentration is thought to be related to myocardial disease. Atrial fibrillation features in some patients with mild mitral valve prolapse and they have normal ratios (Conway *et al.*, 1994).

Whether heart failure associated with uncontrolled AF is associated with a precipitous fall in the PCr/ATP ratio is unknown but studies of such patients may help to explain one of the biochemical mechanisms of acute heart failure. No studies are reported describing the effects of persistent ventricular tachycardia on PCr/ATP but a low ratio may well be found which can be expected to recover once sinus rhythm is re-established.

9. HYPOXAEMIC CONGENITAL HEART DISEASE

Children with hypoxaemic congenital heart disease (CHD) due to valvular and other causes are prone to increased morbidity due to heart failure and death from pump failure. These patients frequently develop right and left ventricular hypertrophy and the metabolic features of such tissue perfused by hypoxaemic blood may be relevant to the long-term outcome. The effect of chronic hypoxaemia may be different from that produced acutely.

Further CHD studies are important since in conditions such as pulmonary stenosis the metabolic improvement resulting from valvular and other surgery may be worth determining so as to monitor cardiac function and assess prognosis.

Keevil *et al.* (1993) reports that amongst three patients with cyanotic congenital heart disease the PCr/ATP ratio was low (0.69 ± 0.02 (SE)) and significantly different from that found in three normal children (1.5 ± 0.3 (SE)).

10. INFILTRATIVE CONDITIONS: AMYLOIDOSIS AND GRANULOMATOUS CARDIOMYOPATHY

Amyloidosis, sarcoidosis and granulomatous connective tissue disorders are rarely encountered diseases associated with the destruction of normal myocardium. Patients usually present with symptoms of fluid retention either due to impaired systolic function (congestive cardiomyopathy) or due to diastolic failure. The pathological pattern in end-stage disease is often similar to ischaemic cardiomyopathy and the destruction of myocytes leads to a decrease in the volume of actively metabolizing cells. Masuda *et al.* (1992) reported on one patient with cardiac amyloidosis in whom the PCr/ATP ratio was low. Whether such findings are a feature of the underlying condition or whether they represent more general changes is unclear. The symptoms of such patients mirror those with impaired left ventricular function due to valvular

and other causes as outlined above. The signal from the damaged heart may derive from the non-infarcted segments or those parts of the myocardium which have not been affected by inflammatory or other conditions. The metabolic demand on such tissue is greater and so the reduced PCr/ATP ratio may be a common response unrelated to the primary pathology affecting the heart. The mechanism of the abnormal PCr/ATP ratio is unknown but may relate to an increased requirement for ATP in the individual segments. This could imply that a new steady state is achieved where the baseline PCr is low, consistent with the hypothesis that the heart is more prone to acute deterioration when uncontrolled AF or other arrhythmias place increased stress on the myocardium.

11. CHANGES IN CREATINE PHOSPHATE: THE EFFECTS OF DRUG THERAPY AND DIAGNOSTIC POTENTIAL

Few cardiac ^{31}P MRS studies of high-energy phosphates following therapeutic intervention have been reported. This is likely to reflect the difficulties encountered in localizing the same part of the myocardium during repeat studies. No difference in the PCr/ATP ratio was observed using dobutamine on the myocardial metabolism of patients with dilated cardiomyopathy (Schaefer, 1993a). Neubauer *et al.* (1992) reported that β-adrenergic blocking agents improve patient symptomatology corresponding with an increase in the PCr/ATP ratio. High-energy phosphate metabolism may respond particularly well to converting enzyme inhibition and studies of Captopril and other such agents are indicated.

^{31}P MRS shows promise as an investigation prior to valvular heart surgery. Heart failure is an important indicator to proceed to surgery for mitral and aortic valve disease. The optimum timing is often difficult to determine, particularly when objective measurements of damage are associated with few symptoms. Much work has been performed to try and identify the key haemodynamic or other factor which allows categorization of the severity of heart failure. Little success has been achieved using echocardiographic parameters, various haemodynamic measurements such as the cardiac index (Leith *et al.*, 1984), cardiac output, left ventricular end-diastolic dimension, mean velocity of circumferential shortening, % dP/dt and the ejection fraction (Benge *et al.*, 1980; Franciosa *et al.*, 1981; Port *et al.*, 1981; Francis *et al.*, 1982) – as outlined below (Chapter 10). The PCr/ATP ratio represents a new index which must be fully characterized for evaluating the patient with heart failure. It is the factor which may determine the exercise capacity since changes in PCr due to exercise may limit individual patients (and such alterations in PCr might not be reflected by altered haemodynamics, etc.). In conditions such as mitral valve disease where the cause of heart failure is surgically treatable, low PCr could be an indication to proceed to surgery. Procrastination in patients

with valvular heart disease predisposes to deterioration either before, during, or after surgery. This may explain the well-recognized outcome in some patients who succumb despite mitral valve replacement. The avoidance of "overt congestive heart failure" (Hancock, 1990) by the identification of impending metabolic deterioration is the ideal and the PCr/ATP index can be expected to constitute a worthwhile addition to the clinical judgement currently based on echocardiographic measurements such as the degree of cardiac dilatation (Henry *et al.*, 1982) and calculating the individual risk factors (Lund, 1990). Clearly such a non-invasive test would be more acceptable than preoperative biopsy (Krayenbuehl *et al.*, 1989).

12. SUMMARY

Magnetic resonance spectroscopy represents a potentially new diagnostic and management tool for patients with a wide range of myocardial diseases. Many technical problems still hinder widespread use of the method. However, results from animal models indicate that clear changes in high-energy phosphorus metabolism occur and the reliable identification and full characterization of these in the human heart is the challenge for the future era of cardiac spectroscopy.

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10

Skeletal Muscle Metabolism in Heart Failure

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Key words: skeletal muscle, metabolism, heart failure, creatine phosphate, exercise, transplantation, perfusion, ischaemia, creatine

1. BACKGROUND HAEMODYNAMIC AND OXYGEN CONSUMPTION STUDIES

Two of the main complaints reported by patients suffering from chronic heart failure are fatigue and reduced exercise tolerance. The direct mechanism of these symptoms has been examined extensively ever since the earliest days of invasive haemodynamic measurement (McMichael, 1947; Cournand, 1952). No simple pressure abnormality was found which might easily explain the symptoms although an elevated left ventricular end-diastolic pressure is a frequent, though not universal, finding (e.g. Mason *et al.*, 1970; Patterson *et al.*, 1972). Some groups attempted to relate symptoms such as breathlessness to the cardiac index (Leith *et al.*, 1984) and ejection fraction (Strauss *et al.*, 1971; Schelbert *et al.*, 1976; Franciosa *et al.*, 1981), but such measurements at rest vary considerably between reported studies and often only show clear

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differences from normal when the patients have severe disease. The exercise capacity is not related to any resting abnormalities of cardiac output, stroke volume, systemic vascular resistance, left ventricular end-diastolic dimension, mean velocity of circumferential shortening, % dP/dt and ejection fraction (Benge *et al.*, 1980; Port *et al.*, 1981; Franciosa *et al.*, 1981; Francis *et al.*, 1982). Even in studies where the haemodynamic abnormalities were assessed during exercise, no clear relationship between central changes and exercise capacity was demonstrated, and indeed it is only possible to show a significant difference in the cardiac index during exercise when the severity of heart failure is markedly different [New York Heart Association (NYHA) class I versus class IV heart failure: 6.12 ± 0.43 vs 3.31 ± 0.31: Franciosa *et al.*, 1979].

These observations indicate that factors other than central haemodynamics alone determine the symptomatology of heart failure. An indication as to a possible mechanism follows from studies in which the exercise tolerance was related to the maximum oxygen consumption ($\dot{V}O2_{max}$). Weber *et al.* (1987) demonstrated that patients can be classified into four groups related to the $\dot{V}O2$ (class A, greater than 20 ml/kg/min; class B, 16–20 ml/kg/min; class C, 10–16 ml/kg/min; class D, less than 10 ml/kg/min). Patterson *et al.* (1972) developed a similar classification and both Franciosa *et al.* (1981) and Higginbotham *et al.* (1983) found that the maximum oxygen consumption was 13.7 ± 1.0 ml/kg/min or 14.8 ± 3 ml/kg/min, respectively, in patients with moderate heart failure (NYHA class II or class II–III).

2. ABNORMALITIES IN MUSCLE METABOLISM

The identification of a connection between exercise tolerance and maximal oxygen consumption may argue for a relationship between symptoms and muscle metabolism. High-energy phosphates are some of the key metabolites which are central to muscle contraction. Adenosine triphosphate (ATP), creatine phosphate (PCr) and inorganic phosphate (P_i) can be measured non-invasively using ^{31}P magnetic resonance spectroscopy (MRS). Hence a number of groups have examined PCr and ATP in heart failure using this technique. Any alteration in intracellular biochemistry is likely to be linked to changes in the peripheral circulation, both with respect to nutrient supply and the removal of the metabolic products. Indeed, there is good evidence for reduced peripheral blood flow at rest and during exercise in the renal circulation (Merrill, 1946; Tauxe *et al.*, 1981; Cleland *et al.*, 1986; Ribstein and Mimran, 1986; Conway *et al.*, 1989b) and in forearm and leg muscle (Zelis *et al.*, 1968, 1974; Longhurst *et al.*, 1974; Cowley *et al.*, 1986) of patients with severe heart failure. Although questions remain about the blood flow to peripheral organs in mild and moderate heart failure, any degree of chronic hypoperfusion could affect the muscle metabolic processes, either at the control or substrate levels.

Since the mid-1980s several groups have examined skeletal muscle PCr

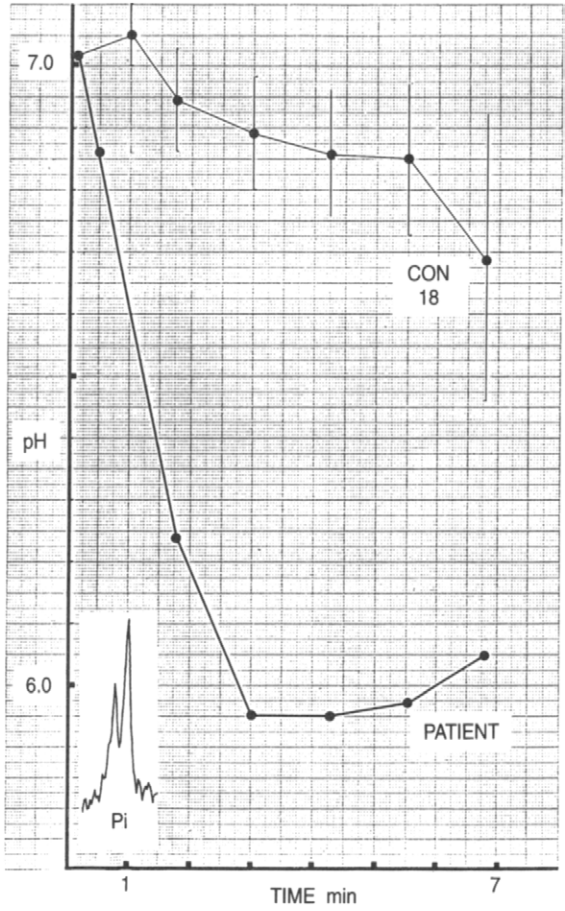

Figure 10.1. The fall in pH in the flexor digitorum of a patient with congestive heart failure due to a dilated cardiomyopathy. The P_i peak, which is also shown, is clearly split. This indicates the likely presence of two populations of cells with different intracellular pHs, a feature sometimes seen in the heart failure patient.
CON: Controls, n 18. PATIENT: Patient with CHF.

during exercise in patients with heart failure. Not only have abnormal ratios of PCr to other phosphate metabolites such as P_i and ATP been observed, but large falls in pH – such as that shown (Fig. 10.1) – were documented. Early studies such as this led to consideration of potential mechanisms underlying the metabolic abnormalities and since then studies examining differing factors such as blood flow (Wilson *et al.*, 1984a & b), disuse atrophy (Mancini *et al.*, 1992) and the therapeutic consequences of transplantation (Stratton *et al.*, 1994) and training (Adamopoulos *et al.*, 1993) are reported.

In the original studies of forearm skeletal muscle, ^{31}P MRS was performed using small-bore 1.9 T magnets (Wilson *et al.*, 1985; Massie *et al.*, 1987a & b). Both studies examined high-energy phosphorus metabolism of the forearm flexor muscles such as flexor digitorum superficialis (Taylor *et al.*, 1983). The studies differed in design from one another since Wilson *et al.* (1985) used a 4.5 cm surface coil during wrist flexion exercise every 5 s for 7 min with 10 min rest periods between each 1, 2 or 3 J workload. In contrast, Massie *et al.* (1987a & b) used a 2.5 cm surface coil with repetitive bulb squeeze or continuous incremental (250 g/min) exercise until exhaustion. Furthermore, while the Oxford group examined the changes immediately during recovery from the full exercise protocol, Wilson *et al.* (1985) exercised the patients and controls until the amplitude of the PCr and P_i peaks were equivalent and then observed recovery at longer time intervals (1.0 min vs 32 s intervals).

The two groups studied around 39 male, middle-aged, NYHA class II–III patients suffering from treated, stable and chronic compensated heart failure with similar ejection fractions (e.g. 24 ± 7 and $23\pm7\%$). Both groups characterized the patients by bicycle ergometry exercise capacity and in the study by Wilson *et al.* (1985) the $\dot{V}O2$ was 14.3 ± 2.7 ml/min/kg. Wilson *et al.* (1985) analysed the spectra according to peak height while the area of the spectral peaks was measured by the Oxford investigators.

2.1. Skeletal muscle metabolism in forearm flexors in controls

Control measurements (Wilson *et al.*, 1985) confirmed the findings of previous investigators (Chance *et al.*, 1981; Taylor *et al.*, 1983) which demonstrated decreased skeletal muscle PCr with exercise. They also showed that such changes were linearly related to the power output during the last 4 min of steady state exercise. The pH decreased significantly at heavier workloads and it was only during such exercise that normal subjects experienced muscle fatigue (Fig. 10.2). The PCr changes were graphically depicted in the Oxford studies and the mean $PCr/(PCr+P_i)$ fell by approximately 10% during the first exercise and remained at this level until exercise 7, after which it fell progressively. Controls demonstrated similar, though more marked, changes during the initial minutes of steady state pre-incremental exercise when the $PCr/(PCr+P_i)$ fell to approximately 70% of the resting value and plateaued at approximately 60% by exercise 3 and 4 (Fig. 10.3). Examination of the relationship between the pH and the $PCr/(PCr+P_i)$ showed that the $PCr/(PCr+P_i)$ at which the pH fell to less than 6.9 was 0.61 ± 0.13 (Conway, 1994).

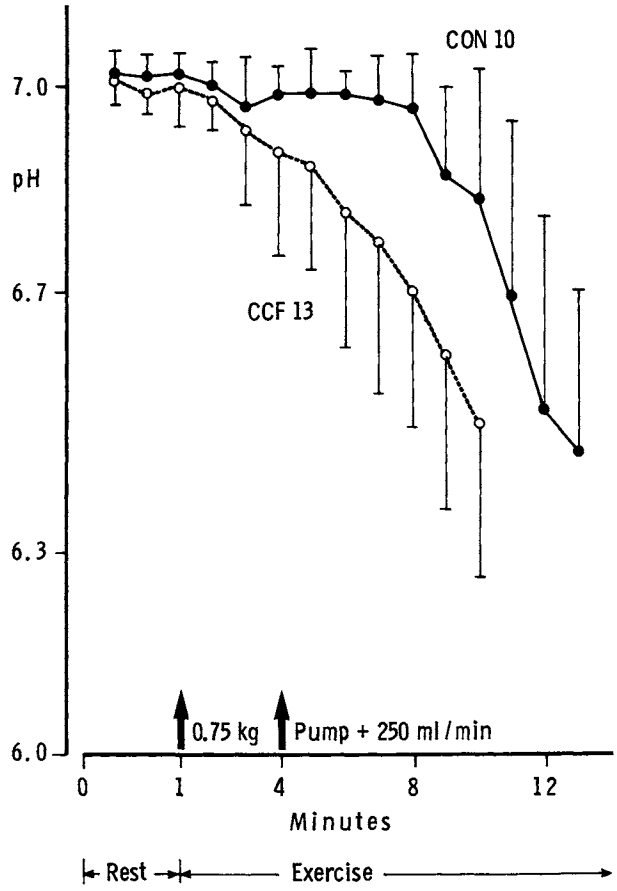

Figure 10.2. The change in pH during an incremental exercise protocol in 10 controls (CON 10) and 13 patients with heart failure (CCF 13) (mean±SD).

2.2. Skeletal muscle metabolism in forearm flexors of patients with heart failure

Skeletal muscle ^{31}P MRS spectra are normal at rest in patients with heart failure (Wilson *et al.*, 1985; Massie *et al.*, 1987a,b). However, during exercise marked PCr, P_i and intracellular pH changes are seen. Wilson *et al.* (1985) identified a difference in the slope of the heart failure P_i/PCr curve with a linear relationship between the work output and the P_i/PCr measured over the last 4 min of exercise. Thus, for a power output of 0.4 W the control P_i/PCr was approximately 0.4 compared to approximately 1.4 in a heart failure patient, indicating lower PCr and higher concentrations of P_i. Also, for similar power outputs patients had lower pH, e.g. the pH at 0.2 W in patients was similar to

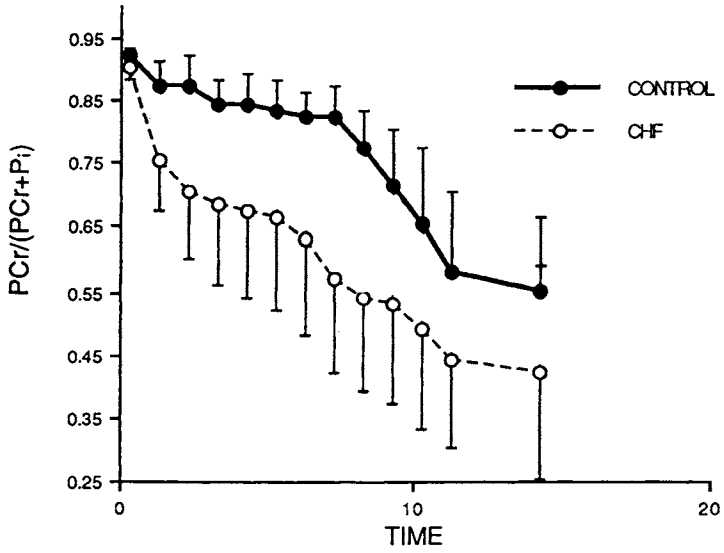

Figure 10.3. The changes in PCr (represented as $PCr/(PCr+P_i)$) in a group of 10 controls and 13 patients with heart failure (CHF) during incremental exercise (mean±SD).

that at 0.4 W in controls. Patients described symptoms of fatigue at all workloads.

Massie *et al.* (1987a,b) observed similar changes [described in millimoles and in terms of the $PCr/(PCr+P_i)$ ratio]. Bulb squeeze exercise decreased the $PCr/(PCr+P_i)$ ratio to approximately 40% of resting values in the heart failure group (Conway, 1994). During the final three spectra at the low workload (100 mmHg pressure) the curves became significantly different owing to the lower patient PCr. During the final minute of exercise the curves converged. In the original cohort of patients studied using the incremental exercise protocol (Fig. 10.3; Conway, 1994) the $PCr/(PCr+P_i)$ quickly decreased to about 25% of resting values within the first minute until exercise 7, following which the rate of fall increased. The difference in mean $PCr/(PCr+P_i)$ between controls and heart failure was highly significant from exercise one ($p<0.01$), through exercise 7 ($p<0.001$). It was less significant during the later increments of exercise as the control metabolite ratios decreased (e.g. exercise 9, $p<0.01$). The pH (Fig. 10.2) was significantly lower from exercise 5 ($p<0.03$), through exercise 8 ($p<0.002$) and exercise 9 ($p<0.03$). The maximal work capacity of the heart failure patients was lower and the maximum weight lifted incrementally by the patients was significantly reduced (2.3±0.5 vs 3.1±0.6 kg). This was not due to differences in load since when the results were analysed in an extended group of patients according to normalized loads the difference still applied (Massie *et al.*, 1987b). Massie *et al.* (1987b) found no relationship between the metabolic changes and the age, etiology, duration

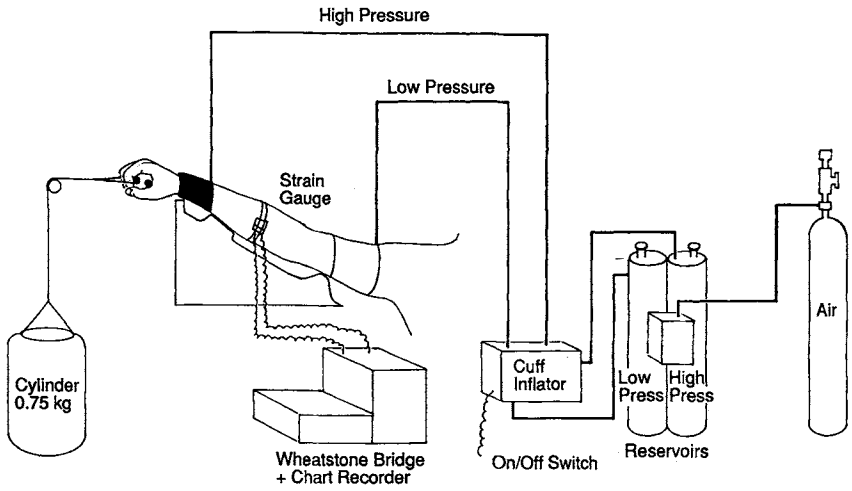

Figure 10.4. Experimental arrangement for the performance of venous occlusive plethysmography.

of disease or ejection fraction. There was a relationship between the pH changes and the NYHA class and bicycle exercise capacity such that NYHA class III/IV patients had lower pH and those with exercise capacity greater than 100 W had more normal pH during forearm exercise at 50% of maximal load.

3. PERIPHERAL BLOOD FLOW DURING EXERCISE: RELATIONSHIP TO METABOLIC CHANGES

A number of studies have been performed which were directed at finding the cause of these changes. Chronic prolonged mild hypoperfusion related to possible preferential diversion of the reduced cardiac output to more vital organs is likely to be relevant (Conway *et al.*, 1989a). This is difficult to demonstrate and so most attention has been directed at measuring blood flow during limb exercise. Weiner *et al.* (1986a) measured forearm blood flow within 2 h of ^{31}P MRS studies in controls and 21 male patients with heart failure using venous occlusion plethysmography. The exercise was performed at three levels of work (0.2, 0.4 and 0.6 W) with intervening 10 min rest periods. No blood flow abnormality was detectable even in the nine patients who had abnormal P_i/PCr to power output slopes and low pH. These findings were confirmed (Massie *et al.*, 1987b; Conway *et al.*, 1989) during incremental exercise (Figs 10.4–10.6). The patient exercise duration was decreased compared with controls – but the forearm blood flow was equivalent during the period of

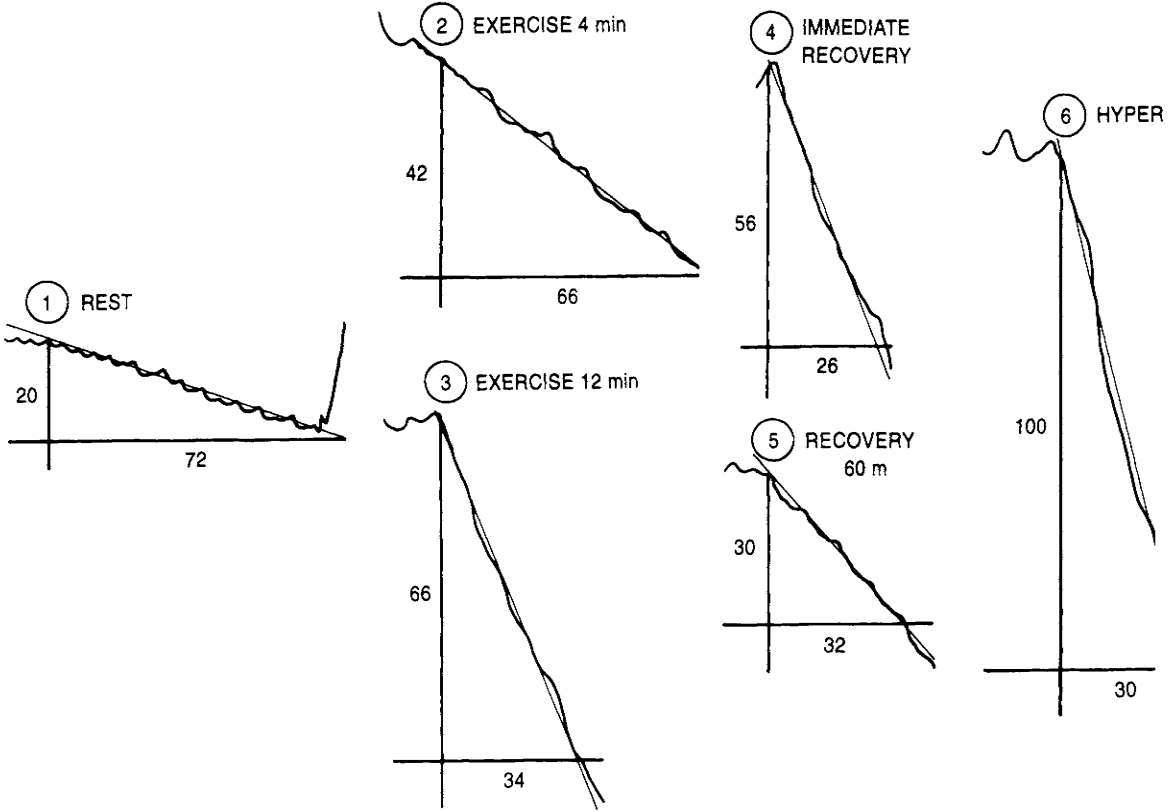

Figure 10.5. Original recording of venous occlusion plethysmographic measurements in a patient with heart failure, NYHA grade II. Paper speed: 1, 100 mm/min; 2–6, 300 mm/min. HYPER = hyperaemia.

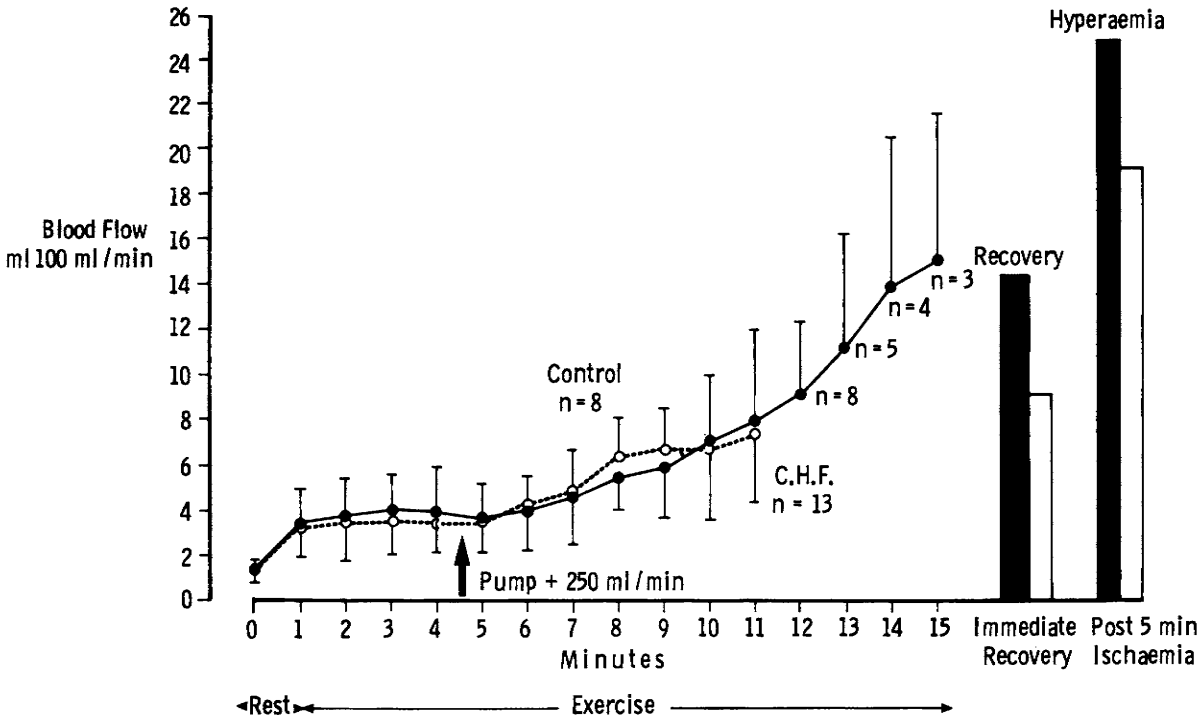

Figure 10.6. Blood flow during incremental exercise in controls and patients with heart failure. The hyperaemic response to 5 min of blood flow occlusion is also depicted.

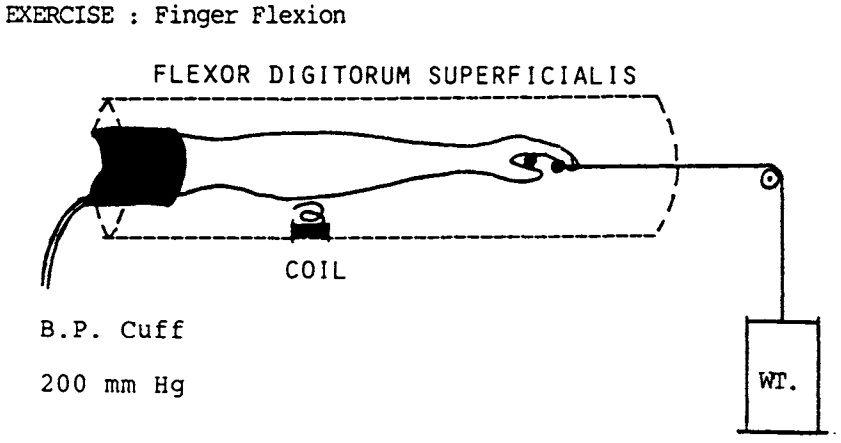

Figure 10.7. Experimental arrangement for ischaemic forearm exercise.

comparable exercise. Forearm vascular resistance is also similar in heart failure compared with controls. Massie *et al.* (1987b) noted that forearm blood flow rose faster in patients with lower pH during submaximal exercise, suggesting a relationship between increased tissue acidity and tissue perfusion.

Measurements of high-energy phosphates during ischaemic exercise (Massie *et al.*, 1988) support these observations. Using steady state finger flexion exercise at 33% of maximal capacity in nine male patients drawn from their original study population, the Oxford group identified differences in metabolism between controls and patients (Figs 10.7–10.10; Conway *et al.*, 1989a). Such findings further support the proposal that metabolic abnormalities are not directly related to circulatory abnormalities during exercise.

Thus, measurement of blood flow while increasing the workload shows that even patients with impaired systolic pump performance are able to increase perfusion to the individual limb during the period in which the exercise can be performed to levels comparable with normal. Increasing cardiac output and limb blood flow using dobutamine (Wilson *et al.*, 1984a) fails to alter exercise duration or muscle lactate excretion, suggesting further that blood flow *per se* does not determine fatigue and muscle metabolic changes. Indeed, in a recent study using a femoral vein thermodilution catheter, Wilson *et al.* (1993) found that a significant percentage of a group of 34 patients with heart failure had normal leg blood flow during upright bicycle exercise even though the femoral lactate increased abnormally.

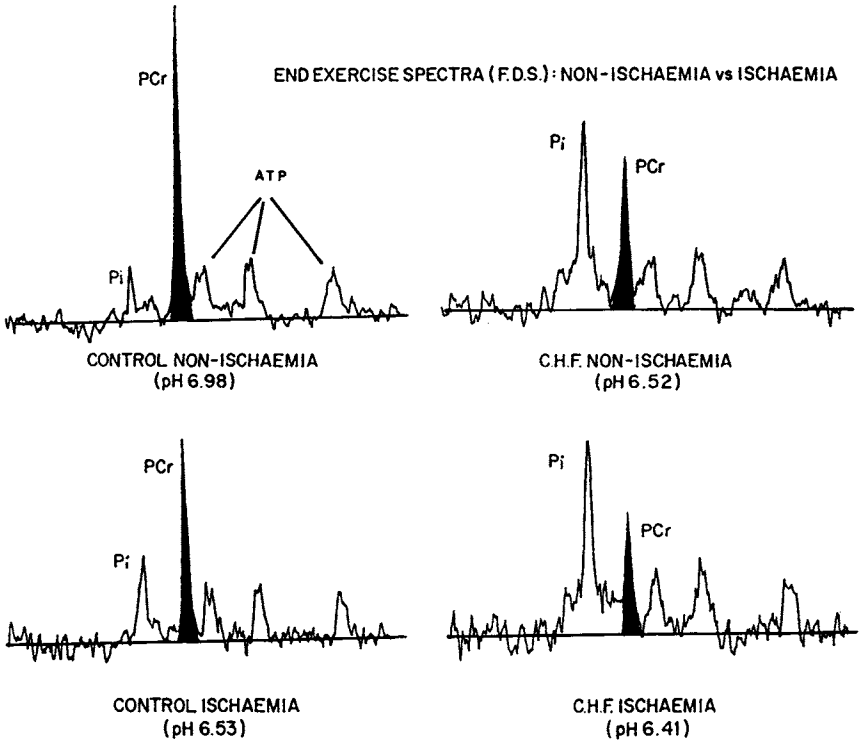

Figure 10.8. Changes in spectra of the flexor digitorum superficialis (F.D.S.) during ischaemic exercise.

4. SKELETAL MUSCLE METABOLISM AND BLOOD FLOW IN THE CALF MUSCLE IN CONTROLS AND PATIENTS WITH HEART FAILURE AT REST AND DURING EXERCISE

Metabolic abnormalities are known to occur in the calf muscle of patients with heart failure (Mancini *et al.*, 1988; Arnolda *et al.*, 1990; Marie *et al.*, 1990). Mancini *et al.* (1988) examined 20 patients – similar to those described above – after the completion of either stair climbing or plantar flexion exercise. Each exercise period lasted 3 min (plantar flexion) or 5 min (stair climbing) with intervening 10 min rest periods. The metabolic effects of exercise were "frozen" by inflating a pneumatic cuff on the thigh to suprasystolic pressures. Clear differences in the ratios of PCr and P_i were identified in the patients compared to controls with a steeper slope for the patient P_i/PCr versus $\dot{V}O2$ identified even when the $\dot{V}O2$ was normalized for body weight. In the studies by Marie *et al.* (1990) the workload needed to produce a given value for the

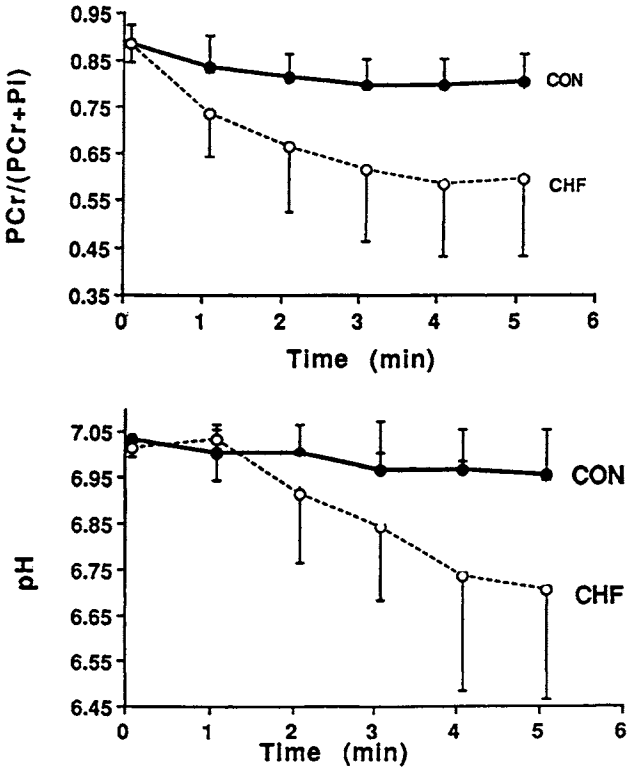

Figure 10.9. Changes in PCr (represented as $PCr/(PCr+P_i)$) and pH during non-ischaemic constant load exercise in controls (CON) and patients with heart failure (CHF) (mean±SD).

$PCr/(PCr+P_i)$ was measured during both aerobic and anaerobic exercise. At the PCr concentration produced no abnormality of pH was found.

Arnolda *et al.* (1990) monitored the $PCr/(PCr+P_i)$ during an incremental exercise protocol. This group measured leg blood flow during exercise within the magnet at the time of ^{31}P magnetic resonance spectroscopy (Fig. 10.11). There was no significant change in foreleg blood flow but abnormal PCr levels were observed in patients compared with controls (Fig. 10.12). Recently Minotti *et al.* (1990) reported that there is no relationship between exercise blood flow and muscle endurance.

These observations on metabolism and blood flow indicate that high-energy phosphate changes occur despite adequate flow to the legs and arms. This indicates that fatigue during mild to moderate exercise in patients with heart failure is unrelated to nutrient or substrate delivery at the time of exercise. An inherent abnormality of cellular metabolism leading to lower PCr and greater ATP utilization appears to occur for a given amount of work.

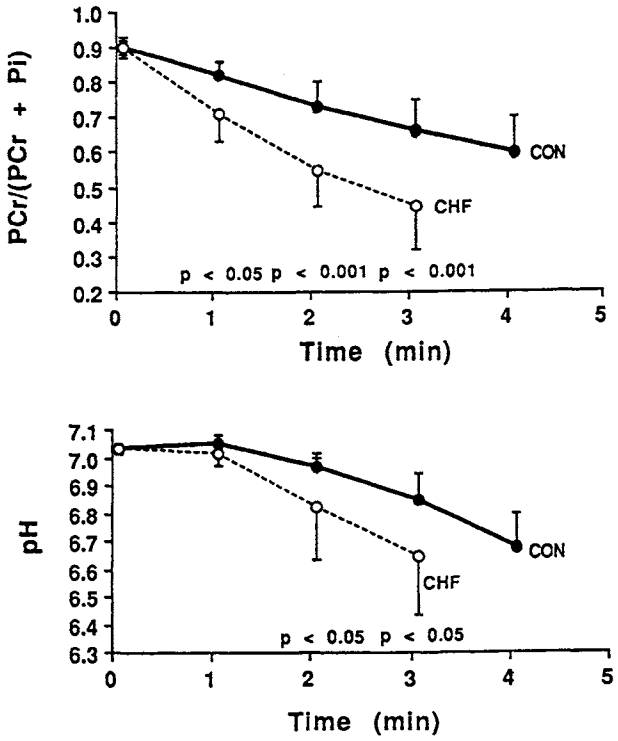

Figure 10.10. Changes in PCr (represented as PCr/(PCr+P_i)) and pH during ischaemic constant load exercise in controls (CON) and patients with heart failure (CHF) (mean±SD).

5. SKELETAL MUSCLE METABOLISM DURING RECOVERY FROM EXERCISE

Recovery of metabolism after exercise was monitored in most studies. Wilson *et al.* (1985) used a 2 min period of exercise to produce a PCr/P_i ratio of approximately 1.0 and then followed recovery over 1 min periods. The slope of the P_i/PCr recovery was significantly greater in the patients compared with controls, indicating that oxygen delivery is not impaired. Massie *et al.* (1987a,b) found a similar recovery pattern, although some patients had a prolonged timecourse – particularly those subjects with marked metabolic changes during exercise. Post-exercise metabolism was also similar in the patients compared to controls following ischaemic forearm exercise (Massie *et al.*, 1988). Recovery of leg muscle metabolism following the "metabolic freeze" experiments of Mancini *et al.* (1988) was delayed by approximately 1 minute whereas no significant difference was observed by Marie *et al.* (1990) or Arnolda *et al.* (1990).

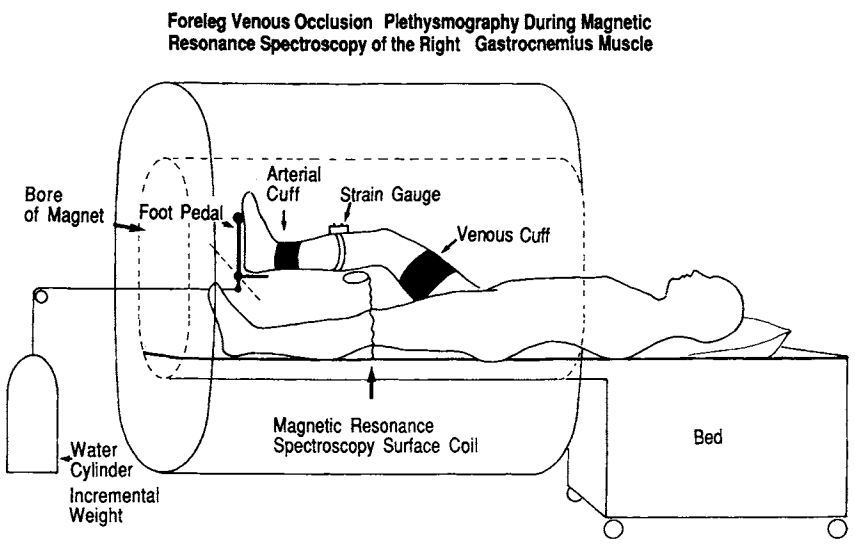

Figure 10.11. Experimental arrangement for the performance of foreleg exercise in a magnet.

Kemp *et al.* (1996) retrospectively examined skeletal muscle metabolism in heart failure of the Oxford patients using pH and PCr concentration changes during recovery to calculate the maximum rate of oxidative ATP synthesis Q_{max}. The measurements show that the Q_{max} decreased with an increased glycolysis in the heart failure group. Animal studies in the postinfarction rat model from this group of investigators also demonstrate slow PCr recovery from exercise (Thompson *et al.*, 1994).

6. MECHANISMS OF METABOLIC ABNORMALITIES IN SKELETAL MUSCLE IN HEART FAILURE

The mechanism of the changes in abnormal skeletal muscle metabolism in heart failure is unknown. Muscle atrophy was identified amongst 62 heart failure patients by Mancini *et al.* (1992) based on calf muscle MRI and other studies. A weak correlation between work slope – based on ^{31}P MRS measurements in 15 patients with heart failure – and muscle volume was detected. However, the overall conclusion was that the alteration in metabolism is likely to relate to factors other than muscle atrophy. Massie *et al.* (1987, 1988) allowed for possible differences in muscle mass by normalizing the load for patients versus controls and demonstrated that the metabolic abnormalities persisted. Thus, a reduction in the population of mitochondria is unlikely to be

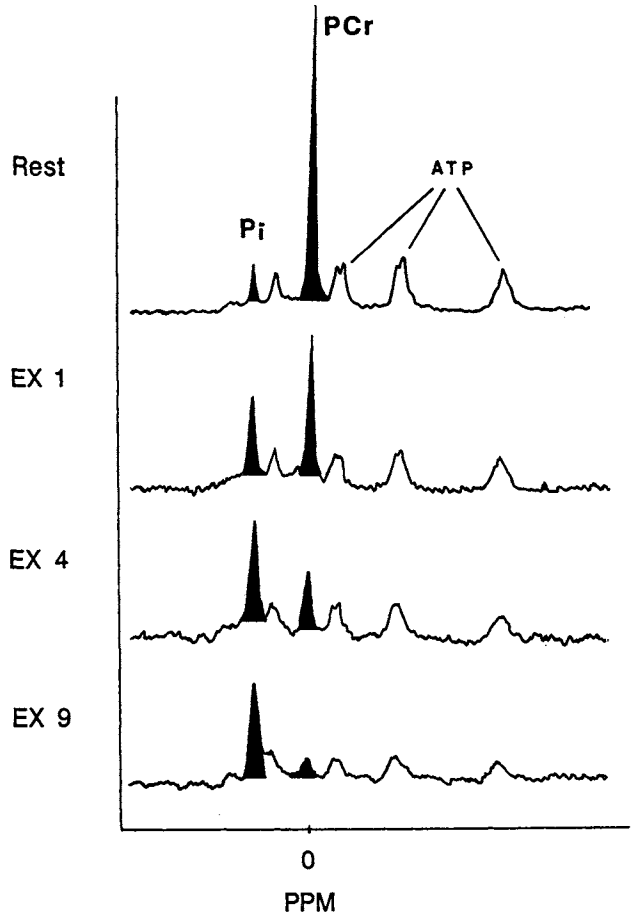

Figure 10.12. Changes in foreleg muscle spectra during exercise in a patient with NYHA class II heart failure. There is a marked fall in the amplitude of the PCr with exercise.

central to the observed changes, especially since the changes were observed in forearm muscle which is less prone to atrophy.

From the earliest days of ^{31}P MRS investigations into heart failure, concerns about inactivity as a mechanism of the changes in metabolism have been raised (Wilson *et al.*, 1985). Possible abnormalities related to ventilation (Wilson and Ferraro, 1983), impaired oxygen delivery caused by shunting around capillary beds or a block to diffusion (Massie *et al.*, 1987b) and fibre type changes are commonly considered (Dunnigan *et al.*, 1987; Lipkin *et al.*, 1988; Mancini *et al.*, 1989; Sullivan *et al.*, 1990). An increase in Type IIB fast-twitch glycolytic fibres is described. This may be important since these fibres

are more susceptible to acidosis and fatigue than Type I fibres. The former also display greater PCr depletion and higher P_i during similar electrical stimulation (Saltin and Gollnick, 1983). Biochemical abnormalities which may be relevant to the $PCr/(PCr+P_i)$ such as reduced oxidative capacity (Drexler, 1992) and decreased aerobic enzyme activity (Yancy *et al.*, 1989; Sullivan *et al.*, 1990) are reported.

Other factors which may contribute to the changes in muscle metabolism in heart failure are: (1) circulating catecholamines (Kawai *et al.*, 1983; Bayliss *et al.*, 1987; Tomita *et al.*, 1975) – isoprenaline produces greater PCr utilization and lower pH during high-level stimulation of rat skeletal muscle (Challis *et al.*, 1988); (2) myoglobin (Korner, 1959), the content of which may be lower in heart muscle failure compared with controls; and (3) decreased muscle capillarization (Yancy *et al.*, 1989) and other effects similar to those seen during immobilization and detraining (Patel *et al.*, 1969; Sargeant *et al.*, 1977; MacDougall *et al.*, 1980). A single individual factor alone may not explain the observations and indeed small changes in a number of factors most probably combine to produce the abnormality.

7. REVERSING THE CHANGES IN SKELETAL MUSCLE METABOLISM IN HEART FAILURE

7.1. Exercise and training

Early consideration of the possible effects of training on forearm muscle followed from the initial Oxford MRS studies and in one well-characterized heart failure patient the outcome following a month of daily bulb squeeze exercise was examined (L. Arnolda and M.A. Conway, personal communication). A modest improvement in the $PCr/(PCr+P_i)$ and pH was identified. Significantly improved high-energy phosphorus metabolism and pH were reported in calf muscle by Adamopoulos *et al.* (1993) in 12 patients recruited to examine the effects of bicycle ergometer exercise on symptomatology, cardiovascular and oxygen consumption changes in chronic heart failure. The patient population was similar to that described in other calf muscle studies outlined above (Arnolda *et al.*, 1990). Observations consistent with these findings are reported by Minotti *et al.* (1990) using 15 min wrist flexion exercise of the non-dominant arm for 30 days. The metabolic improvement was independent of peripheral or central cardiovascular responses. The mechanism underlying such beneficial changes has not been defined but training enhances normal muscle capillary density and mitochondrial enzyme activity and this may be relevant in heart failure also (Saltin and Gollnick, 1983). The old advice that patients with chronic heart failure should restrict physical activity was recently questioned (Drexler, 1992).

7.2. Cardiac transplantation

Cardiac transplantation is now recognized as an important mode of therapy for patients with severe heart failure. Those who are severely limited or bed-bound prior to surgery return to active lifestyles after transplantation with reduced fatigue and improved, though subnormal, exercise tolerance (Kavanaugh *et al.*, 1988). Surprisingly, this did not improve skeletal muscle metabolism (Stratton *et al.*, 1994) and indeed in early post-transplantation (<6 months) a significantly lower pH and PCr/(PCr+P_i) is seen compared to pretransplantation. The cause of the persistent metabolic abnormalities is unknown, but the severity of the illness before surgery and the side-effects of cyclosporine have been considered (Stratton *et al.*, 1994). No improvement in high-energy phosphorus metabolism occurs even late after transplantation (>6 months) despite improved symptomatology (NYHA class 2.7 ± 1.3 pre- vs class 1.1 ± 0.7 post-transplantation) and the absence of transplant rejection.

7.3. Pharmacological therapy

The effects of pharmacological therapy on skeletal muscle metabolism in heart failure have received little attention. Agents which lead to better exercise tolerance such as converting enzyme inhibitors are now known to reduce mortality from heart failure and myocardial infarction (e.g. SOLVD, 1991; ISIS 4, 1995). The maximal effect on symptoms occurs at around 3 months and ideally, studies designed to examine the benefits of vasodilator therapy will require such a placebo period followed by a long washout.

The potential for therapeutic intervention in the metabolism of patients with heart failure using creatine has recently begun to be examined (Andrews *et al.*, 1994; Gordon *et al.* 1995). Some encouraging results are reported of the effects of PCr on muscle mass recovery following orthopaedic leg surgery (Satolli and Marchesi, 1989; Pirola *et al.*, 1991; Agnese *et al.*, 1992) and in muscle strength (Dal Monte *et al.*, 1976), and the symptomatic recovery of athletes after cycling races (Tegazzin *et al.*, 1991) (see also Chapter 13).

The relationship between the metabolic abnormalities, the improvement with training or pharmacological therapy and the eventual mortality in patients with heart failure had not been examined and this can only be reliably answered with a large prospective study. Some indication of a possible relationship may yet be derived by pooling the measurements from the population already studied and reported in the literature, e.g. patients with relatively normal metabolism may survive longer than those displaying severe abnormalities. Conversely, altered metabolism may be unrelated to factors which determine longevity in heart failure.

8. SUMMARY

In summary, a greater understanding of the mechanism underlying fatigue and reduced exercise tolerance has been gleaned from skeletal muscle metabolism studies in patients with heart failure using ^{31}P MRS. Further application of the technique in association with innovative physiological, biopsy and molecular biological studies should further elucidate the mechanisms of symptoms for which the clinician, in the out-patients' department, often has little to offer. The discovery of changes in PCr metabolism raises the possibility that creatine and PCr therapy may have a role in the future management of these patients. Whether these agents will be adequate on their own, or whether administration in combination with "facilitator" agents will be required, is unclear as yet. However, the observation of biochemical abnormalities *in vivo* which are central to, or at least a marker of, inefficient muscle biochemistry (and perhaps heart) metabolism presents a promising avenue of investigation as we approach and commence the next millennium.

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11

Clinical Experience with Creatine Phosphate Therapy

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Key words: creatine phosphate, cardioplegic solutions, myocardial infarction, heart failure, arrhythmia

1. INTRODUCTION

The clinical application of creatine phosphate (PCr) for cardioprotection during heart surgery and myocardial ischaemia is based on the results of a series of pharmacological studies in animal models. These were initiated by Parratt and Marshall (1974), who demonstrated that perfusion with PCr protected the isolated guinea-pig atrium subjected to anoxia, thereby preventing the decrease in contractile strength induced by reduced oxygenation. Since then numerous studies extending these observations have been reported.

The effect of PCr on the incidence and duration of coronary ligation-induced ventricular fibrillation (VF) was studied by Marshall and Parratt (1974) in the dog model and by Fagbemi *et al.* (1982) in rats. A marked reduction in VF was observed. Hearse *et al.* (1986) reported prevention of reperfusion arrhythmias and improved functional recovery in the working rat heart

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Table 11.1. Creatine phosphate (concentration 10 mM) added to cardioplegic solutions.

Patient group	Type of surgery	Reference
Crystalloid cardioplegic solution (St Thomas' 1 and St Thomas 2)		
200 PCr+120 C (2)	Valve disease & CABG	Tronconi *et al.* (1987)
19 PCr+21 C (2)	Valve disease	Pastoris *et al.* (1991)
25 PCr+25 C (2)	Valve disease & CABG	Chambers *et al.* (1991)
79 PCr+72 C (2)	CABG	Mastroroberto *et al.* (1992)
15 PCr+14 C (2)	Valve disease	Thorelius *et al.* (1992)
59 PCr+54 C (2)	Paediatric heart surgery	Cossolini *et al.* (1993)
22 PCr+22 C (1)	Valve disease	Chambers *et al.* (1993)
18 PCr+18 C (2)	CABG	Pauletto *et al.* (1993)
Blood cardioplegic solution		
41 PCr+37 C	Valve disease	Semenovsky *et al.* (1987)

CABG, coronary artery bypass grafting; C, controls; PCr, treated with PCr. Total number of patients treated with PCr=478; total number of controls=383.

model when PCr was added to cardioplegic solutions. The potential for further myocardial protection through the addition of PCr to these solutions was demonstrated in the rat heart during cardiopulmonary bypass and ischaemic arrest under conditions of normothermia or hypothermia by Robinson *et al.* (1984). Such protective effects of PCr were shown to be additive to those provided by other cardioplegic agents in dose–response studies and the optimal effect on the recovery of minute-work, aortic flow, coronary flow and systolic stroke volume was obtained with solutions at a concentration of 10 mM PCr. Extensive studies on the cardioprotective effects of PCr have been performed by Saks and others at the Cardiology Research Centre, Moscow, and these have included pharmacokinetics, haemodynamic and pharmacological aspects of cardioprotection, direct ATP and PCr measurement using nuclear magnetic resonance (^{31}P NMR) and histological assessment based on the colloidal lanthanum technique (Sharov *et al.*, 1986, 1987). These studies on animals form the basis for the clinical application of PCr which has now been evaluated in heart surgery, cardiac failure and acute myocardial infarction clinical trials.

2. HEART SURGERY

The cardioprotective effects of PCr added both to cyristalloid and blood cardioplegic solution peroperatively have been extensively studied in adults and children during surgery such as valve replacement, coronary artery bypass grafting and the repair of congenital heart defects (Table 11.1). (The observations of Chambers *et al.* (1991, 1993) are described on the next page.)

In all these investigations, PCr was added to the cardioplegic fluids at a concentration of 10 mM in accordance with the dosage derived from animal studies (Robinson *et al.*, 1984).

The first human trials were conducted by Semenovsky *et al.* (1987) and D'Alessandro *et al.* (1987). In the former, the addition of PCr to blood cardioplegic fluids resulted in much better restoration of sinus rhythm and a decreased need for defibrillation (Fig. 11.1, upper panel). In contrast to controls, in whom the myocardial ATP content decreased by 25% at the end of the operation, the PCr-treated group maintained the concentration of high-energy compounds at preoperative levels (Fig. 11.1, lower panel). Ultrastructural analysis demonstrated good preservation of the sarcolemmal structure and this was clearly seen using the lanthanum method – despite the high degree of heterogeneity of the human heart material studied. Very similar results were obtained by D'Alessandro *et al.* (1987), who demonstrated that in the PCr-treated group there are significant differences in the number of watts required for defibrillation, the time to recovery of cardiac activity after aortic declamping, inotropic drug support in the postoperative period and the presence of electrocardiographic anomalies. Results from other studies support these findings and Mastroroberto *et al.* (1992) observed that in patients undergoing myocardial revascularization the PCr-treated group had a significantly lower need for inotropic support, a lower incidence of major and minor arrhythmias, a higher cardiac index, and a reduced cardiac enzyme release postoperatively compared with the control. In patients undergoing coronary artery bypass grafting for ischaemic heart disease, Pauletto *et al.* (1993) – utilizing PCr 10 mM added to cyristalloid cardioplegia – observed a significantly lower prevalence of severe arrhythmias (Lown class IVB) and complete heart block. Reduced myocardial enzyme leakage was also noted (Table 11.2).

Cossolini *et al.* (1993) utilized PCr-enriched cardioplegia in newborn infants and paediatric patients (9 days to 13 years, mean age 33.9 months) undergoing open heart surgery for congenital heart disease. After reperfusion, the PCr-treated group showed a significantly higher spontaneous recovery of sinus rhythm (64% vs 33%, $p<0.001$), decrease in A-V block (20% vs 54%, $p<0.001$) and postoperative arrhythmias (10% vs 24%, $p<0.05$). Upon reperfusion, VF was also significantly less frequent (15% vs 31%, $p<0.05$), with fewer DC shocks required to convert to sinus rhythm. The PCr-treated group also required significantly lower doses of inotropic drugs ($p<0.05$). All of these trials confirm the cardioprotective effect of PCr added to both cyristalloid and blood cardioplegia and the effects are summarized in Table 11.3.

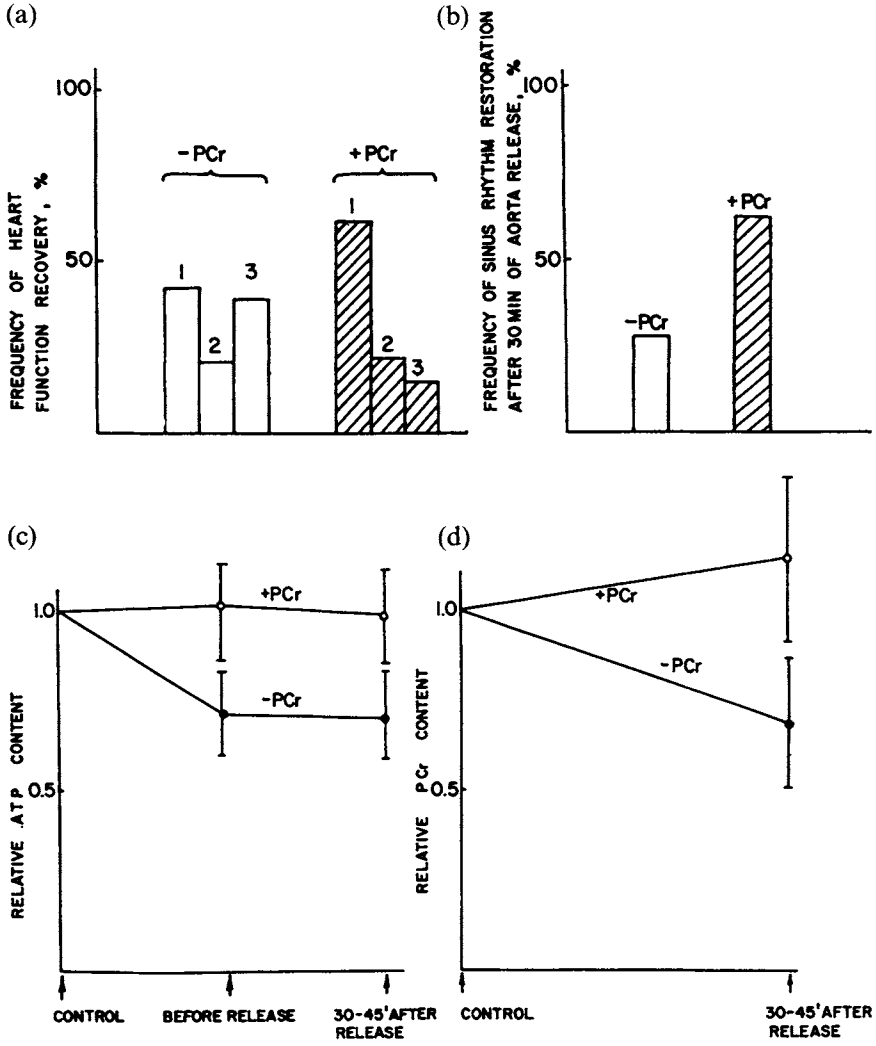

Figure 11.1. (a) Recovery of cardiac function in the presence and absence of creatine phosphate (8–10 mmol/l). (1) Spontaneous; (2) single defibrillation; (3) repeated defibrillation). (b) Recovery of sinus rhythm in the presence and absence of creatine phosphate (8–10 mmol/l). (c) and (d) The effect of PCr cardioplegia on the tissue content of ATP (c) and creatine phosphate (d) before (control) and after aortic cross-clamping to induce ischaemia. From Semenovsky *et al.* (1987), with permission.

Table 11.2. Prevalence of arrhythmias and A-V block in patients who underwent coronary artery bypass grafting for ischaemic heart disease.

Lown class	PCr		C	Degree of block	PCr		C
I	22.2%	n.s.	5.6%	A-VI	5.6%	n.s.	5.6%
II	11.1%	n.s.	0	A-VII	0	n.s.	16.7%
III	5.6%	n.s.	0	A-VIII	5.6%	$p<0.02$	38.9%
IVA	16.7%	n.s.	11.1%	DC shock	1.9±0.45	n.s.	12±0.35
IVB	44.4%	$p<0.02$	83.3%	Ventricular fibrillation	16.7%	n.s.	33.3%
V	0	—	0				

PCr, creatine-phosphate-treated; C, controls; n.s., not significant.
From Pauletto *et al.* (1993).

Table 11.3. Effects of adding creatine phosphate to cardioplegic solution.

- Better recovery of spontaneous sinus rhythm
- Reduction of postoperative arrhythmias (III–IV Lown) and A-V block (III)
- Reduction in DC shocks and total pulses for defibrillation
- Lower doses of inotropic drugs
- Reduction of myocardial enzyme leakage (CK, MB-CK)

Table 11.4. Creatine phosphate in acute myocardial infarction.

Patient group	Dose (g/day, i.v.)	Treatment (days)	Reference
18 PCr, C[a]	2	10	Pedone *et al.* (1984)
31 PCr+13 C	8	5	Scattolin (1987)
30 PCr+30 C	10	1	Ruda *et al.* (1988)
61 PCr+59 C	12	3	Raisaro *et al.* (1989)
31 PCr+31 C	10 (I°) 2 (II°–VI°)	6	Camilova *et al.* (1991)
89 PCr+97 C	8	5	Reimers (1994)

[a] Double-blind, crossover vs placebo.
Total number of patients treated with PCr=171; total number of controls 151.
° NYHA Class

3. MYOCARDIAL INFARCTION

The cellular mechanism underlying the cardioprotective effects of PCr (Saks and Strumia, 1993) and experimental animal research *in vivo*, demonstrates reduction in the extent of damage in experimental acute myocardial infarction (AMI) lesions (Sharov et al., 1986), suggest that PCr may be useful during the early hours post myocardial infarction. Many trials (Table 11.4) have been con-

ducted on patients with AMI utilizing protocols similar to that proposed by Ruda *et al.* (1988). In this study 60 patients aged 35 to 70 years (mean 56.0±1.5 year) admitted to the coronary care unit with AMI within 6 h of the onset of chest pain were studied. The patient population was carefully selected to exclude those with (1) acute (Killip class III or IV) or chronic heart failure; (2) a history of previous myocardial infarction, chronic arrhythmias, or both; (3) bradycardia (heart rate <50 beats/min); (4) hepatic or renal insufficiency; or (5) antiarrhythmic therapy before hospitalization. No patient in either group received heparin, nitroglycerin, β-adrenergic blocking agents, cardiac glycosides, glucose-insulin-potassium, thrombolytics, or diuretics. Patients for whom any of these drugs became necessary were excluded from the trial. Morphine was given for pain relief.

Patients fulfilling these criteria were randomized into two groups: group A received PCr (n=30), and group B served as control (n=30). Immediately after enrolment, 24-h Holter monitoring was performed. Electrocardiograph (ECG) leads V1 and V5 were monitored to allow precise correlation of the ECG with time. Creatine phosphate was given as a 2 g bolus injection, followed by a 2-h infusion at the rate of 4 g/h. Thus, the full PCr dose for one patient was 10 g. [Previous pharmacokinetic studies showed that this method of PCr administration maintains the plasma drug concentration at approximately 0.2 mmol/l (Sharov *et al.*, 1986).] A continuous intravenous infusion of PCr was necessary to maintain the PCr level in blood due to its very rapid clearance at a rate constant equal to 0.16–0.18 min. The control group received sodium chloride isotonic solution according to the PCr administration regimen. Conventional antiarrhythmic therapy was begun only for treatment of rhythm disorders causing detrimental haemodynamic effects. Data in such instances were excluded from analysis.

Twenty four-hour Holter monitoring showed (Fig. 11.2A) a significant decrease in the frequency of ventricular premature beats (VPBs). In the PCr-treated group the total number of VPBs over 24 h was 690±179 vs 2468±737 in the control group ($p<0.02$). During this period the number of paroxysms of ventricular tachycardia (Fig. 11.2B) was 6±2 in the treated group and 97±35 in controls ($p<0.01$). No side-effects or complications were found after PCr administration. A similar effect on ventricular ectopy was observed by Fagbemi *et al.* (1982) in the experimental AMI and it prevailed, not only during PCr intravenous infusion, but also after ceasing administration. Such a property of PCr may be explained by transient myocardial accumulation and also by preservation of the cardiomyocyte membrane structure and electrophysiological characteristics. The membrane protection could be due to stabilization of the phospholipid membrane in the acute phase of myocardial infarction and in particular due to the suppression of lysophosphatidylcholine accumulation. In other acute myocardial infarction trials, PCr infusion took place over a more prolonged period, according to the following scheme: day 1, 4 g bolus intravenous injection, followed by an intravenous infusion at a rate of

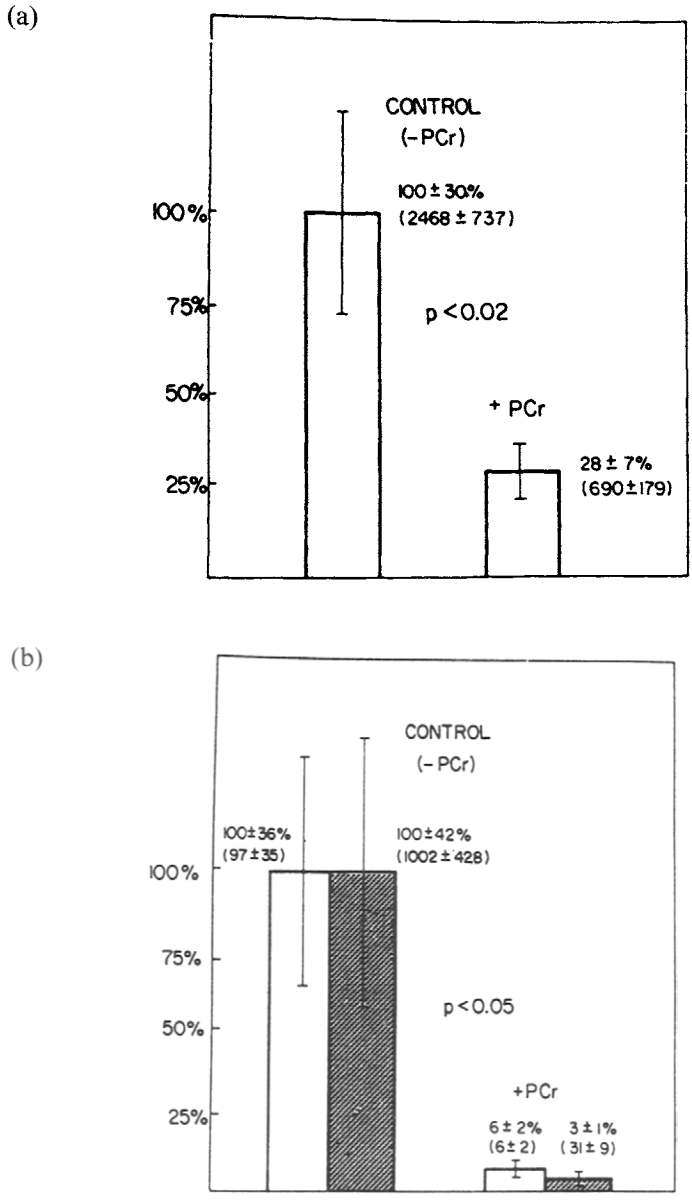

Figure 11.2. (a) The total number of ventricular premature beats (VPBs) in a PCr-treated group of patients during a period of monitoring compared with a control group (100%). (The actual numbers of VPBs are in parentheses.) (b) The number of paroxysms of VT (open areas) and the number of VPBs occurring during episodes of VT (filled areas) in a PCr-treated group compared with a control group (100%). (The actual numbers of VT and VPBs in VT are shown in parentheses.) From Ruda *et al.* (1988), with permission.

Table 11.5. Creatine phosphate in chronic heart failure.

Patient group	Dose (g/day, i.v.)	Duration (days)	Acute and subacute haemodynamic studies	Reference
20 PCr	1	1	Open label	Smilari *et al.* (1987)
15 PCr	6	5	Open label	Ferraro *et al.* (1990)
13 PCr	8	3	Double-blind placebo crossover	Ferraro *et al.* (1995)
11 PCr	1	7	Open label	Strozzi *et al.* (1992)
20 PCr	1	7	Open label	Scattolin *et al.* (1993)

Total number of patients treated with PCr = 79.

4 g/h over 2 h; days 2–6, 4 g intravenous injection twice daily. Using this posology in a randomized multicentre study on 235 patients with AMI, Reimers *et al.* (1994) observed markedly reduced creatine kinase (CK) and MB-CK release – statistically significant for the patients with MB-CK peak greater than 100 U/l – consistent with other trials where reduced enzyme release and arrhythmias were observed in the PCr-treated group (Cini *et al.*, 1987; Coraggio *et al.*, 1987; Raisaro *et al.*, 1989; Camilova *et al.*, 1991). Based on these findings, some investigators conclude that PCr may be a potentially important anti-arrhythmic drug for AMI.

4. HEART FAILURE

The third cardiovascular condition in which the effects of PCr have been carefully investigated in man is chronic heart failure (CHF). The haemodynamic effects were characterized by Cafiero *et al.* (1994), Galyautdinov *et al.* (1993) and other investigators listed under Table 11.5.

Ferraro *et al.* (1990, 1995) studied the effects of PCr in patients with chronic heart failure. In the later study, a double-blind crossover design protocol (PCr and placebo) was employed involving an acute PCr/placebo intravenous infusion (6 g diluted in 50 ml NaCl 0.9%, over 10 min) followed by a short-term PCr/placebo treatment (3 g diluted in 50 ml NaCl 0.9% over 10 min, twice daily for 3 days). Thirteen hospitalized CHF (New York Heart Association (NYHA) Class II–III) patients (12 M, 1 F, mean age 52 ± 8 years) were studied. All were on stabilized conventional pharmacological therapy for CHF which remained unchanged during the study period. Echocardiographic measurements were performed at baseline, after acute infusion and after the end of short-term therapy. No significant changes occurred after placebo but the PCr-treated group demonstrated a significant increase in ejection fraction

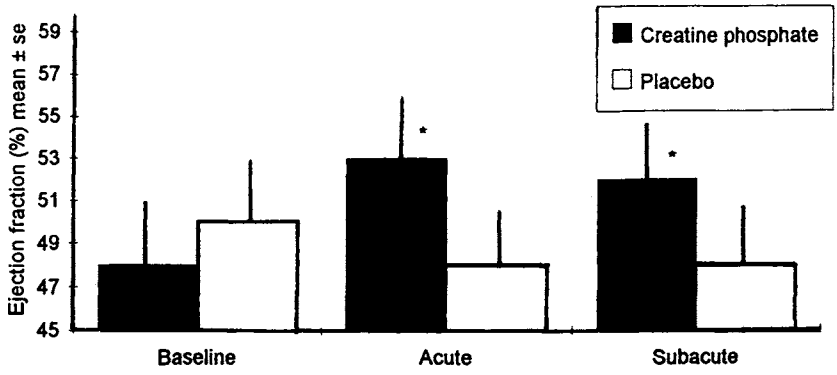

Figure 11.3. The effect of PCr compared with placebo on the ejection fraction in 13 patients with chronic heart failure at baseline, following an acute infusion and 3 days after short-term (subacute) infusion. Acute test, PCr 6 g i.v.; subacute test, PCr 6g/day for 3 days. *$p<0.05$ vs baseline (modified from Ferraro *et al.*, 1996).

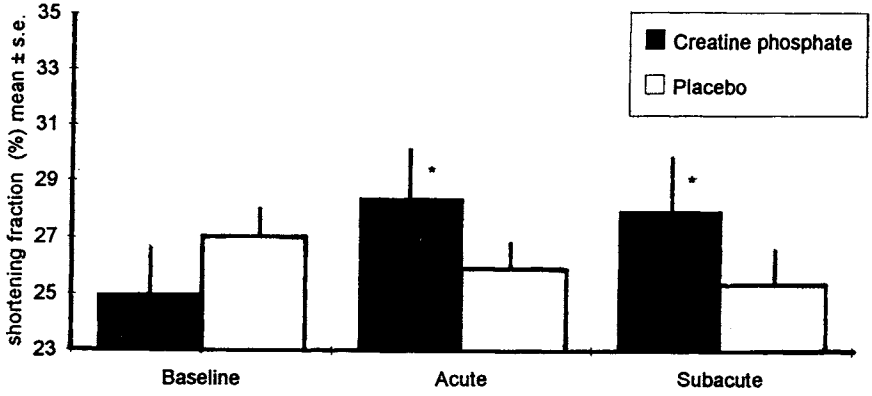

Figure 11.4. The effect of PCr compared with placebo on the fractional shortening in 13 patients with chronic heart failure at baseline, following an acute infusion and 3 days after short-term (subacute) infusion. Acute test, PCr 6 g i.v.; subacute test, PCr 6 g/day for 3 days.* $p<0.05$ vs baseline (modified from Ferraro *et al.* 1996).

(Fig. 11.3) and fractional shortening (Fig. 11.4) and reduced end-systolic diameter and calculated systemic vascular resistance.

In primary ischaemia, diastolic dysfunction may be the first alteration to appear. The effect of PCr on diastolic parameters has been studied (Scattolin *et al.*, 1993). Acute infusion of PCr (5 g i.v.), to 20 patients suffering from chronic ischaemic cardiomyopathy, produced a general improvement in diastolic function parameters as measured by echo-Doppler. There was a statistically significant modification in the isovolumetric relaxation time and the

Table 11.6. Creatine phosphate in chronic heart failure.

Acute haemodynamic (echocardiography) studies showed
Significant increase of: • ejection fraction • shortening fraction • slope of the first half of deceleration rate
Significant decrease of: • isovolumetric relaxation time

Table 11.7. Creatine phosphate in chronic heart failure.

Patient group	Dose (g/day)	Duration (days)	Drug therapy	Reference
508 PCr+499 C	2 i.v. 0.5 i.m.	14 30	PCr+usual treatment	Grazioli *et al.* (1992)
40 PCr+20 C	8 i.v.	21	PCr+digitalis, diuretics	Andreev *et al.* (1992)

The total number of patients with heart failure treated with PCr in these studies is 1307 compared to 974 controls.

protodiastolic deceleration slope rate. The effects of PCr on echocardiographic measurements of cardiac function are summarized in Table 11.6.

A number of clinical trials (Table 11.7) are reported to date on the therapeutic effect of PCr in CHF (Grazioli and Strumia, 1989; Grazioli *et al.*, 1992; Strozzi *et al.*, 1992; Andreev *et al.*, 1992; Galyautdinov *et al.*, 1993) The details of two studies are in Table 11.7. One of these was a multicentre trial conducted to evaluate the effects of PCr, in addition to conventional therapy with digitalis, diuretics and nitrates (Grazioli *et al.*, 1992). Clinical symptoms and NYHA classes, electrocardiographic (ECG) signs of ischaemia, and the use of sublingual nitroglycerin were monitored. A total of 1007 patients were studied, of whom 508 were randomized to receive PCr intravenously (1 g twice daily) for 2 weeks, followed by a 1-month course of therapy with PCr intramuscularly (500 mg four times per day). The control group comprised 499 patients who were treated with conventional therapy alone. Both groups were comparable with regard to sex, age, study period, type of drugs used and the aetiology of CHF. During the study period, the number of patients in NYHA classes III and IV decreased significantly in the PCr group compared with the control (Fig. 11.5). The main symptoms and signs of ischaemia (angina pectoris, need for sublingual nitroglycerin and T wave inversion on ECG), and the incidence of VPBs improved significantly in the PCr-treated patients.

Andreev *et al.* (1992) examined the effects of PCr and digoxin in 67 patients aged over 60 years with CHF of NYHA class II–III of ischaemic aetiology. Clinical symptoms, haemodynamic indices, exercise tolerance, Holter monitoring, electrocardiography, impedance cardiography, echocardiography and

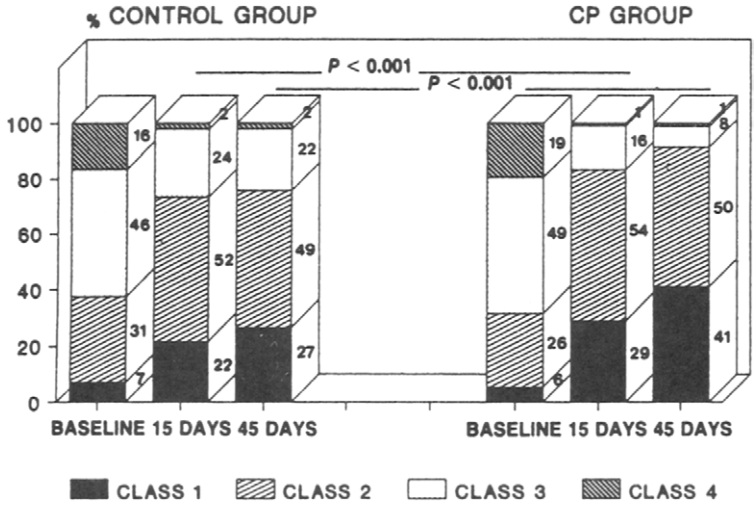

Figure 11.5. Changes in the NYHA classification of patients with heart failure treated with PCr. From Grazioli *et al.* (1992), with permission.

bicycle ergometry were evaluated at baseline and after 3 weeks of treatment. Compared with digoxin, PCr significantly decreased systemic vascular resistance and tended to normalize both systolic and diastolic blood pressure. The PCr also reduced the number of VPBs (particularly paired beats) and the number of episodes of paroxysmal ventricular tachycardia. Combined treatment with digoxin plus PCr resulted in an increase in left ventricular ejection fraction and a decrease in systemic vascular resistance. This combination led to a further improvement in the patients' clinical status with a decrease in the severity of dyspnoea and frequency of anginal attacks and a reduction in the need for nitroglycerin. The use of digoxin with PCr was also found to reduce significantly the frequency of ventricular extrasystoles and the incidence of paroxysmal ventricular tachycardia. A substantial improvement in exercise tolerance was noted. No side-effects were observed. The authors concluded that PCr, and especially the combination of PCr and digoxin, are effective and safe treatments for NYHA class II–III congestive heart failure associated with ischaemic heart disease.

The main results obtained with prolonged PCr treatment in CHF patients are summarized in Table 11.8.

5. SUMMARY

More than 5 years of clinical trials with PCr in cardiac surgery and for the treatment of acute myocardial infarction have provided sufficient data to support the contention that exogenous administered PCr is a cardioactive

Table 11.8. Creatine phosphate in chronic heart failure.

Chronic administration of creatine phosphate associated with usual stabilized treatments showed significant: • improvement in NYHA score • improvement in ECG scores of myocardial ischaemia • reduction in nitrate consumption • reduction in arrhythmias

drug which affords effective myocardial protection from acute ischaemic injury and perioperative ischaemic damage. Its application as a cardioplegic additive (10 mM) and for intravenous infusion leads to significantly better functional recovery following ischaemia, during the postinfarction period and upon reperfusion. Studies in patients with CHF show that PCr significantly improves left ventricular systolic and diastolic function. Multicentre trial results demonstrate that the administration of exogenous PCr to patients with heart failure effectively improves clinical symptoms and haemodynamics with better cardiac performance, fewer patients in the higher NYHA classes, reduced ventricular arrhythmias and an improvement in the severity of angina. Furthermore, high doses of PCr appear to improve cardiac function, even when patients are stabilized on conventional pharmacological therapy for CHF.

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12

Creatine Phosphate Added to St Thomas' Cardioplegia

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Key words: creatine phosphate, cardioplegic solutions, myocardium, ischaemia, coronary artery bypass, arrhythmia

1. INTRODUCTION

Cold chemical crystalloid cardioplegia has proven to be a safe, inexpensive and effective method for myocardial protection during cardiac surgery. One of the most commonly used crystalloid cardioplegic solutions, both in experimental studies and clinically, is the St Thomas' Hospital solution. This is an "extracellular" type preparation which is formulated to be similar to extracellular fluid and is available as St Thomas' No 1 (STH1) and St Thomas' No. 2 (STH2) fluids (Hearse *et al.*, 1981). The three major components of cardioplegic fluids are designed for achieving: (1) chemical arrest; (2) hypothermia and (3) additional protection. Preservation of the high-energy phosphate content of the ischaemic myocardium is one of the main objectives of cardioplegia and a number of compounds such as adenosine and inosine have been shown to confer a protective effect (Levitsky and Feinberg, 1975; Foker *et al.*, 1980; De Witt *et al.*, 1983). Other agents such as high-energy phosphate

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compounds (ATP and creatine phosphate, PCr) have also been shown to protect the myocardium from anoxia and rhythm disorders (Parratt and Marshall, 1974; Fagbeni *et al.*, 1982). Consequently, it had been suggested that the addition of high-energy phosphate compounds to St Thomas' cardioplegia may confer additional protective effects.

2. EXPERIMENTAL STUDIES

2.1. Methods

Experimental work is reported on the effects of high-energy phosphates in cardioplegia by Robinson and colleagues (1987). In these studies the isolated perfused working rat heart preparation was used to investigate the effects of adding exogenous creatine phosphate and/or ATP to STH2.

2.2. Experimental model

In the experimental model, the rat is heparinised before rapid excision of the heart which is then mounted on a stainless steel cannula via the aorta and perfused in the Langendorff mode with oxygenated perfusion medium (modified Krebs–Henseleit bicarbonate buffer: pH 7.4, containing 11.1 mmol/l glucose and gassed with 95% O_2 and 5% CO_2). After a short equilibration period, during which the left atrium is cannulated, the heart is converted into a working preparation in which cardiopulmonary bypass, is simulated by clamping the left atrial cannula. Coronary artery perfusion is achieved from a reservoir situated 100 cm above the heart. The heart continues to beat but performs no external work. Ischaemia is induced by clamping the aortic line, and preischaemic cardioplegic solution is infused via a side arm of the aortic cannula from a reservoir situated 60 cm above the heart.

2.3. Cardioplegic solution

The composition of STH2 used in the studies is shown in Table 12.1. Creatine phosphate was added to the solution at concentrations ranging from 0 to 50 mmol/l and because the creatine phosphate (Schiapparelli Searle, Torino, Italy) is supplied as the disodium salt, the sodium concentration of the cardioplegic fluid was adjusted accordingly.

Table 12.1. Composition of St Thomas' Hospital cardioplegic solution (STH).

Compound	Concentration (mmol/l)	
	STH1	STH2
NaCl	144.0	110.0
KCl	20.0	16.0
$MgCl_2$	16.0	16.0
$CaCl_2$	2.4	1.2
$NaHCO_3$	—	10.0
Procaine hydrochloride	1.0	—
pH	5.5–7.0	7.8
Osmolarity (mOsmol/l H_2O)	300–320	324

2.4. Experimental protocol

Hearts were perfused in the Langendorff mode for 5 min and then converted to the working mode for 20 min when control indices of cardiac function (aortic flow, coronary flow, aortic pressure) were measured. The cardioplegic solution was infused for 3 min (at the appropriate temperature for the ensuing ischaemia) and the heart was then subjected to either 40 min of normothermic (37°C) global ischaemia or 240 min hypothermic (20°C) global ischaemia (with multidose re-infusion of cardioplegia every 30 min). Reperfusion was started in the Langendorff mode for 15 min to simulate supportive bypass. The incidence of spontaneous sinus rhythm was determined together with the need for electrical defibrillation. The hearts were converted to the working mode for 20 min and postischaemic cardiac function recovery was measured and expressed as a percentage of the preischaemic control function. Thus, each heart acted as its own control. In all studies, there were at least six hearts per group and results are expressed as mean ± standard error of the mean.

3. RESULTS

3.1. Dose–response: normothermic ischaemia

The results for postischaemic recovery of aortic flow in hearts subjected to STH2 containing 0, 5, 10, 15, 25, 35 and 50 mmol/l creatine phosphate (PCr) are shown in Fig. 12.1. In the control hearts, the recovery of aortic flow was 21.1 ± 5.4% and PCr addition caused a significant ($p<0.05$) dose-dependent improvement such that at the optimal concentration of 10 mmol/l PCr the recovery of aortic flow was 82.5 ± 3.7%. At higher concentrations the recovery

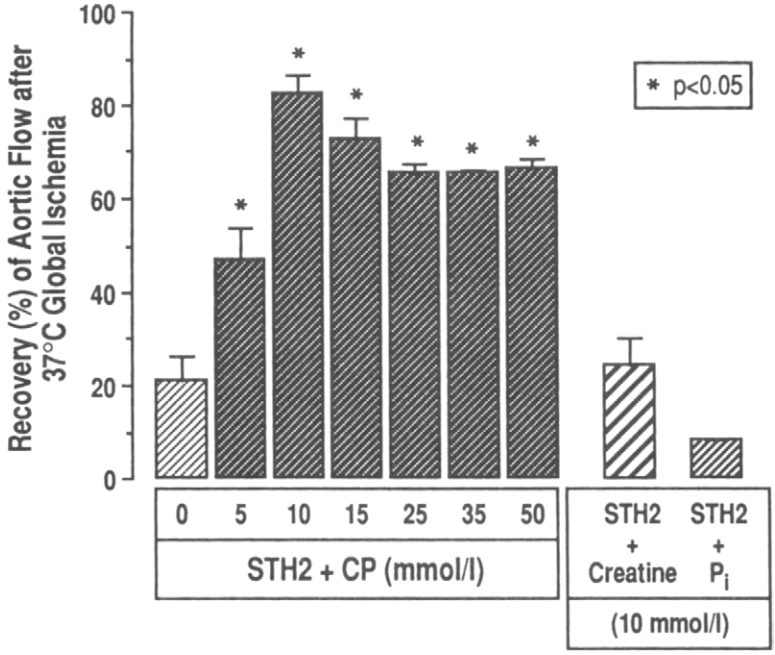

Figure 12.1. The recovery of aortic flow in response to different concentrations of PCr in a STH2 solution. The effects of creatine alone and phosphate (P_i) alone in association with the STH2 solution are also shown.

declined slightly, plateauing at around 65%, but was still significantly higher than the control value ($p<0.05$).

A similar profile was seen in relation to the incidence of spontaneous sinus rhythm (Fig. 12.2), with only 60% of control hearts exhibiting spontaneous sinus rhythm whereas there was 100% recovery in hearts treated with PCr in concentrations of 10, 15 and 25 mmol/l.

The effects of creatine and inorganic phosphate (both at concentrations of 10 mmol/l) were also investigated. The results showed (Fig. 12.1) that creatine had no effect on postischaemic recovery of function – aortic flow recovered to the same extent as control (24.2±5.9%). Inorganic phosphate, however, reduced postischaemic recovery such that only one of the six hearts recovered some aortic output (8.3% of preischaemic function).

3.2. Hypothermic ischaemia

When the hearts were subjected to extended periods of hypothermic (20°C) global ischaemia and multidose infusions of STH2 (either with or without 10 mmol/l PCr), postischaemic recovery of aortic flow was significantly ($p<0.05$)

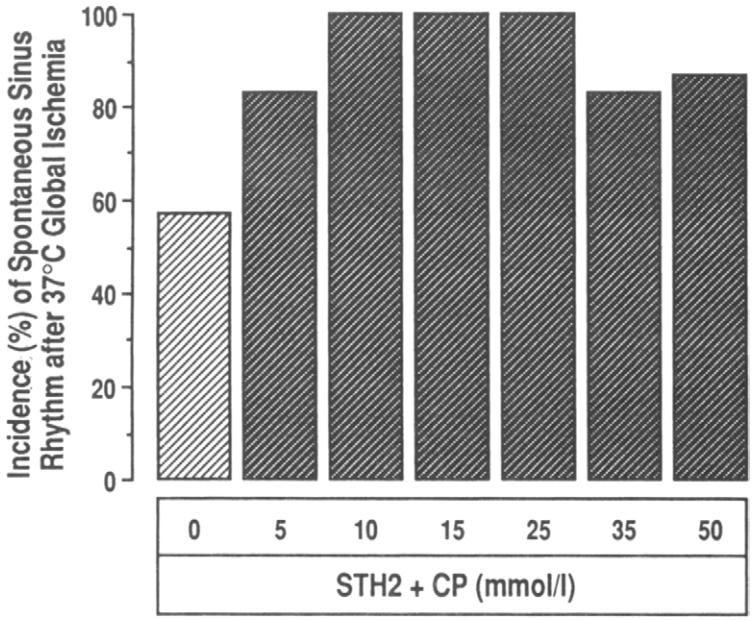

Figure 12.2. The incidence of sinus rhythm during perfusion with STH2 solution in the presence and absence of PCr.

improved from a control value (without PCr) of 33.1±8.3% to 77.9±4.2% using the optimal concentration (10 mmol/l) of PCr.

3.3. Synergistic effect of ATP and creatine phosphate

In another series of studies the dose-dependent effects of adding ATP to STH2 were investigated. Similar dose-dependent beneficial effects occurred and the optimal ATP concentration was established as 0.1 mmol/l. Using a virtually identical protocol to that described above, the effects of combining ATP and PCr (at their optimal concentrations of 0.1 and 10 mmol/l) were studied. After a period of hypothermic (20°C) global ischaemia which was extended to 270 min in this study (with reinfusions of STH2 every 30 min), the recovery of aortic flow in control hearts was 26.8±8.4%. Significantly ($p<0.05$) improved recovery was observed in those hearts subjected to STH2 containing either ATP or PCr alone (58.0±4.7% and 57.9±4.3%, respectively). When ATP and PCr were combined, however, there was a further enhancement in postischaemic recovery of aortic flow to 79.7±1.1%, and this value was significantly ($p<0.05$) greater than either of the individual additives alone.

4. CLINICAL STUDIES

Two clinical studies have been performed by Chambers and colleagues (Chambers *et al.*, 1991, 1993). In the first study the patients were mainly undergoing coronary artery bypass surgery (Study A), and in the second all patients were undergoing valve replacement (Study B).

4.1. Methods

In both studies some of the methods used were essentially the same, but in each study different individual methods were also investigated.

4.2. Cardioplegic solutions

The composition of the cardioplegic solutions used is in Table 12.1. In Study A, STH2 was used whereas in Study B, STH1 was employed. For Study A, PCr (Neoton) was dissolved in the sodium bicarbonate (10 ml 8.4% sodium bicarbonate solution) used to buffer the solution. For Study B, a 10-ml aliquot of the final cardioplegic solution was withdrawn, added to the PCr (Neoton), and the solution so obtained was subsequently added back to the cardioplegic solution. The final concentration of PCr was 10.0 mmol/l. This was previously shown to be the optimal concentration by Robinson *et al.* (1984).

4.3. Surgical procedures

In each study, 50 patients were randomized into two equal groups comprising those receiving cardioplegia alone or those receiving cardioplegia supplemented with PCr. Standard anaesthetic and surgical techniques were used. Cardiopulmonary bypass was established using a bubble oxygenator in Study A and a membrane oxygenator in the other study. The systemic flow rate was initially 2.4 l/min/m^2, and when the aorta was clamped the systemic flow rate was reduced to 1.5–1.75 l/min/m^2 in order to minimize non-coronary collateral flow to the myocardium. Most patients were cooled to around 25–28°C. An initial arresting volume of 1.0 l of cardioplegic solution was infused into the aortic root or directly into the coronary ostia. In Study A, further infusions of cardioplegia (500 ml) were made at intervals of 30 min and the heart was topically cooled by running 4°C Hartmann's solution over the surface whereas in Study B only one infusion of cardioplegia was made and the heart was cooled by surrounding it with iced-saline slush.

4.4. Biopsy procedures

Full thickness left and right ventricular biopsies (TruCut biopsy needle) were taken: (1) immediately before aortic cross-clamping; (2) immediately before removal of cross-clamp and (3) 10–15 min after aerobic reperfusion in Study A. In Study B, they were taken to correspond to (1) and (3) only. The left ventricular biopsy was divided into endomyocardial and epimyocardial halves and then chilled using techniques described elsewhere (Chayen and Bitensky, 1991). Quantitative polarization microscopy was used for birefringence assessment of myocardial protection (Braimbridge and Cankovic-Darracott, 1979; Chayen *et al.*, 1985). This technique involves measuring birefringence changes in longitudinal muscle fibres in air and then, in the same fibres, in response to buffer containing ATP and calcium.

4.5. ECG analysis

Patients were analysed for: (1) the incidence of spontaneous sinus rhythm; (2) the number of DC shocks required for reversion to spontaneous sinus rhythm and (3) the incidence of postoperative arrhythmias. In Study A, Holter monitoring was performed for 48 h to measure for ventricular tachycardias (VT) and supraventricular tachycardias (SVT). In Study B, VT and SVT were monitored routinely during the patients' stay in ITU.

4.6. Creatine kinase analysis (Study A only)

Arterial blood samples were collected preoperatively (sample 1), perioperatively (samples 2–6) and postoperatively for 4 days (samples 7–13). Total creatine kinase (CK) and the cardiac specific enzyme (CK-MB) were measured in each sample using an immuno-inhibition technique (*N*-acetyl cysteine activated kit from Boehringer, London).

4.7. Clinical and haemodynamic parameters

The preoperative, intraoperative and postoperative status of the patient was measured with respect to haemodynamics, inotropic status and clinical outcome. In study B, a Swan Ganz catheter (Baxter Healthcare) was inserted during anaesthesia and cardiac function measured by the thermodilution technique preoperatively, perioperatively and postoperatively for 24 h (during convalescence in ITU).

4.8. Statistics

Statistical analyses were carried out using the Student's *t*-test and the Chi square test. Values of $p<0.05$ were considered significantly different.

5. RESULTS

5.1. Study A

Fifty patients were randomized into two groups – those receiving STH2 alone and those receiving STH2 plus 10 mmol/l PCr. The patient groups (STH2 and STH2+PCr) were similar in relation to age (54.6±2.3 and 55.4±2.1 years, respectively), preoperative ejection fraction (64.5±2.1 and 64.7±2.8%, respectively), the number of cardioplegic infusions (2.9±0.3 and 2.4±0.3, respectively), bypass duration (103.5±12.2 and 104.8±60 min, respectively) and aortic cross-clamp duration (80.7±9.4 and 87.1±5.9 min, respectively).

5.1.1. Clinical outcome

Clinically, there were no differences between the two groups. Three patients (12%) in each group had low cardiac output (defined as requiring adrenaline for inotropic support) and three patients from the STH2 alone group required dobutamine support. No patient in either group died.

5.1.2. Arrhythmias

All patients had ventricular fibrillation immediately after reperfusion requiring defibrillation but there were no differences between groups in the mean number of DC shocks required to restore sinus rhythm. No differences were observed in VT or SVT in the first 24 h or in the second 24 h postoperatively.

5.1.3. Myocardial infarction (enzyme release)

The mean total CK and CK-MB started to increase immediately after the onset of cardiopulmonary bypass and the total CK peaked 24 h postoperatively. It subsequently fell rapidly to normal values on day 3. The CK-MB reached a peak 2 h postoperatively and then declined to preoperative values by day 2 (Fig. 12.3). There were no differences between the two groups.

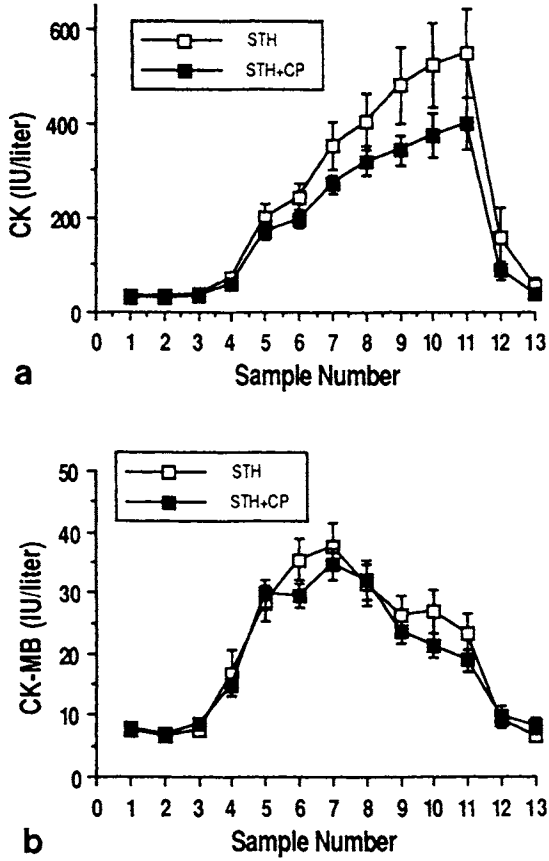

Figure 12.3. The CK-MB release wth STH solution alone and in the presence of PCr. From Chambers *et al.* (1991), with permission.

5.1.4. Myocardial protection

The birefringence change in response to ATP and calcium is shown is Fig. 12.4. There was a significant ($p<0.05$) increase in birefringence change at end-ischaemia and post-ischaemia in the endomyocardial biopsies (Fig. 12.4a) and the right ventricular biopsies (Fig. 12.4c indicating apparent improvement in contractility).

5.2. Study B

Fifty patients were randomized into two groups in the Valve Study comprising those receiving STH1 alone and those receiving STH1 plus 10 mmol/l PCr. The patient groups (STH1 and STH1+PCr) were similar in terms of age

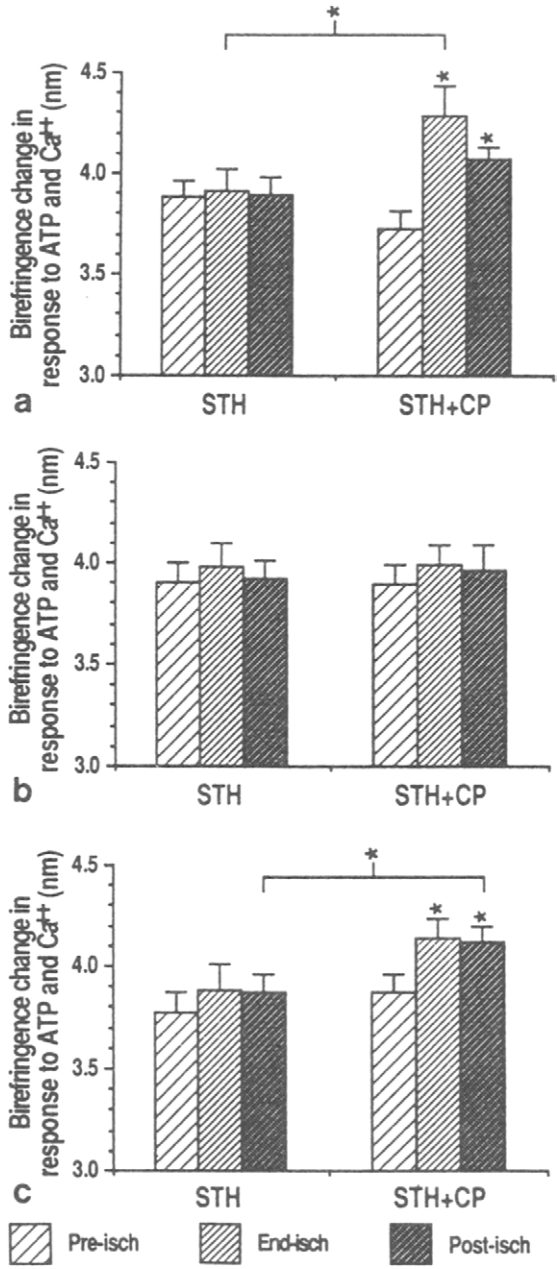

Figure 12.4. Birefringence changes in endo- and epi-myocardial LV biopsies and in RV biopsies in relation to perfusion with STH solution with and without PCr; **a**, LV Endomyocardial biopsies; **b**, LV Epimyocardial biopsies; **c**, RV Biopsies. Adapted from Chambers *et al.* (1991) with permission. *$p<0.05$ in comparison with pre-ischaemic sample and between STH and PCr groups.

(64.3±2.1 and 62.6±2.2 years, respectively), New York Association class (NYHA 2.4±0.2 and 2.6±0.2, respectively), volume of cardioplegia infused (968±14 and 1033±43 ml, respectively), aortic cross-clamp duration (62.7±2.7 and 61.1±2.3 min, respectively) and bypass duration (82.9±3.8 and 80.2±3.2 min, respectively).

There were no fatalities in either group. The incidence of inotropic support was similar in both groups, with 10/25 (40%) in the STH1 alone group compared with 7/25 (28% in STH1 plus PCr). The duration of inotropic support, however, was significantly ($p<0.002$) lower in the STH1 plus PCr group (16.5±1.1 h) compared with the STH1 alone group (24.8±7.3 h).

5.2.1 Arrhythmias

There was a significant ($p<0.01$) increase in spontaneous sinus rhythm (Fig. 12.5) from 8% with STH1 alone to 40% in those patients receiving PCr as an additive to STH1. This was associated with a significant ($p<0.02$) reduction in the mean number of DC shocks required to revert to sinus rhythm (1.40±0.14 to 0.88±0.15, respectively) and a corresponding significant ($p<0.02$) reduction in the mean total joule dosage (34.4±3.7 to 22.0±3.5 J, respectively).

5.2.2. Cardiac function

There was a general trend towards improvement in the cardiac index (Fig. 12.6) during the initial 24 h postoperatively. Although there were no significant differences between the two groups, there was a tendency to better function in the patients treated with PCr in the early postoperative period (0.5 and 1 h).

5.2.3. Myocardial protection

The birefringence change in response to ATP and calcium (expressed as a percentage of the preischaemic value) suggested an improvement in the endomyocardial and epimyocardial left ventricular biopsies (Fig. 12.7). These differences, however, were not significant and no differences were observed between the groups in relation to the right ventricular biopsies either.

6. DISCUSSION

The protective properties of cardioplegic solutions depend on three main components: chemical arrest, hypothermia and additional protection. The St Thomas' Hospital cardioplegic solutions (STH1 and STH2) contain elevated concentrations of potassium to induce rapid diastolic arrest of the myocardium (Hearse *et al.*, 1981). In addition to chemical arrest, hypothermia

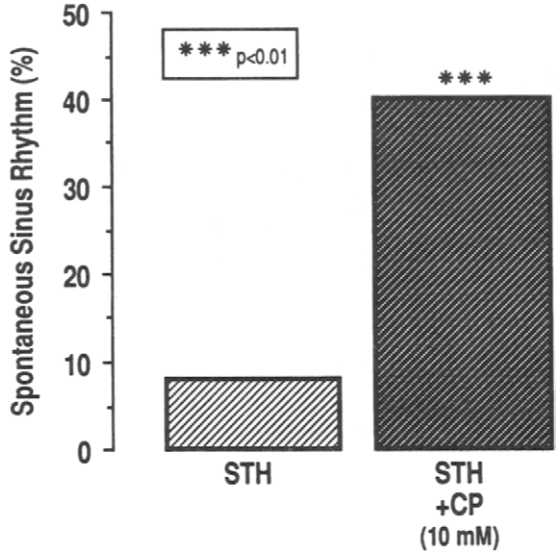

Figure 12.5. The recovery of sinus rhythm in the presence and absence of PCr with STH.

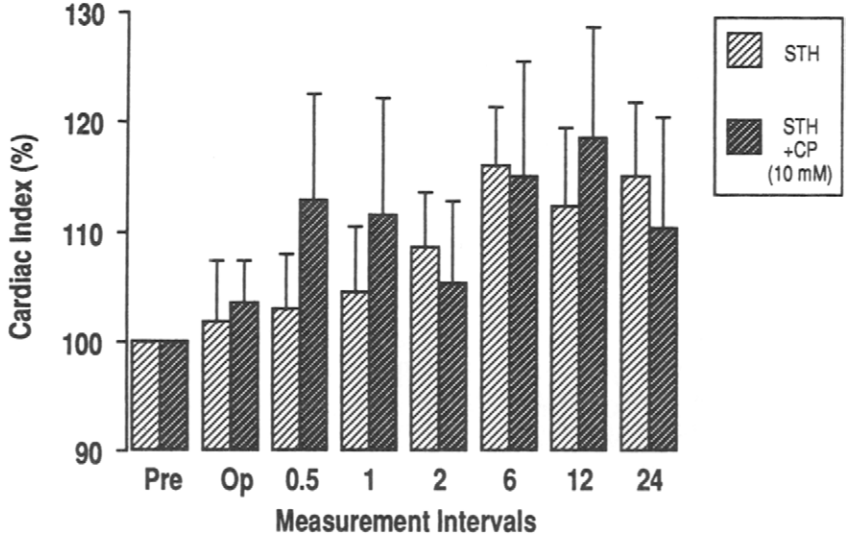

Figure 12.6. The cardiac index before, during and after surgery in relation to perfusion with STH solution with and without PCr.

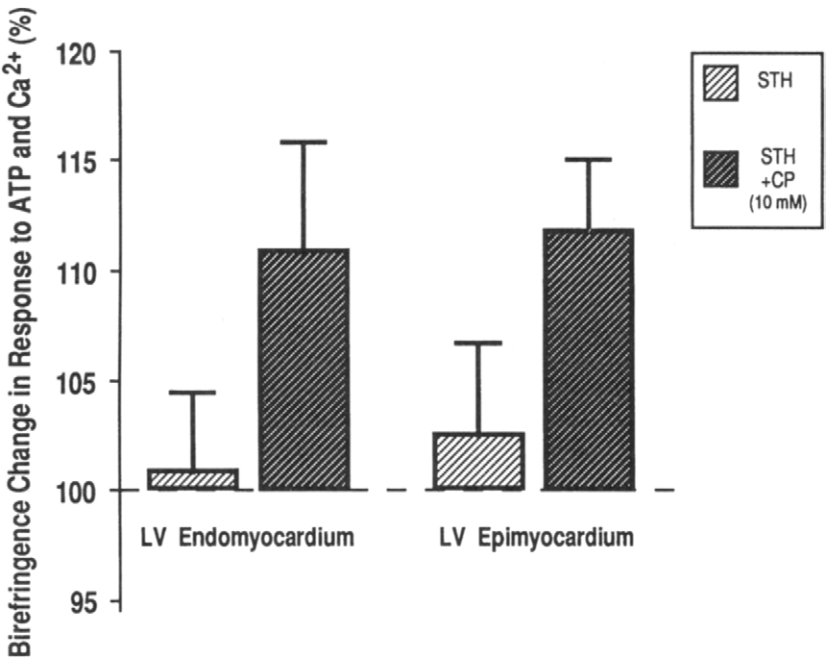

Figure 12.7. The birefringence change in LV epi- and endo-myocardium. The marked improvement associated with STH containing PCr is seen.

confers significant myocardial protection which has been shown to be additive (Hearse *et al.*, 1980; Rosenfeldt *et al.*, 1980).

The third component of cardioplegia – additive protection – has been observed with a large number of compounds added to the basic cardioplegic solution. In order to preserve the high-energy phosphate content of the myocardium, attempts have been made to manipulate nucleotide synthetic pathways by adding compounds such as ribose (Pasque *et al.*, 1982), adenosine and adenosine deaminase inhibitor (EHNA) (Foker *et al.*, 1980) and AICAr (Galinances *et al.*, 1992). A number of studies have suggested that exogenous ATP and PCr exert protective effects on the myocardium (Siska *et al.*, 1969; Parratt and Marshall, 1974; Hearse *et al.*, 1976; Chaudry and Baue, 1980; Fagbemi *et al.*, 1982) and the experimental and clinical studies reported in this chapter support these earlier findings.

6.1. Myocardial protection

Experimentally, PCr conferred significant additive protection to the St Thomas' No. 2 solution, with a large improvement in postischaemic recovery of function at the optimal concentration of 10 mmol/l. This value is similar to that

previously shown by Parratt and Marshall (1974) to give protection to anoxic guinea-pig atria. It was also identical to the PCr concentration (10 mmol/l) shown by Hearse and colleagues (1976) to exert significant protective effects when used in a study describing the characterization of a cardioplegic solution.

In clinical studies it is difficult to observe the benefit of an additive to a cardioplegic solution that already provides good protection. Thus, in Study A, in which only gross clinical outcome and mortality were examined as parameters for cardiac function, no detectable difference between the two groups was observed. Cardiac function, as determined by the thermodilution technique, was measured in Study B and no significant differences were observed at any point within the first 24 h postoperatively. It has been suggested that there is a period of transient dysfunction during the early postoperative phase (Breisblatt *et al.*, 1990). No alterations consistent with such a concept were detected in either group of patients. Indeed, in the group treated with PCr in STH1, Study B, cardiac function appeared to show a trend towards improvement in the early (first 60 min) postoperative period. However, the patient population is small for such a clinical study (50 patients in total).

In both clinical studies, quantitative polarizing microscopy was used to measure birefringence changes in the myofibrils in response to ATP and calcium. This technique, which measures the (apparent) ability of the myofibrils to contract, has been shown to be a sensitive method of measuring changes in cardiac function (Braimbridge *et al.*, 1979; Chayen *et al.*, 1985; Darracott-Cankovic *et al.*, 1987). Both studies demonstrated an improvement, measured at the cellular level, suggesting that PCr was exerting a beneficial effect on the ventricular myocardium. Larger changes were seen in Study A than in Study B; this may relate to the use of STH2 in the former, since this has been shown experimentally (Ledingham *et al.*, 1987) to have superior protective properties to STH1. (However, the duration of ischaemia in Study A was approximately 50% longer than in Study B, and this may be relevant to the findings.) In addition, the patients had ischaemic heart disease, which may render the myocardium more amenable to a compound providing additional protection.

6.2. Myocardial arrhythmias

Although PCr was shown to have significant protective effects on myocardial function after ischaemia, it was also demonstrated to exert antiarrhythmic properties by reducing the necessity for defibrillation (Robinson *et al.*, 1984). This confirmed previous reports of the antiarrhythmic properties of PCr (Fagbemi *et al.*, 1982), which have been supported by studies in the regionally ischaemic rat heart in which reperfusion-induced arrhythmias were reduced by treatment with PCr (Hearse *et al.*, 1986).

Previous clinical studies have also demonstrated the antiarrhythmic properties of high-energy phosphate compounds. Adenosine triphosphate is known

to have significant antiarrhythmic effects, which are removed by slowing conduction from the atrioventricular node, and terminating supraventricular tachyarrhythmias (Greco *et al.*, 1982; Belardinelli *et al.*, 1984). Creatine phosphate has also demonstrated antiarrhythmic properties in the clinical setting. Pedone and colleagues (1984) showed that, in patients with ischaemic heart disease, premature ventricular extrasystoles could be reduced by the administration of PCr over a 10-day period. Semenovsky and colleagues (1987) demonstrated an increased incidence of spontaneous sinus rhythm in patients treated with PCr as an additive to blood cardioplegia. Also, PCr was associated with a reduction in the number of DC shocks necessary to revert the heart to sinus rhythm.

The results of Semenovsky *et al.* (1987) were confirmed in Study B but not by the results of Study A in the present series. This could relate to differences in design between the studies. Thus, in Study A none of the patients from either group had spontaneous sinus rhythm and all required defibrillation. There were no differences, however, in the number of DC shocks required to revert the hearts to sinus rhythm. In addition, PCr had no effect on postoperative arrhythmias. In contrast, PCr added to STH1 in Study B exerted similar properties to those described by Semenovsky *et al.* (1987) with a significant increase in the incidence of spontaneous sinus rhythm and a significant reduction in the mean number of DC shocks required for reversion to sinus rhythm. Postoperative arrhythmias were also reduced in the PCr-treated patients. Similar findings to those described in Study B have been described by Pauletto and colleagues (1993), in which PCr produced a significant reduction in severe arrhythmias and in the incidence of atrioventricular block postoperatively. Interestingly, these results were obtained in patients undergoing coronary artery bypass surgery – a similar group of patients to those in Study A.

7. SUMMARY

In summary it seems reasonable to conclude from the studies described above that PCr, as an additive to St Thomas's cardioplegia, exerts a beneficial effect. This benefit has now been observed in a number of experimental and clinical studies, with effects on both postischaemic function and postischaemic arrhythmias. Owing to the difficulty of obtaining conclusive data in clinical studies, further large-scale clinical trials are warranted.

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13

Uses of Creatine Phosphate and Creatine Supplementation for the Athlete

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Key words: ADP, ATP, anaerobic, creatine kinase, exercise, muscle energetics

1. INTRODUCTION

In this chapter, some of the latest information regarding the uses of creatine (Cr) and creatine phosphate (PCr), and how they may be used as a modality in sports medicine are summarized. We will examine the current knowledge regarding the application of Cr and/or PCr with a focus on its utility in training and exercise.

The function of the Cr/PCr system inside the muscle cell is closely linked to the high-energy phosphate metabolism that underlies energy production. Creatine is phosphorylated by the enzyme creatine kinase (CK) according to the following reaction:

$$\mathrm{ATP + Cr \overset{CK}{\rightleftharpoons} PCr + ADP + H^+}$$

This reaction is readily reversible and may be used to buffer the ATP concentration by phosphorylating ADP. Indeed, 70% of the immediate high-

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energy stores contained within skeletal muscle is in the form of PCr (Greenhaff *et al.*, 1993a, Harris *et al.*, 1992). As much as 60% of the total creatine pool is in the phosphorylated form – PCr (Greenhaff, 1995).

The total Cr concentration in striated muscle is about 30 μmol/g, which represents 4 g of creatine per kg of red muscle. Creatine is synthesized in the liver, pancreas and kidney and released into the bloodstream (Walker, 1979; Wallimann *et al.*, 1992) to be actively taken up by the muscle cells, using the Na^+ gradient (Fitch and Shields, 1966). Creatine is absorbed, unchanged, from the intestinal lumen and passes directly into the bloodstream. The cellular Cr concentration is determined by specific transporters which transport Cr into the cell against its concentration gradient (Bloch *et al.*, 1941; Carpenter *et al.*, 1983; Moller and Hamprecht, 1989; Balsom *et al.*, 1993a,b). Because of the CK reaction, the concentration of PCr will be dependent upon the ATP and total Cr concentration (see Chapter 4).

The Cr transport protein has a high affinity for Cr and concentrates Cr within the cell (Fitch and Shields, 1966; Moller and Hamprecht, 1989; Walker, 1992). The affinity for Cr (reflected in its Michaelis constant or K_m) is about 30 μM, which is in the range of circulating plasma Cr concentration. Once inside the cell, very little Cr is lost (about 2 g/day in a 75 kg male) (Walker, 1979; Greenhaff, 1995). From this it follows that small increases in plasma Cr (which can occur with Cr supplementation) result in increased transport activity (see Chapter 3).

2. EXPERIMENTAL CREATINE SUPPLEMENTATION

Active Cr uptake has been found in all muscle cells studied including skeletal, heart and smooth muscles. Creatine supplementation is reported to have an anabolic effect (Bloch *et al.*, 1941; Scott *et al.*, 1987; Moller and Hamprecht, 1989; Balsom *et al.*, 1993a and b) and causes increased skeletal muscle protein synthesis (Sipila *et al.*, 1981; Balsom *et al.*, 1993a,b), as seen with increased ^{14}C substrate incorporation into muscle protein. Elevated intracellular PCr stimulates protein synthesis in the microsomes and interestingly, Bessman and Mohan (1992) likened the PCr stimulation of protein synthesis to that of exercise- or insulin-stimulated growth. Conversely, inhibition of normal Cr metabolism with 1-fluoro-2,4 dinitrobenzene prevents normal muscle growth and also decreases protein and lipid synthesis (Carpenter *et al.*, 1983).

During Cr incubation studies on isolated cell and tissue preparations, Clark *et al.* (personal communication) and Odoom *et al.* (personal communication) found that vascular smooth muscle or cultured striated muscle both concentrate Cr inside the cell (see also Chapter 3). Odoom increased total creatine (PCr+Cr) in cultured muscle cells (G8 mouse myoblasts) and demonstrated that its uptake was Na^+ dependent and cAMP mediated. Similar experiments using isolated vascular smooth muscle showed a striking two- to four-fold

increase in intracellular Cr concentration and increased PCr (Scott *et al.*, 1987). From the above studies it is apparent that Cr is taken up by living tissue and that increasing its intracellular concentration has multiple benefits.

3. ORAL CREATINE SUPPLEMENTATION

Harris *et al.* (1992, 1993) have shown that feeding Cr at 4–20 g/day increases intracellular Cr and PCr. This is confirmed by Balsom *et al.*, who showed that after ingestion, both serum Cr and the amount of Cr taken into the muscle increase, leading to higher PCr levels (Scott *et al.*, 1987; Balsom *et al.*, 1993a,b). This extra PCr enhances anaerobic output in the working skeletal muscle (Fig. 13.1), but there is no effect on maximal force (Harris *et al.*, 1993). These authors also found that elevated PCr produced an increase in anaerobic burst capacity. Another important benefit of an elevated muscle PCr concentration is that it provides the extra energy needed for work before glycolysis and glycogenolysis can be activated (Greenhaff *et al.*, 1994; Greenhaff, 1995).

Creatine administration and the concomitant increase in muscle PCr concentrations, was shown by Greenhaff *et al.* (1993a,b, 1994, 1995) to produce a striking increase in the rate of PCr resynthesis after exercise. The latter has been correlated to the rate of power recovery (Bogdanis *et al.*, 1995). Resynthesis of PCr is essential during repeated bouts of exercise. It is important to realize that Cr does not readily leave the cell. The loss of Cr from cultured skeletal muscle cells is less than 3% per day (Bloch *et al.*, 1941), which closely matches the amount of creatinine produced non-enzymatically by living human muscle (Rose, 1933). [The main mechanism whereby Cr is lost is via conversion to creatinine, which is an irreversible non-enzymatic process (Walker, 1979).] Therefore Cr efflux from the cell is considered to be negligible and hence the concentration of Cr is not at risk of becoming depleted by exercise. Thus, the advantages of Cr administration are that the cellular creatine concentration is stable and not prone to being lost (Bloch *et al.*, 1941; Walker, 1979; Wallimann *et al.*, 1992).

The rapid resynthesis of PCr is likely to be oxidative in origin (Saks *et al.*, 1985; Saks and Strumia, 1993). One might then ask, do increased PCr and Cr cause an increase in oxidative phosphorylation? Due to the presence of a specific creatine kinase isoenzyme within the mitochondria (Saks *et al.*, 1985), it appears very likely that the added Cr will increase PCr resynthesis. This is because mitochondrial creatine kinase (Mi-CK) is found on the outer surface of the inner mitochondrial membrane and is functionally coupled to oxidative phosphorylation (Wallimann *et al.*, 1992). Thus, the Mi-CK system stimulates mitochondrial respiration because of elevated intermembrane space [ADP]. The result is that instead of 60 μM ADP being required for half-maximal stimulation of oxidative phosphorylation, only 10 μM is needed in the presence of active Mi-CK and Cr (Saks *et al.*, 1985; Wallimann *et al.*, 1992).

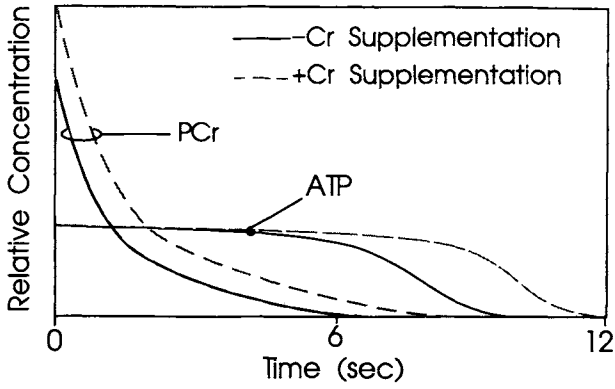

Figure 13.1. Creatine loading. A schematic representation of what happens to ATP and PCr concentrations during a 10–12s sprint. The solid lines represent what happens under control conditions. As the [PCr] falls [ATP] is maintained relatively constant until [PCr] reaches very low levels. Note that after 10 s of maximal anaerobic exercise such as a sprint, both ATP and PCr can be decreased. The dashed lines represent the same 10–12 s sprint but after [PCr] has been increased by supplementation with Cr. Again, [ATP] is maintained relatively constant with a prolonged [ATP] plateau. The delayed fall in [ATP] is because of extra PCr available to buffer ATP. To the sprinter this is equivalent to having extra energy for a push during the last 20 m of a 100 m sprint.

One potential concern relating to the apparent increase in anaerobic metabolism caused by Cr supplementation is that the athlete may produce more lactate and therefore be prone to lactic acidosis (Bogdanis *et al.*, 1995). Recently, Stroud *et al.* (1994) reported that oral Cr supplementation had **no** effect on blood lactate production. These authors also found that the respiratory gas exchange was unchanged. The $\dot{V}O2$ in control and Cr supplementation groups remained unchanged during incremental exercise lasting 90 min with 15 min of recovery. Therefore, despite an apparent shift towards anaerobic metabolism, there is no observable change in the usual anaerobic variables in athletes using oral Cr supplementation (Straud *et al.*, 1994). There also appears to be little benefit of added Cr for endurance performance (Balsom *et al.*, 1993a). Indeed, once the athlete has reached a steady state energy output, the benefit from added PCr would be minimal. There may, however, be some increase in the rate of recovery from exercise or a decrease in muscle stiffness (see below).

Greenhaff *et al.* (1993a,b, 1994, 1995) found that following oral Cr supplementation there was greater peak torque production during the final few contractions in a series. Oral Cr did not influence plasma lactate levels but these authors did find that PCr was better able to maintain muscle ATP (Fig. 13.1). Creatine supplementation produced significantly decreased plasma ammonia during exercise, indicating that ATP is conserved since the ammonia is partially depleted due to a loss of muscle adenine nucleotide stores (originat-

ing from ATP). This lost ATP is slowly resynthesized but the lower ammonia concentration may reflect the increased ATP which persists in the muscle; thus ATP is better conserved.

4. CONTROL OF NUCLEOTIDE LOSS

The mechanism by which PCr decreases nucleotide loss is due to its ability to phosphorylate ADP rapidly. Creatine phosphate therefore prevents ADP from being elevated by rephosphorylating it via the CK reaction and also by decreasing plasma ammonia. Elevated ADP would have two detrimental consequences for the muscle: a slowing of cross-bridges, and activation of the myokinase reaction. Muscle can use ATP produced by myokinase, but the AMP produced may be deaminated, resulting in ammonia and a loss of one nucleotide. ADP also activates the adenylate kinase reaction:

$$2ADP \rightleftharpoons ATP + AMP$$

Creatine phosphate and the CK reaction therefore prevent intracellular ADP from being elevated. The increased free Cr also stimulates oxidative phosphorylation, as discussed in Chapter 4. The stimulation of respiration by Cr will aid the mitochondria in producing oxidative ATP during stimulation. We can conclude that increasing PCr and Cr supplementation helps maintain muscle ATP and hence increases performance.

Regarding the safety of PCr supplementation, to date no studies involving Cr administration have reported detrimental effects (Greenhaff, 1995). However, the use of Cr or PCr as a therapeutic or other modality must be undertaken only under the supervision of a physician.

5. CREATINE PHOSPHATE

Above, we have seen how PCr is required for normal cellular metabolism and that increased PCr concentrations can enhance high-energy metabolism. PCr has no other known intracellular function because it is not involved in any other reactions. Creatine phosphate does, however, have another action which does not involve enzymes. This relates to its amphipathic (or ionic) nature (Gelfgan *et al.*, 1987; Saks *et al.*, 1987; Sharov *et al.*, 1987; Saks and Strumia, 1993; see also Chapter 5). The ionic characteristics of PCr enable it to bind to the polar phospholipid heads of membranes (Fig. 13.2). This binding stabilizes the membrane phospholipid bilayer by decreasing fluidity (Saks and Strumia, 1993; Sharov *et al.*, 1987) and prevents some of the damage caused by transient ischaemia and hypoxia (which can occur during exercise). Such an effect may decrease the loss of essential cellular substances (such as nucleotides) (Saks and Strumia, 1993).

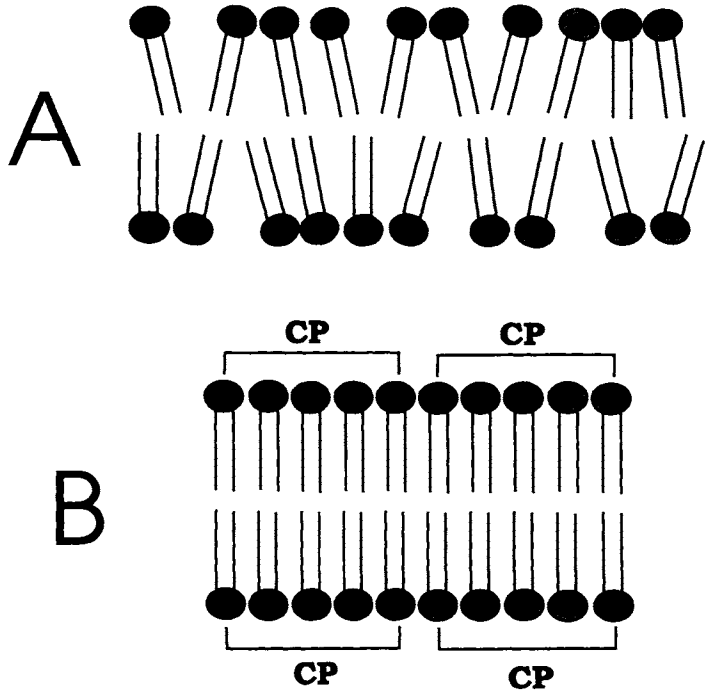

Figure 13.2. Creatine phosphate binding to the membrane. Creatine phosphate will bind to the phospholipid head groups across small patches of plasma membrane. Binding in this way stabilizes the patch of membrane and decreases membrane fluidity. The decreased membrane fluidity helps decrease loss of cytoplasmic contents, thus preserving function. The binding of PCr will occur at the phospholipid head groups on the inside and outside of membranes, with concomitant membrane stabilization to both sides (CP, creatine phosphate).

The membrane-stabilizing capability of intravenously administered PCr has been used in patients as part of the treatment for ischaemia and myocardial infarction (Sharov *et al.*, 1987). It is even used as a cardioplegic cardioprotective agent during heart surgery (Chapter 12). There is also a preserving effect on the intracellular content of soluble enzymes and nucleotides which is attributable to reduced leakage from the cell (Saks and Strumia, 1993; see also Chapter 7).

Other trials report favourable actions of PCr on skeletal muscle function and performance. Creatine phosphate improves maximum anaerobic power, potentiating the ability of the muscle to produce maximum burst output when compared with placebo (Dal Monte *et al.*, 1976). During maximal exercise such as "cronoscalata" (timed uphill cycling), enhanced performance has been shown in amateur cyclists treated with PCr (Tegazzin *et al.*, 1991). In three controlled studies using an isokinetic dynamometer, treat-

ment with PCr increased the rate of muscle strength recovery after intense exercise (Satolli and Marchesi, 1989; Pirola *et al.*, 1991; Agnese *et al.*, 1992). These studies were performed on patients with hypotonotrophy resulting from sequelae associated with knee pathology. The authors, however, did not speculate on its use for healthy individuals or the application as a conditioning aid.

Intravenous PCr has been given to athletes during strenuous endurance training (V.A. Saks, personal communication). Triathletes undergoing a rigorous daily land training schedule were given daily PCr infusions of 2–5 g/day. The athletes were able to train longer and at a greater intensity. They also had a striking reduction in delayed-onset muscle stiffness during this period and PCr administration led to improved overall endurance performance. It was postulated that the decreased stiffness and myalgia seen was due to less muscle damage occurring during training. This may relate to the membrane stabilization by PCr and/or possible improvement in the energetics of the tissue. This result is consistent with the results seen with cardioprotection by PCr in stabilizing membranes (V.A. Saks, personal communication).

One drawback of PCr administration is that it must be given intravenously or intramuscularly because it is readily broken down in the intestinal tract. Even with intravenous administration, rapid degradation to creatine and phosphate occurs in human serum (Saks and Strumia, 1993; see also Chapter 7). As detailed in Chapter 7, the clearance of PCr from blood has two time constants; there is a fast component and a slow component. The latter is probably results from a substantial amount of PCr being bound to membranes and thus being less susceptible to hydrolysis. None the less, intravenous PCr has beneficial effects that Cr alone does not have. Most of these effects are attributed to the membrane-stabilizing action, but when PCr is hydrolysed, it forms inorganic phosphate (P_i) and Cr. As discussed above, Cr is actively taken into the cells and used to supplement the energetic metabolism of muscle. Therefore, intravenous PCr administration has the same benefits as oral Cr administration with the additional membrane effects. **The use of PCr must be undertaken only under the guidance of a qualified physician.** [Hyperphosphataemia did not develop at these doses but may become a concern at higher doses.]

6. SUMMARY

In this chapter the actions of Cr and PCr on muscle metabolism and performance are discussed. Creatine phosphate and Cr constitute an energetic shuttling mechanism which is essential for normal muscular function. Creatine can be supplemented in the diet and is able to enhance anaerobic capacity as well as being anabolic. Along with its energetic role, PCr has the ability to stabilize membranes and protect cells from damage. The membrane

protection afforded by PCr appears to be a biophysical phenomenon at the membrane surface only, but when PCr is degraded, Cr is the product, and this has beneficial effects in the muscle also.

The use and utility of any dietary supplement or other modality is highly variable, must be used cautiously, and must always be kept within limits. Greenhaff *et al.* (1994) reported that three out of eight subjects studied showed no beneficial effects. There was, however, no evidence of performance impairment. What role PCr and Cr supplementation may play in the future of sports medicine remains to be determined.

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14

Creatine and Creatine Phosphate: Future Perspectives

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Key words: creatine, creatine phosphate, arginine, myositis, scurvy, parathyroid, muscular dystrophy, thyroxine, adrenaline, hypothyroidism, thrombolysis, sympathomimetics, myocardial infarction, heart failure

Creatine and creatine phosphate are molecules which are widely distributed in muscle and other organs and their central role is outlined above. Whilst no one doubts their importance to high-energy phosphorus metabolism, questions still remain relating to many biochemical functions and aspects of therapy. This monograph is designed to summarize some of the background knowledge and to stimulate interest in exploring the metabolism and pharmacological properties of these agents.

Both creatine and creatine phosphate have been under study for many years and indeed it is over a century-and-a-half since Chevreul (1835) discovered creatine. The early observations were reviewed by Rose (1933) and Beard (1941) and modern interest was renewed by Walker (1979) and Sipla *et al.* (1981).

Much of what is important for future study has been considered to various degrees in the past. Thus, the relationship between arginine and creatine metabolism, the distribution of the compound in skeletal muscle, the concentration differences in red compared with pale fishmeat, in skeletal compared to heart and smooth muscle, were all studied by earlier investigators. Feeding moderate and large amounts of creatine to rats (5–10% creatine) was long ago shown to increase heart and skeletal muscle creatine concentrations and the importance of the duodenum as the site of absorption identified. Low creatine

Creatine and Creatine Phosphate:
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concentrations were found at autopsy studies of myositis and scurvy. Creatine administration led to reduced urinary phosphate excretion – an effect amplified by the administration of parathyroid extract. In pseudohypertrophic muscular dystrophy the administration of the glycine (gelatin) decreased the loss of creatine owing to possible conversion to creatine. Both adrenaline and thyroxine cause creatinuria, while iodine administration and hypothyroidism induce a complete cessation of creatinuria. Starvation is associated with increased urinary creatine concentrations.

These observations are pointers to fruitful exploration in the future and they identify, not only the conditions which should be examined, but also a technique for studying creatine balance, i.e. urinary collection. The older observations also focus attention on the endocrine system and its role in creatine metabolism. There are some hints (Beard, 1941) of a relationship between sugar metabolism and creatinuria and of the possible role of the posterior pituitary hormones in the transformation of creatinine to creatine.

Although numerous observations on creatine and creatine phosphate metabolism exist, much is yet to be learned as we apply modern detailed methods of measurement and examination. The assessment of the potential of these substances in elite muscle performance is at an early stage yet and the observations on athletes may be relevant to the clinic. Thus, enhancement of muscle metabolism could relieve some of the fatigue due to heart failure. Recovery of recently injured heart muscle post-thrombolysis in acute myocardial infarction may be assisted by the administration of creatine or creatine phosphate, and indeed a trial examining the effects of Creatine phosphate REplenishment in the Acutely Thrombolysed INfarct is already pre-piloted (The CREATINE Project: Michael A. Conway, personal communication). The maximum efficiency of such therapeutic strategies may yet be found to relate to creatine or PCr administration combined with "facilitator" agents such as sympathomimetics and this must represent an important part of future strategy. Detailed examination of the relationship between creatine, the phosphorylated compound and phosphate may also be fruitful since creatine coupled to phosphate is so important to efficient cellular metabolism.

The science of creatine and creatine phosphate metabolism is now moving rapidly and exciting observations will be reported to complement those summarized here. The therapeutic possibilities of agents that are natural and relatively inexpensive means that much effort should be devoted to their detailed examination. The older literature and that outlined in the above chapters will be a good starting point for the interested investigator.

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Chevreul (1835), see Rose (1933).

Assay for Creatine and Creatine Phosphate

The assay for total tissue creatine can be performed using a simple fluorimetric determination as described by Conn (1960). This method combines ninhydrin, at alkaline pH, with guanidine, and monosubstituted guanidines to form a highly fluorescent product. The fluorescence is measured at 525nm. This precise reaction is simple and very specific for creatine (arginine being the only natural substance that reacts significantly under these conditions). The fluorescence produced by creatine is greater than that for other guanidinium compounds, so precision is possible.

Creatine (Cr) and creatine phosphate (PCr) determined with this fluorescence technique because PCr does not react to produce fluorescence. Thus an assay of free Cr paralleled by an assay of total Cr (after hydrolysis of PCr) will allow the determination of free Cr, PCr and total Cr.

CREATINE STANDARD

This assay has an effective range (in that the titration curve is linear) from 1.0×10^{-7}M to 2.5×10^{-5}M. A standard curve should be within this range and centred about the concentration of interest. For samples with concentrations above this range one can simply dilute the samples to an appropriate concentration.

The creatine standard is made from a stock solution of reagent grade Cr. Stock creatine solution is stable, if kept frozen (−20°C), for several weeks.

SAMPLE PREPARATION

For tissue samples, homogenize 5–10mg wet weight (ww) of tissue in 1ml of homogenization buffer. Homogenizing must be done quickly at 0–4°C. Larger tissue mass (up to 100mg ww/ml) are used, where necessary. An example of a typical tissue homogenization buffer is 100mM KH_2PO_4, 1mM EGTA, 10mM Na-HEPES and pH7.1. This is centrifuged at about 1000*g* for 10min at 0°C and the pellet discarded. The supernatant should be used immediately or quickly frozen and stored at −70°C to prevent breakdown of PCr to Cr and phosphate (P_i).

Creatine and Creatine Phosphate:
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ZINC SULPHATE SOLUTION

A 5% (w/v) solution of zinc sulphate is necessary for the creatine assay. This solution is very stable and 50 g of $ZnSO_4 \cdot 7H_2O$, mixed with the sufficient quantity of dH_2O to make 1000 ml, keeps in the refrigerator for several weeks.

BARIUM HYDROXIDE

Ninety grams of $Ba(OH)_2 \cdot 8H_2O$ is mixed with sufficient quantity of H_2O to make 2000 ml. After thorough mixing, the solution is filtered (0.04 μm Millipore filter) and kept at room temperature but protected from CO_2 and open air.

NB: A pH titration of $ZnSO_4$ and $Ba(OH)_2$ is necessary to confirm that equal volumes ($\pm 5\%$ error) will neutralize each other.

NINHYDRIN SOLUTION

A 1% (w/v) solution of ninhydrin is made by mixing 1 g of solid ninhydrin with 100 ml of 70% ethanol.

POTASSIUM HYDROXIDE

A 10% (w/v) KOH solution is prepared by adding a sufficient quanity of 70% ethanol to 100 g KOH to make 1000 ml (the solution will get warm). This solution should be clear. If cloudy, quickly filter the solution but avoid concentrating the KOH.

DETERMINATION OF CREATINE

Mix 1 ml of sample (of appropriate concentration) with 15 ml of distilled H_2O, 2 ml of $Ba(OH)_2$ and 2 ml of $ZnSO_4$ solutions. Prepare a blank with distilled H_2O or appropriate buffer substituted for sample. This solution and the blank are filtered. For the assay, place 2 ml of blank and the standard in a test tube with 1 ml of ninhydrin and mix well. Add 1 ml of KOH at time zero. Measure absorbance at 410 nm excitation with 10 nm slit width and emission of 525 nm using 20 nm slit width. At 8 min, note the absorbance (± 1 min has negligible effects upon the absorbance reading).

NB: Avoid exposing the cuvettes to sunlight and do not leave the cuvette in the excitation beam for more than 20 s before taking the measurement.

The creatine concentration is determined using the standard curve. However, because the standard curve is linear, the creatine concentration can also be calculated directly.

DETERMINATION OF CREATINE PHOSPHATE

Creatine phosphate accounts for a large percentage of total tissue creatine and so complete hydrolysis of PCr is essential for determining total [Cr]. Creatine phosphate is easily hydrolysed to Cr and P_i by combining 500 μl of sample with 50 μl of 1 N HCl and heating to 60°C for 40 min. After cooling, neutralize the sample and commence the assay as described above. Remember to adjust the concentration of Cr after dilution for the acid and base.

This procedure will yield a value for total Cr (Cr_t), where,

$$Cr_t = Cr_f + PCr$$

Cr_f is free creatine which can be determined prior to hydrolysis of PCr. Therefore one can calculate PCr from $Cr_t - Cr_f$. When determining PCr in this way it is important that the determination of Cr_f be done with minimal spontaneous hydrolysis of PCr and P_i. This can be guarded against by using pH-neutral buffers and keeping the samples at –70°C until assay.

DECREASING CONTAMINANT INTERFERENCE

Interference from naturally occurring and other compounds can occur to give false reading of Cr. These materials tend to be guanidino compounds and arginine. Many of the contaminating compounds can be removed by running the sample through an ion-exchange resin. Amino acids and guanidino contamination are greatly reduced in this way.

Prefabricated ion-exchange columns are readily available, but in many cases, ion exchange is not required. For example, in whole blood the Cr is 10 times that of guanidino acetic acid, while the resulting fluorescence from guanidino acetic acid is less than half that of Cr. Therefore guanidinoacetic acid contamination under normal conditions will only cause an error of about 3%.

Arginine is another contaminant for the Cr assay. Its fluorescence is about one-fifth that of creatine. Except in cases of proteinuria, arginine contamination during a urine Cr assay would be less than 10%. In tissue such as muscle, brain and heart, the Cr concentration is 5–30 μg/ml. This concentration is again 10 times the concentration of other contaminants. Thus even for crude homogenates from tissue samples (providing they are properly buffered), Cr_f can be determined as well as Cr_t with little interference from guanidino compounds.

OTHER ASSAY METHODS

There are other techniques to assay for Cr and PCr in biological samples. We have seen in Chapters 3 and 8 that PCr can be visualized using ^{31}P NMR spectroscopy. In experimental conditions this PCr concentration is used to determine Cr_f from Cr_t. Conversely, ^{1}H and ^{13}C NMR may be used to determine Cr in some tissues.

Thus NMR is also be used to determine PCr, Cr_f and Cr_t in samples of interest. This technique, however, requires the availability of the spectrometer, magnet, sufficient concentrations of metabolites and it is also less precise and sensitive than fluorescence methods. NMR has the distinct advantage that it can be used on living (isolated perfused) samples, *in vivo*, and is non-invasive.

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Glossary

14C: carbon 14 isotope
Hypertrophy: increase in the size of cells or tissue
Fibre type: determined from the sensitivity of myosin ATPase to extremes of acidity. Types I (slow fatigue resistant), IIA (fast twitch oxidative) and IIB (fast twitch glycolytic)
Isometric: Development of tension while the muscle is prevented from producing movement
Isotonic: muscle contraction taking place with normal contractile movement
K_m: the concentration of a substance to half maximally stimulate the reaction
Magnetic resonance spectroscopy: technique for non-invasive measurement of the concentration of intracellular molecules based on the acquisition of signal from nuclei such as phosphorus
Mitochondria: intracellular organelle central to energy production
Myofibrils: muscle fibres
NAD: nicotinamide adenine dinucleotide oxidized
NADH: nicotinamide adenine dinucleotide reduced
OD 340: optical density at 340 nanometers
PEP: phosphoenolpyruvate
Pronex: exercise in the face-down or prone position
Rate of respiration: the velocity of oxygen consumption
Stoichiometrically: being related by ratios of a reaction
Vmax: maximal rate of reaction

Index

Note – Page numbers in *italics* refer to figures and tables.