ALTERATIONS IN CARDIAC MEMBRANE ACTIVITIES DURING THE CALCIUM PARADOX IN ISOLATED PERFUSED RAT HEARTS

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

by

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September, 1982

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BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to my wife, Linda, our son,

Christopher and our Children

yet to come

ACKNOWLEDGEMENTS

My first wish is to thank my supervisor and dear friend Dr. N.S. Dhalla. The opportunity to work with a man of your character and scientific genius is one I will forever treasure. I will always be grateful for your guidance, assistance, friendship and at times, immeasurable patience. Thankyou most sincerely; without you this investigation would have been impossible to complete.

To my graduate committee members, Drs. V.M. Panagia and P.K. Singal; our interractions over the last few years have in no small way accounted for the success of my research endeavors.

I would also like to acknowledge the essential role played by my fellow students and departmental colleagues in the completion of this thesis. If for no other reason, I thank you because you are largely responsible for establishing the vibrant and highly professional environment in which I have had the privilege of carrying out my studies. There have been far to many of you over the years to list each one separately. I would however, like to single out Mr. Kalwant (Ken) S. Dhalla. I believe you have become an exceptional technician and teacher and your assistance has been greatly appreciated, my friend.

How can a student survive and succeed without financial assistance? I will always be indebted to the Canadian Heart Foundation for its personal support during the course of my graduate career and to the Manitoba Heart Foundation which has supplied the necessary research grants to Dr. N.S. Dhalla so that the facilities I required were available.

Finally I wish to thank those to which I have dedicated this thesis. Linda you have always been patient with me and certainly I appreciate your typing skills but most of all you have been my closest friend and most avid supporter. As for you Christopher, now and even before you were born, you have given me the motivation to finish what I started in spite of all the obstacles. I love you both.

ABSTRACT

Changes in contractile force (CF), resting tension (RT) and microsomal Ca^{2+}-uptake in isolated perfused rat hearts were investigated under conditions associated with reversible and irreversible stages of the calcium paradox phenomenon. Five minutes of reperfusion with normal (1.25 mM) calcium after 5 minutes of Ca^{2+}-free perfusion, resulted in a marked rise in RT with no recovery of CF and a 63% depression in microsomal Ca^{2+}-uptake. Under some conditions the characteristic increase in RT and decrease in CF and Ca^{2+}-accumulation, were either absent or reduced in comparison to hearts reperfused after 5 min of Ca^{2+}-free perfusion. This was observed when reperfusion was carried out after 5 minutes of perfusion with 0.025 mM or greater concentrations of Ca^{2+}, after less than 5 minutes of Ca^{2+}-free exposure or after 5 minutes of varying degrees of hypothermic Ca^{2+}-free perfusion. Furthermore, reperfusion-induced increases in RT and decreases in microsomal Ca^{2+}-uptake were also found to be dependent on the duration of reperfusion as well as on the calcium concentration of the reperfusion medium. Microsomes isolated from control, Ca^{2+}-free (5 min) or reperfused (5 min) hearts were found to have similar phospholipid composition, protein profiles (SDS-polyacrylamide gel electrophoresis) and electron microscopic appearance. While Ca^{2+}-free perfusion alone had no effect on any of the parameters studied, reperfusion also depressed microsomal Ca^{2+}-binding, Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activities. Our findings suggest that reperfusion-induced contracture and intracellular calcium overload may be related in part to damage in the ability of the sarcoplasmic reticulum to regulate calcium.

Alterations in sarcolemmal Na^+/K^+ ATPase activity under conditions of the calcium paradox, were examined in order to characterize the nature of the change as well as to analyze the possible existence of a relationship between altered Na^+/K^+ pump function and the characteristic increase in Na^+ and decrease in intracellular K^+ contents, observed in earlier studies on the calcium paradox. One minute of reperfusion with Ca^{2+} after 5 minutes of Ca^{2+}-free perfusion, was sufficient to reduce Na^+/K^+ ATPase activity approximately 53% while Mg^{2+} ATPase, another sarcolemmal bound enzyme, retained 74% of control activity. These changes in enzyme activity were dependent on the duration and Ca^{2+} concentration of the initial perfusion and subsequent reperfusion periods. Under all conditions Na^+/K^+ ATPase was consistently more depressed than Mg^{2+} ATPase activity. Conditions which protect the isolated heart from the calcium paradox also prevented reperfusion-induced enzyme alterations. The mechanism of depression in both enzyme activities was related to an alteration in Vmax. When we compared changes in myocardial Na^+ and K^+ contents to Na^+/K^+ ATPase activity under identical conditions, a significant relationship emerged. At least 60% of control enzyme activity was necessary in order to maintain normal cation gradients. If enzyme activity was depressed by approximately 60-65% the result was a marked increase and decrease in intracellular Na^+ and K^+ contents, respectively. These results support the view that changes in myocardial Na^+ and K^+ contents during the calcium paradox are related to the activity of the Na^+/K^+ pump. The possibility that impaired Na^+/K^+ ATPase activity may augment Ca^{2+} overload via an enhancement of the Na-Ca exchange mechanism is discussed.

The role played by sarcolemmal Ca^{2+} ATPase in the occurrence of massive Ca^{2+} influx and impaired mechanical performance in the calcium paradoxic heart, was also inyestigated. This enzyme is believed to regulate Ca^{2+} channels in the sarcolemma and its activity has been shown to parallel contractile force generation under normal conditions. Decreased recovery of contractile force was found to preceed depression of sarcolemmal Ca^{2+} ATPase in the early stages of the calcium paradox. The results suggest that in more seriously compromised hearts impaired Ca^{2+} ATPase activity may contribute to irreversible failure in contractile force recovery. This belief was supported by the fact that depressed enzyme activity was inversely related to the increase in resting tension; irreversible contracture being a characteristic of severely affected hearts. After 5 minutes of Ca^{2+}-free perfusion, reperfusion for 2 minutes with Ca^{2+} containing medium had no effect on Ca^{2+} ATPase activity in spite of the fact that this perfusion sequence is known to result in calcium overload and extensive biochemical and ultrastructural derrangements. The suggestion is made that during the initial phases of reperfusion, Ca^{2+} ATPase retains its function and so will continue to favour calcium influx and the development of intracellular calcium overload. Subsequent impairment in ATPase activity as the duration of reperfusion was prolonged, appeared to be a consequence of calcium overload. Depressed Ca^{2+} ATPase activity was due to a decrease in Vmax. These alterations in enzyme activity were not related to contamination of the sarcolemmal preparation by other subcellular constituents nor were they due to major changes in membrane phospholipid composition.

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I. INTRODUCTION AND STATEMENT OF THE PROBLEM

The term "calcium paradox" was first coined by Zimmerman and Hulsmann (1) and Zimmerman et al (2) to describe a series of observations resulting from the reintroduction of calcium to an isolated rat heart previously perfused with a medium devoid of calcium. Rather than allowing for a recovery in contractile activity lost during the Ca^{2+}-free period, reperfusion with Ca^{2+} was associated with marked and irreversible contracture, a loss of intracellular proteins and severe morphological changes. This initial description of the calcium paradox phenomenon has intrigued researchers and stimulated much investigation over the past sixteen years such that the relevence of this observation now spans several areas of basic and clinical science. Needless to say a complete characterization of the events leading to and arising from the calcium paradox is essential.

The basic principal underlying the calcium paradox phenomenon is one of altered calcium regulation by the myocardium. It was initially believed that the effects of reperfusion were due to a massive influx of calcium (2,3,4). Subsequently it was demonstrated that Ca^{2+}-free perfusion altered sarcolemmal permeability characteristics (1,5) as well as decreasing myocardial calcium contents (6) while reperfusion was associated with an intracellular calcium overloading (6). Intracellular calcium deficiency and overload are believed to be related to the contractile failure and cardiac cell death seen in several pathological situations (4,7-11), therefore the calcium paradox offers a unique model for studying the effects of these conditions.

The major sites responsible for the regulation of myocardial cell Ca^{2+} and in turn mechanical performance, are undoubtedly sarcolemma and sarcoplasmic reticulum (12,13). The thrust of the present investigation was therefore towards elucidating the effects of Ca^{2+}-free perfusion (calcium deficiency) and reperfusion (calcium overload) on the biochemical activities of these membrane systems; sarcoplasmic reticulum has the ability to accumulate intracellular calcium while sarcolemmal Ca^{2+} ATPase is believed to govern the influx of extracellular calcium (12). Furthermore, the degree of calcium paradoxic effects on contractile performance are dependent upon the temperature, duration of perfusion and calcium concentration present, both before and during reperfusion (5,6,14,15). This has enabled us to investigate the relationships between membrane function and contractile activity under reversible and irreversible stages of the calcium paradox.

Finally, earlier work (6) has demonstrated that the calcium paradox is not merely associated with altered cellular calcium but other myocardial cations, notably Na^+ and K^+, also change dramatically. In light of these observations we have also investigated the effects of the calcium paradox on sarcolemmal Na^+/K^+ ATPase; the enzyme responsible for maintaining the levels of cellular Na^+ and K^+ (16). With these measurements and our earlier determinations of myocardial cations we planned to assess the possible existence of a cause and effect relationship.

In general then, this study was designed to more fully characterize the nature of the cationic changes which occur during the course of the calcium paradox in addition to shedding light on the mechanisms responsible for the mechanical derrangements which typify the development of intracellular calcium overload.

II. REVIEW OF THE LITERATURE

THE CALCIUM PARADOX PHENOMENON

A. Definition and Description

Perhaps the single greatest problem for present day researchers studying the calcium paradox is deciding on a suitable definition for the phenomenon. In the sixteen years since the first report of the calcium paradox, numerous facets of the paradox have been documented and most are now recognized as being integral characteristics, thus making an all inclusive definition virtually impossible. What seems most appropriate is to restrict the definition and basic description to that originally put forth by Zimmerman and Hulsmann (1) and Zimmerman et al (2) with the provision that those working in the field be aware of the additional aspects when interpreting their findings.

Zimmerman and Hulsmann (1) observed a rapid cessation of contractile activity when the isolated rat heart was perfused with Ca^{2+}-free medium. Electrical activity however was maintained. This electromechanical dissociation supported earlier observations by Ringer (17) and Mines (18). During the Ca^{2+}-free period it was noted that the heart maintained its red colour and various myocardial proteins like myoglobin, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) remained within the cell. However, when the normal perfusate was reintroduced after more than 2 minutes of zero Ca^{2+} perfusion, a series of marked changes occurred. Contractile activity was not restored and the hearts became irreversibly contracted; electrical activity disappeared; the hearts became pale and mottled. They called this the "calcium paradox". Additional findings included the demonstration of myoglobin, LDH , CPK and high energy phosphate compounds in the

perfusate shortly after the onset of reperfusion. They concluded that the cell membrane had lost its ability to retain intracellular constituents.

In a subsequent report, Zimmerman and his colleagues (2) examined the morphological changes resulting from the calcium paradox in rat hearts. Up to one hour of Ca^{2+}-free perfusion produced no structural alterations at the light microscopic level but a dilated transverse tubular system was appreciated using the electron microscope. In spite of our desire to avoid complicating the initial observations of Zimmerman et al with subsequent findings, this last point does deserve some additional attention. Other studies have revealed separation of the interalated discs after 10-50 minutes of Ca^{2+}-free perfusion (3,18,19,23) while still others (19-22) have seen separation of the basal lamina from the sarcolemma after 20-40 minutes of Ca^{2+}-free exposure of rabbit and rat hearts. Ca^{2+}-free exposure for 60 minutes has been shown to disrupt myofibrillar integrity (24). In two recent reports, one in rabbit (25) and one in rat (15) myocardium, as little as 5 minutes of Ca^{2+}-free perfusion resulted in separation of the glycocalyx, a decrease in intramembrane particles, alterations in the middle lamina, intercalated disc and golgi apparatus as well as a loss of heterogenic staining properties of the nucleoplasm. Therefore the ultrastructural picture of Ca^{2+}-free perfusion is not as simple as first indicated by Zimmerman et al (2). However, there is essentially universal acceptance (3,4,5,26) of their morphological analysis of reperfused hearts. The reintroduction of calcium resulted in the patchy loss of normal cellular organization. Mitochondria became spherical and swollen and the major components

of the contractile apparatus were absent. Intercalated discs were largely unrecognizable and in those cells not completely disrupted, prominent contracture bands were observed. Another observation was the presence of electron-dense bodies deposited in the mitochondria. This material was believed to be calcium which prompted the proposal that reperfusion resulted in an excessive influx of calcium and turned attention towards calcium movements during the course of the calcium paradox.

B. Intracellular Calcium Overload and Deficiency

Intracellular calcium deficiency and overload are believed to be intimately related to the contractile failure and cardiac cell death seen in several pathological conditions (4,7-11,24,27,32). In 1975 Yates and Dhalla (3) suggested that the isolated heart perfused with Ca^{2+}-free medium forms an interesting model for studying the pathogenesis of intracellular calcium deficiency while reperfusion of calcium deprived hearts supplies a model in which to study intracellular calcium overload. This initial hypothesis was substantiated when it was demonstrated (6) that indeed Ca^{2+}-free perfusion depletes myocardial Ca^{2+} contents while reperfusion results in an intracellular calcium overloading. The questions then are from what locations is calcium lost during the period of Ca^{2+}-free perfusion and from where does the excess calcium originate and enter the myocardial cell at the onset of reperfusion? While both issues will be dealt with in subsequent sections the later warrents brief mention here in light of a recent review by Grinwald and Nayler (33). They concluded that there is still no certainty regarding the routes of Ca^{2+} entry during the Ca^{2+} paradox nor is the etiology of the reperfusion-induced damage

which follows the massive and uncontrollable entry of Ca^{2+}, clear at present; an important point when one chooses to assess the relevence of the present investigation.

C. Time Requirements and Specificity

(i) Ca^{2+}-free perfusion

Upon introduction of Ca^{2+}-free medium to the isolated perfused rat heart, contractile force falls rapidly such that the eventual cessation of mechanical activity occurs within the first 30 seconds (1,3,4,34,35). This is in contrast to a slower loss of contractile force in cat and rabbit preparations (5,36-40). This loss of contractile function has been shown to occur in the absence of any alteration in surface electrical activity of the heart (1,3,18,41). On the basis of ion fluxes (38,42-44) the loss in contractility can be conceived to be due to decreased calcium entry during excitation of the myocardium which in turn may uncouple excitation from contraction (44-51) as well as alter intracellular Ca^{2+} transport (12,13,52,53).

The dissociation of electrical and mechanical events has also been suggested to be due to the removal of a labile calcium component within the first 30 seconds of Ca^{2+}-free perfusion (19). A decline in tissue and mitochondrial stores of approximately 32% of total Ca^{2+}, appeared to represent the compartment associated with the decrease in contractile force. During the loss of this calcium no structural alterations were apparent. A second more stable compartment, representing 20% of myocardial calcium, was depleted only after 10 minutes of Ca^{2+}-free exposure. In their study (19) this point marked the first sign of ultrastructural damage but as described earlier, ultrastructural alterations have been observed as early as 5 minutes of

Ca^{2+}-free perfusion (15,25) when myocardial Ca^{2+} contents are known to be significantly reduced (6). It should be pointed out that while morphological alterations, especially in sarcolemma, have not been observed when the period of Ca^{2+} deprivation is less than 5 minutes, the data should not be taken to mean that brief periods of calcium-free perfusion are without effect on the myocardium. While some investigators have found no change (18,41), others have pointed to enhanced sarcolemmal permeability to calcium and other cations like K^{+}, after a short period of Ca^{2+}-free perfusion (5,6,40,54). It is possible that Ca^{2+} is removed from the membrane itself during Ca^{2+}-free perfusion which may render intramembranous proteins more labile (55) and so alter membrane function with respect to calcium movements, permeability in general and various membrane bound enzymatic activities. Calcium lack has been shown to change the arrangement of cardiolipin molecules in membranes (54). This is consistent with the view that calcium plays an essential role in membrane integrity (20,54,56,57).

(ii) Reperfusion: the calcium paradox

The time-course of the calcium paradox and effects of reperfusion with Ca^{2+} are not anywhere near as subtle nor controversial as those parameters observed during Ca^{2+}-free perfusion per se. In order for reperfusion to induce the calcium paradox phenomenon, the Ca^{2+}-free period must last anywhere from 2-40 minutes depending on the species and tissue being employed. In isolated perfused rat hearts reperfusion with Ca^{2+} after 2-5 minutes of Ca^{2+}-free exposure results in paradoxical Ca^{2+} necrosis and intracellular Ca^{2+} overload (1,3,6,58,59). Only in one case (60) has the calcium paradox

not been observed in rat heart and the most likely explanation was that Ca^{2+} contamination complicated the Ca^{2+}-free period (61). A similar time course has been reported in rat heart coronary artery smooth muscle (2). Longer periods (10-20 minutes) of Ca^{2+}-free perfusion are required to demonstrate the calcium paradox in rabbit intraventricular septum (5,62) or in rabbit hearts (36). Zimmerman et al (2) were able to demonstrate reperfusion-induced changes in the dog heart deprived of Ca^{2+} for 40 minutes in situ. In a recent report (63) cultured fetal mouse myocardial cells incubated in a Ca^{2+}-free environment for 10 minutes, developed marked morphological alterations and contracture within 1 minute of the reintroduction of calcium. That the calcium paradox is not strictly confined to contractile or electrically active tissue, was demonstrated by Nozick et al (64) when reperfusion produced calcium overload, intracellular enzyme release and ultrastructural damage in kidney parenchyma and vasculature after 20 minutes of Ca^{2+}-free perfusion.

From the foregoing discussion it is obvious that the calcium paradox has very strict time requirements. The extent of changes in ultrastructure (2,3), myocardial calcium contents (1,65), mechanical activity (3,6) and enzyme release (1,21,65) are all heavily dependent upon the durations of Ca^{2+}-free perfusion and reperfusion. In rat hearts reperfusion with Ca^{2+} after less than 2 minutes of calcium free perfusion results in complete recovery in contractile force and the maintenance of normal cellular morphology and composition. With longer periods of Ca^{2+}-free exposure, reperfusion induces progressive alteration in the above parameters as well as significant decreases in myocardial Ca^{2+} contents and ultrastructural alterations due to

Ca^{2+}-free perfusion alone. After 4-5 minutes of Ca^{2+}-free perfusion, 1 minute of reperfusion is sufficient to demonstrate all the characteristics of the calcium paradox. However peak levels of protein release are achieved only after 3 min of reperfusion (65) and the extent of calcium overload plateaus at 10 min of reperfusion (1). Ultrastructural derrangements also become more pronounced as the duration of reperfusion lengthens. The degree of contracture on the other hand, reaches a maximum within 2 minutes after which time a slow fall occurs (3). The explanation for this latter observation is assummed to reside in the extensive structural damage which results in cell-to-cell separation thus making it impossible for the myocardium to maintain the tension generated by the hypercontracted state.

D. Modifiers of the Calcium Paradox

In the previous section those conditions required to demonstrate the full expression of the calcium paradox were outlined. There are however, a wide variety of factors other than time which can prevent, blunt or augment the effects of reperfusion and in so doing they have enhanced our understanding of the mechanisms underlying the genesis of the calcium paradox.

(i) Alterations in the ionic environment

When "trace" amounts of calcium were allowed to contaminate the 'Ca^{2+}-free' buffer, Ruigrok et al (61) demonstrated complete recovery in contractile force upon reperfusion after 4 minutes of Ca^{2+}-free perfusion; conditions usually sufficient to induce the calcium paradox in rat heart. Two subsequent studies have established the critical Ca^{2+} concentration necessary to prevent the calcium paradox.

Crevey et al (5) demonstrated that the separation of membrane superficial laminae after 20 min of 0 Ca^{2+} perfusion of the rabbit intraventricular septum could be prevented if 50 μM Ca^{2+} (inadequate to maintain contractility) contaminated the Ca^{2+}-free medium. Furthermore, the reintroduction of Ca^{2+} resulted in a complete return of normal contractile activity. A similar observation has been made by Alto and Dhalla (6) in isolated perfused rat hearts. Preperfusion with 50 μM Ca^{2+} resulted in only minor Ca^{2+} overloading and 62% contractile force recovery upon reperfusion with 1.25 mM Ca^{2+}. Less protection was afforded by 25 μM Ca^{2+} (47% recovery) but 0.1 mM Ca^{2+} completely prevented the calcium paradox. The results were taken to suggest that trace amounts of Ca^{2+} are necessary to maintain the permeability characteristics of the lipid bilayer and glycocalyx.

The magnitude of reperfusion effects is also dependent upon the amount of Ca^{2+} present. After 5 minutes of Ca^{2+} depletion, at least 0.1 mM Ca^{2+} in the reperfusion buffer is necessary before significant contracture, calcium overload and ultrastructural damage can be demonstrated in reperfused hearts (6,14,66). Increasing the reperfusion Ca^{2+} concentration enhances the above changes suggesting that after Ca^{2+}-free perfusion has disrupted sarcolemmal permeability, the reperfusion effects may depend upon concentration gradient dependent passive Ca^{2+} influx and the magnitude of the resultant intracellular calcium overload. These observations are supported by similar results obtained using cultured fetal mouse myocardial cells (63).

Changing the Na^{+} concentration in the Ca^{2+}-free and reperfusion media also has profound effects on the calcium paradox. Low (35 mM) Na^{+} during Ca^{2+}-free perfusion prevents myocardial Ca^{2+}

depletion and the effects of reperfusion (3,6). It is possible that low extracellular Na^+ by virtue of the Na-Ca exchange mechanism (67,68), may prevent or delay the loss of membrane and/or cellular Ca^{2+} thereby preserving membrane integrity and sarcolemmal Ca^{2+} essential for cardiac contractility (69). Low (35 mM) Na^+ during reperfusion has the opposite effect; reperfusion induced changes are augmented (6). This may be due to the fact that Na^+ is believed to compete with Ca^{2+} for entry through the electrophysiological slow channels (68,70) thereby allowing for enhanced Ca^{2+} influx during reperfusion. On the other hand, reduced extracellular Na^+ in the presence of normal levels of Ca^{2+} during reperfusion, may favor the influx of Ca^{2+} by the Na-Ca exchange mechanisms strictly on a competitive basis. However, intracellular levels of Na^+ and Ca^{2+} both increase during the reperfusion phase. In their recent review (33), Grinwald and Nayler suggested that the increase in intracellular Na^+ during reperfusion may facilitate Ca^{2+} influx via Na-Ca exchange. This may explain why during low Na^+ reperfusion the development of intracellular Ca^{2+} overload preceeds that of Na^+ overload (6). A role for the Na-Ca exchange process in the genesis of intracellular calcium overload and paradoxical calcium necrosis has also been implicated by Goshima et al (63).

The presence of other divalent cations (Sr^{2+}, Ba^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}) during the various perfusion periods also modifies the typical appearance of the calcium paradox. When Sr^{2+} was substituted for Ca^{2+} in the reperfusion medium after 12 minutes of Ca^{2+}-free perfusion of the isolated rabbit heart, Lee and Visscher (37) found a maintenance of assystole with the development of minimal

contracture. However the release of intracellular high energy phosphate compounds, creatine phosphate (CrP), increased to the same extent as with normal Ca^{2+} reperfusion but the remaining intracellular CrP was larger with Sr^{2+} than with Ca^{2+} in the perfusate. They concluded that while Sr^{2+} could substitute for Ca^{2+} with respect to the reperfusion-inducted permeability changes in sarcolemma, it could not replace the reperfusion-induced Ca^{2+} mediated contracture and activation of energy consuming reactions. The addition of various divalent cations to the Ca^{2+}-free medium has yielded some even more puzzling observations, especially with respect to Ba^{2+}. Distortion of the glycocalyx during Ca^{2+}-free perfusion (15,19-22,25) is considered the morphological representation of altered sarcolemmal Ca^{2+} permeability. Nayler and Grinwald (71) found an intact glycocalyx after 10 minutes of Ca^{2+}-free perfusion of rat heart in the presence of 1.3 mM Ba^{2+}. Reperfusion with Ca^{2+}-containing Ba^{2+}-free medium resulted in calcium overload and decreased tissue stores of ATP but the release of intracellular protein was markedly reduced. The fact that sarcolemmal ultrastructure was preserved but massive Ca^{2+} influx still occurred casts doubts over earlier conclusions (5,20,72). It should be noted however that the Ba^{2+} containing Ca^{2+}-free perfusion medium induced a marked contracture and depletion of high energy phosphate stores on its own. Whether these uncharacteristic effects of Ca^{2+}-free perfusion have in some way altered the basic features of the calcium paradox thus making comparisons irrelevent, is unclear at present.

Two recent reports (25,62) have also analyzed the effects of adding divalent cations, known to be partially effective in substituting

for Ca^{2+} but uncoupling excitation from contraction (73), to the Ca^{2+}-free buffer. The presence of Cd^{2+} (50 μM) during the Ca^{2+}-free period was found to protect the glycocalyx in rabbit intraventricular septum and partially protect this preparation from the manifestations of the calcium paradox; 50 μM Ca^{2+} has been shown previously to be completely protective (5). Similar concentrations of Mn^{2+}, Co^{2+} and Mg^{2+} were also partially protective but not as effective as Cd^{2+} or Ca^{2+} itself. The success of each intervention was related to the ability to prevent peeling of the glycocalyx suggesting again a place for basement membrane alterations in explaining the changes in Ca^{2+} permeability. That the different conclusions arrived at by Nayler and Grinwald (71) and Rich and Langer (62) can be explained on the basis of species differences is speculative at best. While rat heart is unquestionably more sensitive to the calcium paradox than rabbit heart (74), more research into the mechanism of increased sarcolemmal permeability will be necessary in order to unravel the mystery.

(ii) Hypothermia

Holland and Olson (4) were the first to demonstrate prevention by hypothermia of paradoxical calcium necrosis in cardiac muscle. Since then (1975) several reports have appeared in the literature (6,21,25,62,76-78) which substantiate and expand these initial observations. When isolated rat hearts were perfused for greater than 3 and up to 30 minutes with Ca^{2+}-free medium cooled to 4°C, reperfusion with Ca^{2+} containing medium and rewarming to 37°C permitted rapid recovery in cardiac function with preservation of normal cardiac ultrastructure and intracellular protein contents (4). They proposed

that during Ca^{2+}-free perfusion at 37°C, sarcolemmal proteins concerned with the "calcium channel" become unstable and are either lost from the membrane or become irreversibly nonfunctional. Hypothermia on the other hand, was expected to slow the rate of change in membrane proteins because of the reduced thermal activity and more viscous nature of the phospholipid bilayer, due to phase transitions (79), at 4°C. Hearse et al (21) have analyzed the effects of hypothermia during both the Ca^{2+}-free and reperfusion periods in isolated rat hearts. They were able to reproduce the results (protein release) of Holland and Olson (4) using Ca^{2+}-free perfusion at 4°C but noted that similar protection was afforded if the Ca^{2+}-free medium was perfused at 30°C. Bulkley et al (76) and Digerness et al (78) have also found 30°C to be inhibitory to the genesis of paradoxical calcium necrosis, high energy phosphate depletion and enzyme release upon reperfusion with Ca^{2+}, respectively . Any further increase in the temperature of the Ca^{2+}-free medium resulted in reperfusion induces enzyme release with a sharp threshold between 34-36°C (21,76). With respect to varying the temperature of the reperfusion solution, the results of Hearse et al (21) and Boink et al (77) revealed that hypothermia during calcium repletion is essentially unable to abolish the calcium paradox, even at 4°C. This supported the hypothesis that critical changes which predispose to the calcium paradox, occur during the Ca^{2+}-free period.

Hypothermic (21°C) Ca^{2+}-free perfusion has also been shown to prevent the depletion of myocardial Ca^{2+} which is characteristic of Ca^{2+}-free exposure at 37°C (6). Therefore, the ability of hypothermia to maintain sarcolemmal permeability characteristics may

be related to delayed depletion of sarcolemmal Ca^{2+} with resultant preservation of sarcolemmal ultrastructure since the connections between the external lamina and surface coat of the cell membrane are ruptured by Ca^{2+} removal (20). The ability of hypothermia to prevent changes in sarcolemmal ultrastructure due to Ca^{2+}-free perfusion, has been analyzed recently (25,62). These authors have demonstrated that the protective effect of hypothermia in the Ca^{2+}-free perfused rabbit septum, is closely related to the protection it affords to the glycocalyx and to a lesser extent, to intramembranous particles in sarcolemma.

(iii) pH

Decreasing the pH of the Ca^{2+}-free medium to 6.8, prolongs the duration of Ca^{2+}-free exposure necessary to evoke the calcium paradox upon reperfusion (80). Increasing the pH to 8.0 has the reverse effect (80). Bielecki concluded that an increased extracellular and intracellular hydrogen ion concentration hampers the massive calcium influx as is the case in other preparations (81,82). It is not clear however, if the altered pH was extended into the reperfusion phase (80) when the development of calcium overload occurs. Finally, whether decreased pH during the Ca^{2+}-free period prevents the loss of myocardial Ca^{2+} or preserves sarcolemmal ultrastructure and permeability, is also unknown at present.

(iv) Drug effects

Kramer et al (83) investigated the effects of 10 mM taurine on the genesis of the calcium paradox in isolated rat hearts. The presence of taurine throughout the Ca^{2+}-free and reperfusion phases retarded the release of creatine kinase from reperfused hearts and

improved contractile force recovery. Since taurine has been shown to interact with specific sarcolemmal membrane proteins (84) and exhibit membrane stablizing activity (85), these authors speculated that taurine may enhance sarcolemmal Ca^{2+} binding and so inhibit the effects of Ca^{2+}-free perfusion (ie Ca^{2+} depletion) on sarcolemmal structure. While these results are intriging, a detailed analysis of the perfusion protocols raises concern about the appropriateness of the calcium paradox model employed and so therefore, the validity of the observations. Under 'control' conditions, that is induction of the calcium paradox in the absence of drug, 10-11 minutes of Ca^{2+}-free perfusion was required before complete failure in contractile force recovery was apparent upon reperfusion. In comparison to other studies (1,4,6,35,65,80) on isolated perfused rat hearts, this duration of Ca^{2+}-free perfusion is at least two times longer than that necessary to induce the calcium paradox. The possibility that some other factor may be modifying the occurrence of the calcium paradox cannot be overlooked.

Reperfusion-induced damage can be reduced when dimethylsulfoxide (DMSO) is present in the reperfusion medium (59) but mechanical arrest is maintained. The presence of DMSO during the Ca^{2+}-free period has no such protective effect against the full expression of the calcium paradox. While the mechanism of action is essentially unknown, Ruigrok et al (59) have postulated a feasible explanation. Reperfusion is known to result in decreased sarcoplasmic reticular Ca^{2+}-accumulating activity (34). DMSO has been shown to stimulate sarcoplasmic reticular Ca^{2+} uptake (86). They suggested that DMSO may counteract the effect of reperfusion on the

sarcoplasmic reticulum. This would reduce the level of free intracellular Ca^{2+} which is believed to be wholly or partially responsible for the reperfusion induced ultrastructural damage (6,8,11,32,33).

Prevention of calcium paradox-related myocardial cell injury with diltiazem, a calcium channel blocker, has also been reported in the literature. Ashraf et al (87) have suggested that diltiazem exerts its protective effect by preventing cell separation and alterations in the gap junctions during Ca^{2+}-deprivation as well as by limiting Ca^{2+} entry when Ca^{2+} is reintroduced into the perfusion buffer. The effect of verapamil on the calcium paradox has caused heated debate in the literature since it was first reported (6) that 0.1 mg/ℓ verapamil in the reperfusion medium had no protective effect on the occurrence of the calcium paradox, after 5 minutes of Ca^{2+}-free perfusion. This was in spite of the fact that this concentration is sufficient to block slow channel transport in normal heart muscle (88) and slow channels maintain a normal slow Ca^{2+} current in cells exposed to Ca^{2+}-free solutions (89). Alto and Dhalla (6) suggested that, because the development of intracellular calcium overload was influenced by the extracellular Na^{+} concentration during reperfusion, the control of the calcium channels was lost during Ca^{2+}-free perfusion rather than the channels per se as hypothesized previously (4). The results with verapamil however, can be used to support either hypothesis or indeed the slow channels may have little or no significance with respect to the final outcome of calcium paradox (33).

Subsequent studies have both demonstrated (58,90,91) and

disregarded (65,92) the protective effect of verapamil. In an attempt to reconcile these differences we would like to draw attention to three recent articles (33,93,94). In summary, regardless of the concentration of verapamil employed, the eventual manifestations of reperfusion following several minutes of Ca^{2+}-free exposure cannot be prevented. The immediate events of reperfusion (within 1-2 minutes) especially massive calcium influx, may be partially blunted by calcium blockers indicating that one of the routes of calcium entry may involve the slow channels. However, as the duration of reperfusion is prolonged the damage induced by the reintroduction of Ca^{2+} is so pronounced that uncontrollable calcium influx is inevitable. This results in an intracellular calcium overload which in turn likely leads to further activation of various mechanisms deleterious to the structure and function of the myocardial cell.

E. Energy Metabolism in the Calcium Paradox

The postulate that lesions in those processes concerned with energy production and utilization are closely related to the pathogenesis of heart failure, has prompted much research into the field of myocardial metabolism (95,96,97). Dhalla et al (98) have shown that calcium plays a critical role in the regulation of myocardial metabolism. In so much as the calcim paradox phenomenon is associated with intracellular calcium changes and major ultrastructural derrangements, it is conceivable that the reperfusion-induced effects discussed thus far may in some fashion relate to alterations in energy metabolism. Indeed another characteristic feature of the calcium paradox, though not expressly described in the initial observations

of Zimmerman and colleagues (1,2), is the change in myocardial high energy phosphate compounds which occurs during the calcium-free and reperfusion periods. In earlier sections we have alluded to the fact that the development of intracellular calcium overload is associated with an almost immediate depletion in high energy phosphate stores while Ca^{2+}-free perfusion per se, results in little or no change in the intracellular levels of CrP or adenosine triphosphate (ATP). These alterations in intracellular high energy phosphates during the calcium paradox were first described by Lee and Vischer (37) and then more thoroughly by Boink et al (35). Since then many investigators have found similar results in a variety of preparations subjected to the calcium paradox (21,71,76,87,90,99). The preservation of energy rich compounds during Ca^{2+}-free perfusion most likely relates to mechanical quiescence. It is generally accepted that perfusion-induced depression in CrP and ATP is due to the turning on of various energy consuming reactions by excess intracellular calcium, within the first minute of reperfusion (35,99), after which time a further decline in high energy phosphate compounds is accounted for by continuing hydrolysis, release of CrP and ATP into the effluent (1,35) and inhibition of mitochondrial oxidative phosphorylation. This latter situation can be conceived to result from excessive, energy-dependent calcium uptake by mitochondria which occurs in preference to the phosphorylation of ADP (100). Support for this comes from earlier reports in which excessive mitochondrial Ca^{2+} accumulation was first suggested (2) and then demonstrated (34) to occur during the calcium paradox as well as a recent report in which mitochondria, isolated from reperfused hearts, were found to have defective oxidative

phosphorylation activity (99). Similar conclusions regarding the roles of Ca^{2+}, mitochondria and high energy phosphate metabolism in the pathogenesis of various forms of heart failure and damage, have been made by other investigators (7,29,31,32,75,101,102,103).

While the effects of reperfusion and intracellular calcium overload on energy metabolism are relatively straight forward, the observation that the calcium paradox is energy-dependent (104,105) has undoubtedly complicated our understanding of the mechanisms leading to calcium overload and paradoxical calcium necrosis. Ruigrok and his colleagues (104,105) demonstrated that the calcium paradox as documented by CPK release and ultrastructural changes, could not be produced in hearts previously depleted of ATP and lacking electron transport suggesting that mitochondria play an important role in the origin of the calcium paradox. They emphasized that while high energy phosphate depletion may be a crucial reaction in the genesis of cardiac necrosis caused by intracellular calcium overload, the decline in CrP and ATP are a result and not the immediate cause of the calcium paradox. It is also worth noting that both ATP hydrolysis and mitochondrial calcium accumulation can generate hydrogen ions but a role for cytoplasmic acidification in myocardial cell necrosis is still controversial (33,35).

F. Cardiac Myocytes

The isolation of intact, beating and viable cardiac myocytes has been plagued with difficulties recently recognized to be related to the calcium paradox phenomenon. The basic isolation procedure involved perfusing hearts with Ca^{2+}-free medium since it was recognized that this protocol would effect separation of cell to cell

connections (3). This would be followed by perfusion with enzymes like trypsin, collagenase and hyaluronidase to further weaken the extracellular lattice work so that agitation and other similar mechanical processes could yield preparations of individual rod-shaped cells. Unfortunately, upon exposure to solutions containing physiological levels of calcium, many cells would round up and exhibit abnormal metabolism and permeability characteristics either immediately or within 1 hour thus making their usefulness rather limited (106-111). In one of these preparations (110) however, somewhat better calcium tolerance was achieved, probably due to a small (25 μM) amount of Ca^{2+} present during some of the Ca^{2+}-free perfusions. The mechanism of this protection is best explained on the basis of results in the calcium paradox (5,6) where trace amounts of Ca^{2+} have been found to prevent reperfusion induced damage in whole tissue preparations.

Since 1976 several approaches have been utilized in an attempt to improve the quality of the isolated myocyte preparation. Clark et al (112) incorporated DMSO and taurine into one of their Ca^{2+}-free media and found improved Ca^{2+} tolerance in their final cell suspension. Both taurine (83) and DMSO (59) have some protective effects against the calcium paradox, as described earlier in this report. Powell (113) has suggested that using hyaluronidase, which can alter the glycocalyx, focused on by so many investigators of the calcium paradox, may lead to Ca^{2+} intolerance, so he has excluded it from his procedure with positive results. Altschuld et al (114) have also reported the isolation of viable myocytes and they document the presence of at least 25 μM Ca^{2+} throughout their procedure.

Similar success was achieved by Kao et al (115) by employing both taurine and 50 μM Ca^{2+} in their perfusion medium and there is also reason to believe that the presence of small amounts of Ca^{2+} partially account for the calcium tolerance of myocytes isolated according to Wittenberg and Robinson (116) and Montini et al (117). In an interesting report, especially with regards to understanding the calcium paradox, Haworth et al (118) have described a successful mycocyte isolation procedure which employs trypsin in the final incubation and separation step. They suggest that trypsin cleans out the extraneous membrane shreds, which keep susceptible gap junctions (intercalated disc regions) open, thus allowing these potential Ca^{2+} channels to close. In so doing the reintroduction of calcium does not result in calcium overload and contracture. Taking this postulate further, they hypothesized that the site of massive calcium influx in the calcium paradox may be the intercalated disc and not related to separation of the basement membrane as suggested by others (5,20,21).

It is thus obvious that past and future research on isolated cardiac myocytes and the calcium paradox phenomenon have and will help in improving the former and understanding the later.

G. Cardioplegia

In their initial study, Zimmerman et al (2) raised concern over the very low calcium ion concentrations used in perfusion media during cardiac bypass. Others (119,120) have also suggested that the use of Ca^{2+}-free solutions during open heart surgery may be hazardous by making the heart susceptible to the calcium paradox. While

it is beyond the scope of this report to eyaluate all the cardioplegic literature to date, it is worth noting that some clinically used cardioplegic solutions are acalcemic (121,122) while others have found success by combining the protective effects of chemical cardioplegia (in the presence of Ca^{2+}) with hypothermia (123,124).

H. Current Status

In conclusion, reperfusion of Ca^{2+}-deprived hearts results in altered myocardial cation contents notably intracellular calcium overload, massive enzyme and protein release, irreversible loss of active force generation and electrical activity, myocardial contracture, rapid depletion of high energy phosphate stores and extensive ultrastructural damage. These changes are intimately related to defective calcium regulation by the heart, culminating in calcium overloading. Alterations in sarcolemmal Ca^{2+} permeability and enzymes function (99) as well as derrangements in subcellular Ca^{2+} regulation (34), are all apparent during the calcium paradox but the relationship between impaired membrane function and the manifestations of the calcium paradox, especially with respect to mechanical performance, is far from clear. In view of the lack of information in this regard, the present study was undertaken in an attempt to define more completely the roles played by perturbations to sarcolemma and sarcoplasmic reticulum in the manifestations of the calcium paradox phenomenon.

III. METHODS

A. Isolated Heart Preparation

Healthy male Sprague Dawley rats, weighing 300 - 400 grams, were used in the present study. All the animals were kept in environmentally controlled rooms, maintained on standard rat chow ad libitum and given free access to water. The animals were sacrificed by decapitation and their hearts excised and washed in ice-cold, oxygenated Krebs Henseleit (K-H) solution. After trimming of the atria, extraneous fat and connective tissue, the ventricles were arranged for coronary perfusion according to the procedure of Langendorff as described previously (6,14,66,125). Equilibration perfusion was carried out for 15 min with K-H medium containing (mM); NaCl, 120; $NaHCO_3$,25; KCl,4.8; KH_2PO_4,1.2; $MgSO_4$ 1.20; $CaCl_2$,1.25; and glucose, 8.6 (pH 7.4). This solution was continually gassed with 95% O_2 and 5% CO_2 and maintained at 37°C with the exception of the hypothermia experiments. The osmolarity of altered Ca^{2+} solutions or altered Na^+ solutions was adjusted to normal values with appropriate amounts of sucrose. Each preparation was electrically paced at 280 beats/minute with a Phipps and Bird 611 square wave stimulator (event duration: 1.5m sec, amplitude: 60 volts). The coronary perfusion rate was 7.8 ml/min. At the onset of each experiment a resting tension of 2 grams was applied to the heart and contractile force (developed tension) monitored on a Gilson polygraph recorder with a force displacement transducer (FT.03).

B. Isolation of Sarcoplasmic Reticulum (Microsomal Fraction)

At the conclusion of each perfusion sequence the heart was removed from the cannula and placed in a cold 0.25 M sucrose, 1mM EDTA;

20 mM Tris-HCl solution, pH 7.4. Cardiac sarcoplasmic reticulum (microsomal fraction) was isolated by differential centrifugation using the method of Harigaya and Schwartz (126) as modified by our laboratory (66,127). Briefly, after a thorough washing in the 0.25 M sucrose solution the ventricles were minced crudely with scissors and homogenized in 10 volumes (v/w) of 10 mM sodium bicarbonate, 5 mM sodium azide, 15 mM Tris-Hcl, pH 6.8 with a Waring blender for 45 sec at rheostat #5. The homogenate was filtered through 4 layers of gauze and centrifuged at 10,000 x g for 20 min. The supernatant was carefully decanted off and spun for an additional 45 min at 40,000 x g. The resultant pellet was then resuspended in 10 volumes of 0.6 M KCl, 20 Tris-HCl, pH 6.8 and again centrifuged for 45 min at 40,000 x g. This step was repeated twice and the final pellet was suspended in 0.25 M sucrose, 20 mM Tris-HCl, pH 6.8. The protein content was determined by the assay of Lowry et al. (128). Integrity and purity of the microsomal fractions were checked routinely according to criteria described in earlier reports (34,127,129). Endogenous calcium content of the microsomal fraction was determined by atomic absorption (127).

C. Determination of Microsomal Ca^{2+} Uptake and Binding, and ATPase Activities

Calcium uptake (accumulation in the presence of 5.0 mM potassium oxalate at 37°C) and calcium binding (accumulation at 25°C in the absence of a permeant anion) activities were measured using the Millipore filtration technique (127). Briefly, microsomal membranes (0.03-0.06 mg/ml) were preincubated for 3 min in 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP and 20 mM Tris-Hcl, pH 6.8. The reaction was initiated by the

addition of 0.1 mM $^{45}CaCl_2$ and terminated at the appropriate time by filtration.

Total (0.1 mM $CaCl_2$, 10 mM $MgCl_2$), basal (0.5 mM EGTA, 10 mM $MgCl_2$) and Ca^{2+}-stimulated (total-basal) ATPase activities were determined in a similar incubation medium as that used for the uptake studies except that the reaction was initiated by the addition of 4 mM ATP. Inorganic phosphate liberated into the protein-free filtrate was assayed by the procedure of Taussky and Shorr (130). Ouabain-sensitive Na^+/K^+ ATPase activity in the presence of 1 mM ouabain was determined according to the method described earlier (129).

D. Isolation of Sarcolemma

The sarcolemmal fraction was isolated by the hypotonic shock-LiBr treatment technique as basically described by McNamara et al (131,132) but with a further extraction step using Kcl added to the procedure (129). Briefly, at the termination of each perfusion sequence the heart was removed from the cannula and placed in ice cold 10 mM Tris-HCl, 1 mM ethylene-diaminetetracetate (EDTA), pH 7.4. The ventricles were minced with scissors and then homogenized in a Waring blender for 2 x 30 seconds at rheostat #5. The resultant suspension was then filtered through 4 layers of gauze and centrifuged for 10 min at 1,000 x g. After discarding the supernatant the pellet was resuspended in 10 volumes (v/w) of a washing medium. A sequence of washing media were employed (10 mM Tris-HCl, 1 mM EDTA, pH 7.4; 10 mM Tris-HCl, pH 7.4; 10 mM Tris-HCl, pH 8.0; 10 mM Tris-HCl, pH 7.4; 10 mM Tris-HCl, 0.4 mM LiBr, 0.4 mM EDTA, pH 7.4; 10 mM Tris-HCl, pH 7.4; 0.6 M KCl, 10 mM Tris-HCl, pH 7.4; 10 mM Tris-HCl, pH 7.4; 10 mM Tris-HCl, 1mM EDTA, pH 7.4) with each suspension stirred for 15 min (except

the LiBr step where extraction was carried out for 30 min) followed by centrifugation at 1,000 x g for 10 min and resuspension in the next medium. The final pellet was then suspended in the appropriate volume of 1mM Tris-HCl, pH 7.0 and a protein determination carried out (128). Earlier reports (133-137) from our laboratory have characterized this preparation and demonstrated minimal (2-4%) contamination with other subcellular organelles. Endogenous calcium present in our sarcolemmal fraction was determined using atomic absorption (127).

E. Determination of Sarcolemmal Enzyme Activities

(i) Adenosine triphosphatases (ATPases)

For all ATPase determinations the final volume of the appropriate incubation medium was 1 ml at 37°C, with 50 mM Tris-HCl, pH 7.4 and the final protein concentration ranged from 0.05 to 0.07 mg/ml. After 3 min pre-incubation of membrane protein the enzymatic reaction was initiated by the addition of 4 mM ATP (except during those experiments designed to determine K_m where different concentrations of ATP were added) and terminated 10 min later by the addition of 1 ml ice cold 12% trichloracetic acid. The tubes were immediately placed in ice and shortly thereafter centrifuged at 1,000 x g for 10 min. These procedures have been reported earlier (133-135). The concentration of inorganic phosphate in the protein-free supernatant was assayed by the method of Taussky and Shorr (130).

Non-specific ATPase activity was reported as that amount of ATP hydrolysis occurring in the presence of protein and 1 mM EDTA without any added cation. Hydrolysis in the presence of 4 mM $MgCl_2$ minus non-specific ATPase activity represents Mg^{2+} ATPase while that occurring in the presence of only 4 mM $CaCl_2$ minus non-specific hydrolysis was

termed Ca^{2+} ATPase. Total ATPase activity was assayed in the presence of 4 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl. Na^+/K^+ ATPase activity was then calculated by subtracting Mg^{2+} ATPase activity from total ATPase activity. Ouabain sensitive ATPase activity was taken as the Na^+/K^+ ATPase activity inhibited by 2 mM ouabain.

(ii) Adenylate cyclase

Adenylate cyclase activity was assayed using the method of Drummond and Duncan (138) essentially. Membrane protein (50-60 µg) was incubated in a total volume of 0.15 ml containing 50 mM Tris-maleate, pH 8.5, 8 mM caffeine, 2 mM cyclic AMP, 5 mM KCl, 20 mM phosphoenol pyruvate, 15 mM $MgCl_2$, 130 µg/ml pyruvate kinase and 0.4 mM C^{14}-ATP at 37°C. The reaction was terminated by placing the tubes in boiling water for 3 min after the addition of 25 µl 15 mM cAMP. The tubes were centrifuged at 1,000 x g for 10 min and 100 µl of supernatant spotted on chromatographic paper. Descending chromatography (solvent 30:70 of 1 M ammonium acetate and 95% ethyl alcohol) was performed and after 18 hrs the papers were dried and cAMP spots visualized under UV light. These areas were cut out and the C^{14} measured in a Beckman liquid scintillation counter.

F. Electron Microscopic Analysis of Isolated Membranes

Electron microscopic (Zeiss EM 95) examinations of control, Ca^{2+}-free perfused and reperfused heart microsomes and sarcolemma were accomplished by fixing the final pellets in 1% of glutaraldehyde. Further fixation and processing ensued for routine ultrastructural studies (3,14,19).

G. Determination of Membrane Protein Constituents

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

was performed on isolated microsomal and sarcolemmal membranes as per the method of Weber and Osborne (139). Membrane proteins were precipated by 5% TCA, centrifuged and dissolved in 0.1 M sodium phosphate buffer containing 1% SDS, 1% β-mercaptoethanol, 0.015% bromphenol blue and 35% urea. The gel buffer employed contained 0.78% NaH_2PO_4-H_2O , 3.8% Na_2HPO_4-7 H_2O and 0.2% SDS. The monomer solution was 16.7% acrylamide, 0.48% methylenebis acrylamide, while fresh ammonium persulphate was prepared by adding 150 mg to 15 mls H_2O. The gels were made by adding 45 λ of N,N,N',N'-tetramethylenediamine (a crosslinker) to a mixture of 15 mls gel buffer and 13.5 mls monomer solution and quickly pipetting this into the electrophoretic tubes. Polymerization was complete in 2 hrs. The tubes were then placed in the electrophoretic apparatus and protein samples applied to the top of the gels. Electrophoresis was run at 50 mamps/6 tubes using a Buchler Polyanalyst with a buffer reservoir containing twice diluted gel buffer. Fixation was achieved by placing the gels in 40% (V/V) methanol, 7% glacial acetic acid overnight. Gels were then stained for 2 hrs with 0.25 (V/V) coomassie brilliant blue. The destaining solution contained 5% (V/V) methanol and 7.5% (V/V) glacial acetic acid. The resulting bands were scanned at 550 mm in a Unicam SP 1800 ultra violet spectrophotometer. Molecular weights of the protein peaks were estimated by running known molecular weight standards under identical conditions.

H. Phospholipid Analysis of Membrane Preparations

The phospholipid composition of cardiac microsomal and sarcolemmal membranes was measured by suspending 2.0 mg of protein in a 2:1 chloroform/methanol solution for the extraction of phospholipid (140). This

suspension was left overnight at room temperature in a sealed test tube. Phase separation and removal of non-lipid contaminants was then accomplished by the addition of 0.1 N HCl followed by 1 min of stirring and low speed centrifugation. The upper phase was withdrawn and discarded and the lower phase washed by adding synthetic upper phase. Mixing and centrifugation once again followed. This washing step was repeated 3 times and the final lower phase was then mixed with 2-3 droplets of concentrated ammonium. The resultant suspension was evaporated almost to dryness under a nitrogen gas atmosphere and the remaining residue dissolved in 70 µl of chloroform:methanol:H_2O (75:25:2) and spotted on a silica gel plate (Anasil H; 250 µM) previously activated by heating at 110°C for 1 hr. Two directional thin layer chromatography was then performed using a chloroform: methanol: 7 N ammonium hydroxide (12:7:1) solvent for the first run and after drying, chloroform: methanol: glacial acetate: water (80:40:7.4:1.2) for the second, according to the procedure of Pumphrey (141). The plates were dried at room temperature, sprayed with 5% H_2SO_4 and then heated for 15' at 180°C. The spots were visualized under UV light, marked and identified according to Broekhuyse (142), removed from the plate by scrapping and digested in 0.7 ml of 70% perchloric acid at 160°C for 2 hrs. Liberated inorganic phosphate was then determined spectrophotometrically according to Bartlett (143).

I. Statistical Analysis

Statistical analysis of the data was carried out by using the Student's t-test and a P value < 0.05 was taken to reflect a significant difference. Each experimental perfusion was matched with a control heart perfused for the appropriate duration with normal Ca^{2+} containing solution.

IV. RESULTS

A. Effects on Microsomal Ca^{2+}-Uptake

In the first series of experiments an attempt was made to gain information regarding the critical Ca^{2+} concentration of the preperfusion medium for induction of the calcium paradox. The hearts were perfused for 5 min with varying concentrations of calcium followed by reperfusion for 5 min with normal (1.25 mM) Ca^{2+}-containing medium. The results with respect to changes in contractile force, resting tension and microsomal calcium uptake are given in Table 1. Preperfusion per se, had no effect ($P > 0.05$) on microsomal Ca^{2+} uptake. As long as at least 0.1 mM Ca^{2+} was present, reperfusion resulted in the complete recovery of contractile force with no accompanying change in resting tension or Ca^{2+} uptake from control values. However, hearts reperfused after perfusion with 0.05 or 0.025 mM Ca^{2+} recovered contractile force only partially, resting tension rose slightly ($P < 0.05$) and microsomal Ca^{2+} uptake was significantly reduced. A more marked reperfusion-induced increase in resting tension and decrease in Ca^{2+} uptake, along with no contractile force recovery, occurred in those hearts preperfused with Ca^{2+}-free solution for 5 min. It is thus apparent that calcium paradoxic changes begin to appear in hearts preperfused with 0.025 to 0.10 mM Ca^{2+}. Similar results have been found in earlier studies dealing with functional, ultrastructural and myocardial cation changes due to the calcium paradox (5,6).

The effects of different durations of Ca^{2+}-free perfusion on reperfusion Ca^{2+}-triggered alterations were also examined and the results are reported in Table 2. Reperfusion with normal Ca^{2+} after

TABLE 1 Effects of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused with Medium Containing Different Concentrations of Calcium for 5 Minutes

Calcium Concentration (mM)	Ca^{2+}-uptake before reperfusion (nmol Ca^{2+}/ mg protein/ 5 min)	CF after reperfusion (% of control dP)	Increase in RT after reperfusion (% of control dP)	Ca^{2+}-uptake after reperfusion (nmol Ca^{2+}/mg protein/ 5 min)
1.25 (control)	247 ± 11	100	0	256 ± 19
0.50	251 ± 6	100	0	245 ± 8
0.25	241 ± 12	100	0	238 ± 14
0.10	260 ± 10	100	0	239 ± 10
0.05	249 ± 5	63 ± 4*	5 ± 4	150 ± 14*
0.025	249 ± 9	49 ± 6*	10 ± 6*	120 ± 9*
0	251 ± 7	0*	61 ± 7*	95 ± 15*

Results are expressed as mean ± SE of 4 experiments. CF: contractile force, dP: developed tension during the equilibration period, RT: resting tension.

*Significantly different from control ($P < 0.05$).

TABLE 2 Effects of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused with Ca^{2+}-free Medium for Different Intervals

Duration of Ca^{2+}-free perfusion (min)	Ca^{2+}-uptake before reperfusion (nmol Ca^{2+}/mg protein/ 5 min)	CF after reperfusion (% of control dP)	Increase in RT after reperfusion (% of control dP)	Ca^{2+}-uptake after reperfusion (nmol Ca^{2+}/mg protein/ 5 min)
0 (control)	258 ± 11	100	0	269 ± 12
1	249 ± 5	106 ± 6	0	254 ± 13
2	250 ± 12	87 ± 6*	0	209 ± 9*
3	241 ± 15	36 ± 8*	10 ± 6	165 ± 10*
4	260 ± 13	14 ± 5*	30 ± 9*	119 ± 17*
5	255 ± 11	0*	60 ± 11*	92 ± 9*
10	247 ± 16	0*	66 ± 10*	81 ± 6*

Results are expressed as mean ± SE of 5 experiments. CF: contractile force, dP: developed tension during the equilibration period, RT: resting tension.

* Significantly different from control ($P < 0.05$).

1 min of Ca^{2+}-free exposure was associated with a complete return of control contractile force generation with no ($P > 0.05$) alteration in resting tension or microsomal Ca^{2+} transport. Longer periods (2-4 min) of Ca^{2+}-free perfusion were associated with only partial contractile force recovery, increased resting tension and decreased Ca^{2+} uptake activity, when Ca^{2+} was reintroduced. Reperfusion-induced changes in resting tension and microsomal Ca^{2+} uptake were enhanced in hearts perfused with 0 Ca^{2+} for 5 or more min; under these conditions there was no recovery in contractile force. It appeared that the magnitude of change imposed upon cardiac contractile parameters and microsomal Ca^{2+} transport due to reperfusion, was positively related to the duration of Ca^{2+}-free exposure. The presence of 0.5 mM EGTA in the Ca^{2+}-free medium did not alter the time course of effects seen with Ca^{2+}-free perfusion alone.

The next group of experiments was designed to characterize the microsomal fractions isolated from control, Ca^{2+}-free and reperfused hearts. Control hearts were perfused with normal K-H solution for 20-25 min. Ca^{2+}-free hearts were perfused for 15 min with control solution followed by 5 min of Ca^{2+}-free perfusion, while reperfused hearts represent Ca^{2+}-free hearts which were reperfused with 1.25 mM Ca^{2+} for 5 more min. The results for Ca^{2+} uptake by microsomes in the presence of different concentrations of total $^{45}CaCl_2$ for 2 min are shown in Fig. 1. It is apparent that regardless of the amount of Ca^{2+} present in the incubation medium, reperfusion depressed Ca^{2+} uptake approximately 65-75% whereas Ca^{2+}-free perfusion had no significant effect ($P > 0.05$). Changes similar to those shown in Fig. 1 were also observed when the incubation was carried out for

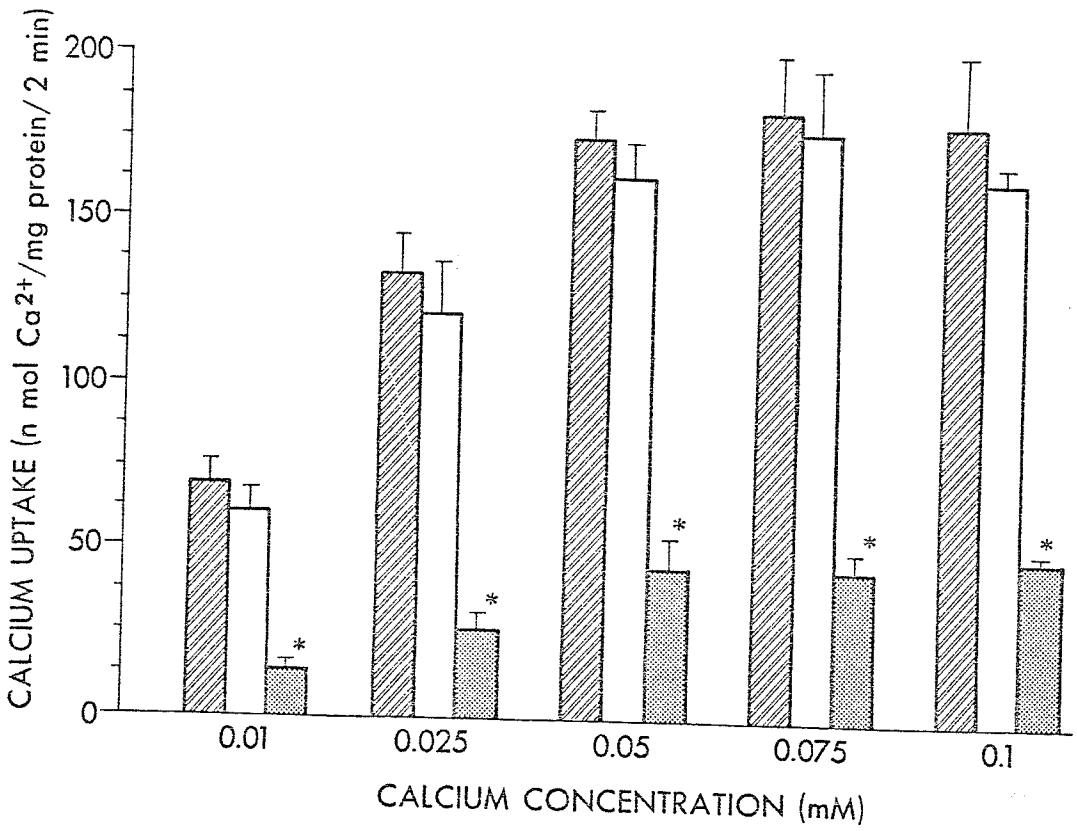

FIGURE 1. Calcium uptake activities at different calcium concentrations, of microsomes isolated from control, Ca^{2+}-free, and reperfused rat hearts. Calcium concentrations in the figure represent the total calcium added to the incubation medium. Calcium uptake by microsomes was determined for a period of 2 minutes. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes each. The control hearts were perfused for 5 to 10 minutes with normal medium following a 15-minute period of equilibration. The results are mean $\pm$ SE of four experiments. ▨ =control microsomes, ▭ Ca^{2+}-free microsomes, and ▦ = reperfused microsomes.

1,5 or 10 min. Such differences in the calcium uptake activities of microsomes from control, Ca^{2+}-free and reperfused hearts were also obtained when the incubation was carried out at different pH values (6.0-7.8) of the medium; optimal pH in each case was 6.8. Likewise, both ATP and Mg^{2+} concentrations used in the incubation medium were optimal for microsomes obtained from control, Ca^{2+}-free and reperfused hearts. The measurements of calcium uptake by microsomes from control and experimental hearts at 15,30,60,120 and 180 sec revealed that the calcium uptake activities in these three preparations were linear during a 2 min period of incubation. Furthermore, the initial rate of calcium uptake by microsomes from reperfused hearts was 60 to 70% lower than that of the control or Ca^{2+}-free preparations (70-90 nmoles/mg/min). Both initial rates and capacities of calcium uptake by microsomes from reperfused hearts were similarly depressed when dfferent concentrations of microsomal protein (.02 to .08 mg/ml) were employed in the incubation medium.

The phospholipid composition and protein profile of control, Ca^{2+}-free and reperfused heart microsomes were also examined. The experimental perfusions had no effect on total extracted phospholipid and as can be seen from Table 3 the relative amounts of each phospholipid moiety remained constant. Microsomes from control and experimental hearts were also shown to have similar protein patterns (Fig. 2) when analyzed by SDS-polyacrylamide gel electrophoresis. While a detailed investigation was not performed, the results suggest that the 100,000 dalton peak, representative of the sarcoplasmic reticular Ca^{2+}-pump ATPase (144), was essentially unaffected. Electron

TABLE 3 Phospholipid Composition of Microsomes Isolated from Control, Ca^{2+}-free and Reperfused Rat Hearts

Phospholipid	Amount of phospholipid (% of total lipid P)		
	Control	Ca^{2+}-free	Reperfused
Phosphatidylcholine	50.0	50.8	51.3
L-phosphatidylcholine	8.5	7.4	8.3
Phosphatidylethanolamine	18.1	17.8	18.1
Sphingomyelin	6.1	6.7	6.0
Phosphatidylserine	3.6	4.8	4.3
Phosphatidylinositol	3.1	3.3	2.6
Diphosphatidylglycerol	2.3	3.1	2.2
Phosphatidic acid	1.8	1.1	0.9
Unidentified phospholipid	6.5	5.0	6.3

Results are expressed as the mean of 3 experiments. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes each.

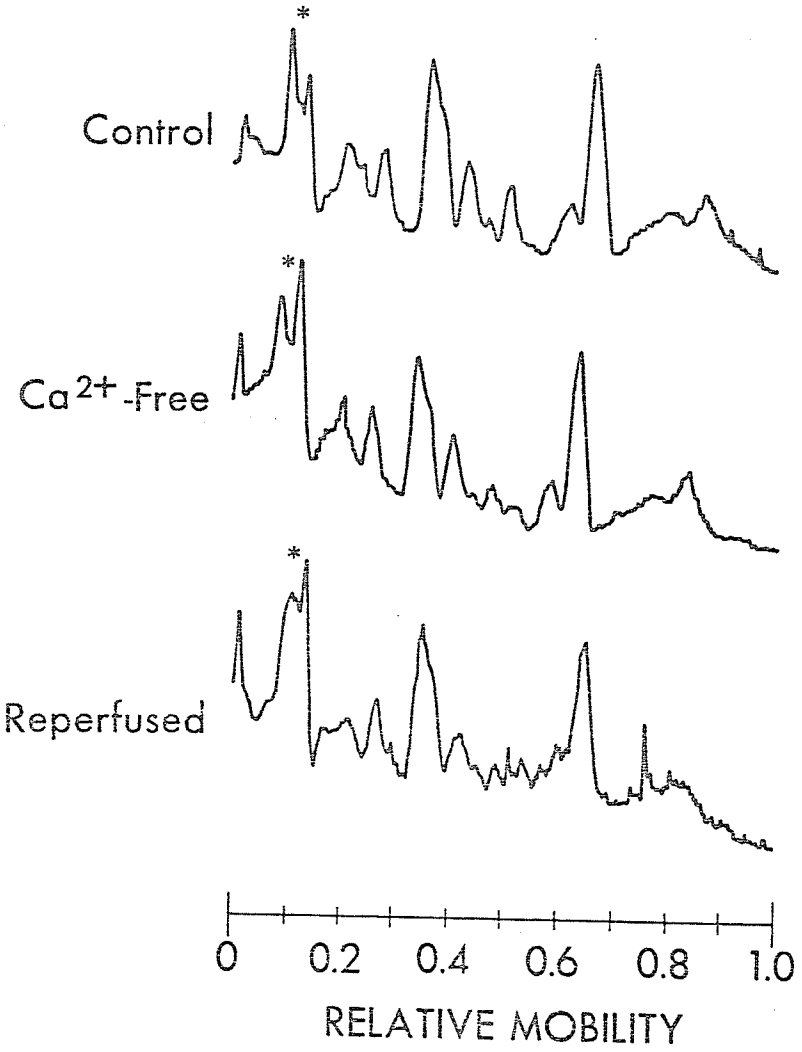

FIGURE 2. SDS-polyacrylamide gel electrophoretic pattern of microsomes isolated from control, Ca^{2+}-free, and reperfused rat hearts. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes each. The tracings are typical for at least four experiments in each case. *Represents the 100,000 dalton peak as described and identified by Jones et al. (144)

microscopic examination of control, Ca^{2+}-free and reperfused heart microsomes revealed that the vesicular appearance of the membranes was similar in all preparations (results not shown). Furthermore, gross contamination by mitochondria and myofibrils was also absent.

Several other biochemical activities were measured in our microsomal preparations and this data is reported in Table 4. Calcium binding was markedly ($P < 0.05$) depressed in reperfused heart microsomes only. When binding was measured in the presence of 5 mM NaN_3 the values were similar ($P > 0.05$) to those in the absence of azide. Since NaN_3 sensitive Ca^{2+}-binding is considered a marker for mitochondria, the slight (12%) inhibition due to azide further suggests the lack of an appreciable amount of mitochondrial contamination. The measurement of cytochrome C oxidase activities (132) in the control, Ca^{2+}-free and reperfused preparations revealed 5 to 7% cross contamination with mitochondrial subfragments in all of these preparations. In addition, the ouabain sensitive Na^+/K^+ ATPase activity was almost negligable in control, Ca^{2+}-free and reperfused microsomes; an observation indicative of a minimal presence of sarcolemmal constitutents. No attempt was made to rule out the possibility of latent ATPase as suggested by Jones et al., (144). Further extraction of the control, Ca^{2+}-free and reperfused preparations with 0.6 M KCl, which is considered to remove myofibrils, did not alter the calcium uptake activities of these preparations. Basal (Mg^{2+} ATPase) and Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities were depressed ($P < 0.05$) in reperfused heart microsomes only. Finally, microsomal protein yield and endogenous calcium contents varied only slightly between preparations.

TABLE 4 Calcium Binding and ATPase Activities, Protein Yield and Endogenous Calcium of Microsomes Isolated from Control, Ca^{2+}-free and Reperfused Rat Hearts

	Control	Ca^{2+}-free	Reperfused
Ca^{2+}-binding (nmol Ca^{2+}/mg protein/5 min)	36.4 ± 2.6	34.9 ± 3.2	21.3 ± 4.3*
Ca^{2+}-binding with 5 mM NaN_3 (nmol Ca^{2+} /mg protein/5 min)	32.1 ± 1.8	31.0 ± 2.6	18.6 ± 1.9*
Mg^{2+}-ATPase (μmol Pi/mg protein/5 min)	12.8 ± 0.6	12.6 ± 0.4	7.8 ± 0.2*
Ca^{2+}-stimulated ATPase (μmol Pi/mg protein/5 min)	0.86 ± 0.10	0.79 ± 0.04	0.21 ± 0.01*
Na^+/K^+ ATPase (Ouabain-sensitive) (μmol Pi/mg protein/5 min)	0.06 ± 0.05	0.07 ± 0.06	0.04 ± 0.01
Protein yield (mg/g heart wt)	0.44 ± 0.02	0.41 ± 0.03	0.49 ± 0.03
Endogenous Calcium (nmol/mg protein)	7.0 ± 1.5	6.0 ± 2.3	8.0 ± 2.1

Values are means ± SE of 4 experiments. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes.

* Significantly different from control ($P < 0.05$).

Calcium binding activities of the microsomal preparations were also measured at 30,60,120,180 and 300 sec intervals of incubation and the results for the control microsomes were 15,31,35,37 and 38 nmoles Ca^{2+}/mg protein (N=2). While the values for calcium binding by microsomes from Ca^{2+}-free hearts were not different from those of the control, 35 to 48% depression was seen in microsomes from reperfused hearts. The values for calcium binding activities of the control and experimental preparations were not affected by the presence of ATP regeneration systems such as 20 mM creatine phosphate and 0.53 µg/ml creatine kinase or 10 mM phosphoenol pyruvate and 130 µg/ml pyruvate kinase in the incubation mixture. The homogenization of ventricles from control, Ca^{2+}-free and reperfused hearts for 15,30 and 60 sec in the Waring blender before isolating the microsomal fractions, also did not affect the results. The preparation of microsomes by another method as described by Lee and Dhalla (34) also revealed a 50% depression in calcium binding activity (control value was 29 nmoles Ca^{2+}/mg protein/5 min) and a 63% depression in calcium uptake activity (control value was 218 nmoles Ca^{2+}/mg protein/5 min) in reperfused hearts whereas the results with Ca^{2+}-free hearts were not different from those for control hearts.

The dependency of reperfusion-induced changes on the duration of reperfusion and the Ca^{2+} concentration of the reperfusion medium was the subject of the next series of experiments. Figure 3 relates resting tension to microsomal Ca^{2+}-uptake in hearts reperfused with normal Ca^{2+} for various times after 5 min of Ca^{2+}-free perfusion. After 1 min of reperfusion an increase in resting tension and decrease in Ca^{2+} accumulation were apparent ($P < 0.05$). Longer periods of

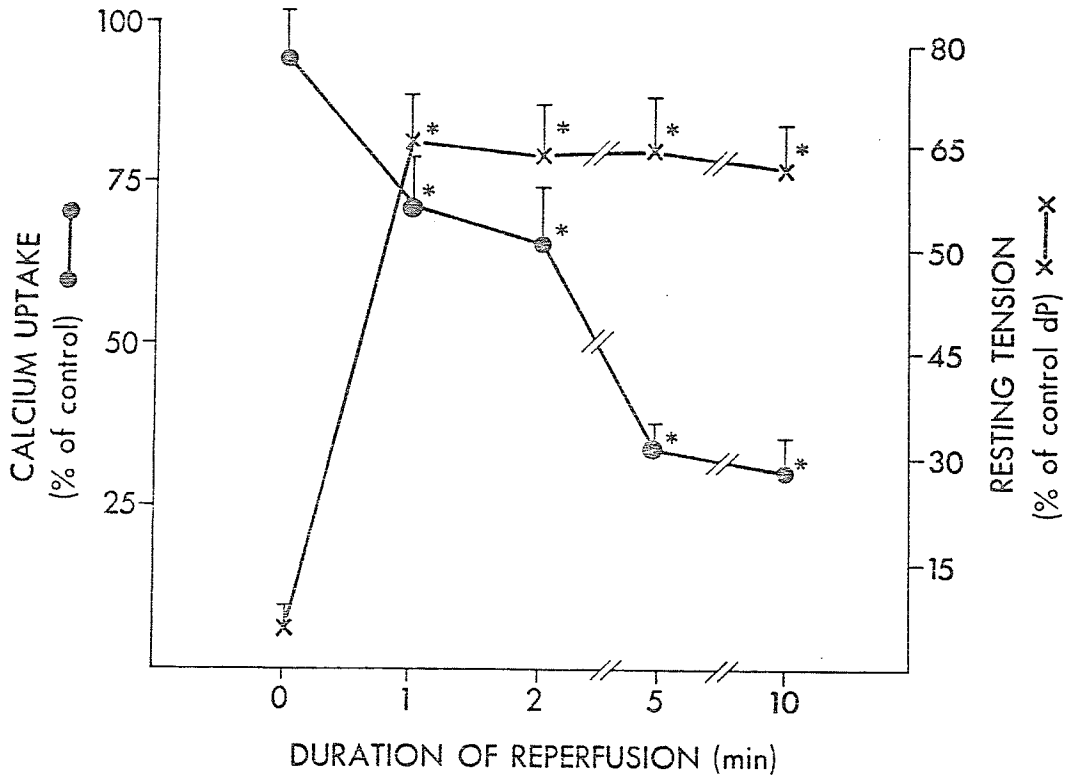

FIGURE 3. Effect of reperfusion for different intervals with medium containing 1.25 mM calcium on microsomal calcium uptake and resting tension of hearts perfused with Ca^{2+}-free medium for 5 minutes. Results are mean ± SE of four experiments for each point. *Significantly different from control ($P < 0.05$). Calcium uptake activity was determined by incubating microsomes for 5 minutes (control value was 255 ± 19 nmol Ca^{2+}/mg protein per 5 min).

reperfusion resulted in further decreases in Ca^{2+} uptake activity while resting tension tended to level off at approximately 65% of control total tension development. Five min of reperfusion with altered Ca^{2+} concentrations after 5 min of Ca^{2+}-free exposure, resulted in an almost inverse relationship between Ca^{2+} uptake and resting tension (Fig. 4). Both the increase in resting tension and decrease in Ca^{2+} accumulation were found to be dependent upon the concentration of Ca^{2+} in the reperfusion medium. It is emphasized that significant ($P < 0.05$) depression in microsomal Ca^{2+} uptake occurred in hearts reperfused with 0.25 mM or greater concentrations of Ca^{2+}.

Hypothermia during Ca^{2+}-free perfusion is known to protect the myocardium from reperfusion-induced damage and paradoxical calcium necrosis (4,6,76). The results in Table 5 demonstrate that reperfusion after 5 min of Ca^{2+}-free perfusion at 20°C allowed for the complete recovery of contractile force and preservation of control resting tension and microsomal Ca^{2+} uptake. If the Ca^{2+}-free solution was maintained at 25°C, normothermic (37°C) reperfusion produced significant inhibition of contractile force recovery and microsomal uptake. Increasing the temperature further resulted in the typical paradoxic changes seen in this study. As was the case in all the previous experiments, Ca^{2+}-free perfusion even at 20°C, had no significant effect per se on Ca^{2+}-uptake activity while contractile force was absent usually within 20 sec.

In earlier reports from our laboratory (3,6,34) it has been shown that by reducing the Na^{+} concentration from 145 mM to 35 mM during the period of Ca^{2+} depletion, some of the changes associated

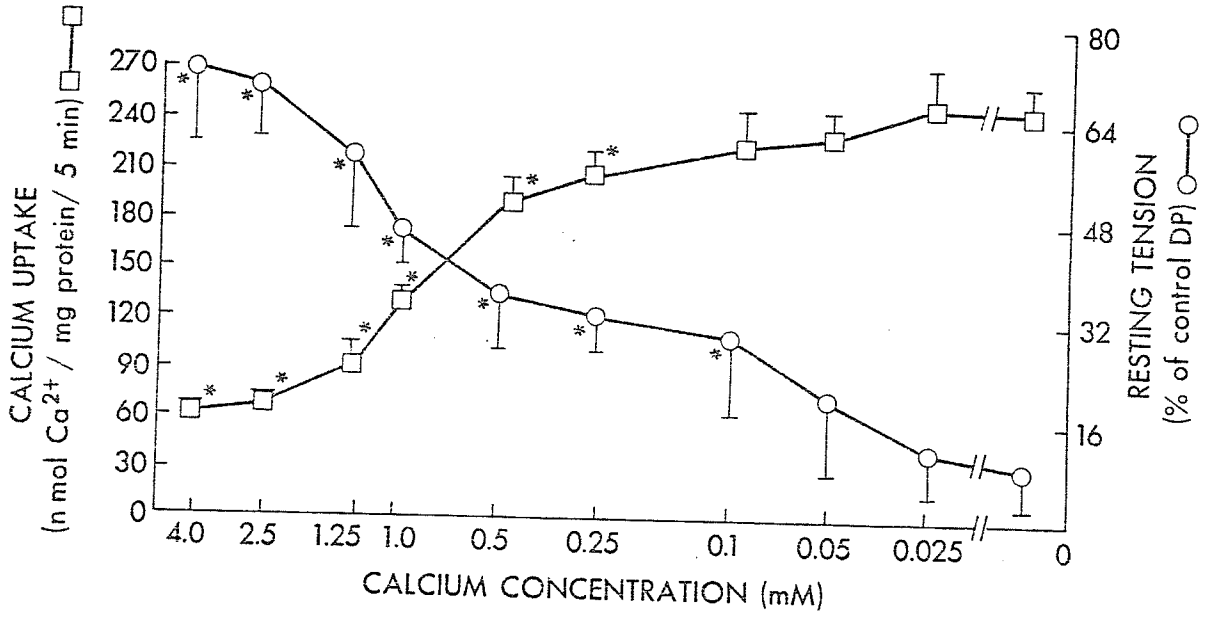

FIGURE 4. Effect of reperfusion for 5 minutes with medium containing different concentrations of calcium on microsomal calcium uptake and resting tension of heart perfused with Ca^{2+}-free medium for 5 minutes. Results are mean ± SE of four experiments for each point. *Significantly different from control ($P < 0.05$). The control value for calcium uptake activity was 240 ± 17 nmol Ca^{2+}/mg protein per 5 min.

TABLE 5 Effect of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused with Ca^{2+}-free Medium for 5 Minutes at Different Temperatures

Temperature of Ca^{2+}-free medium (°C)	Ca^{2+}-uptake before reperfusion (nmol Ca^{2+}/mg protein/ 5 mg)	CF after reperfusion (% of control dP)	Increase in RT after reperfusion (% of control of dP)	Ca^{2+}-uptake after reperfusion (nmol Ca^{2+}/mg protein/5 min)
Control	249 ± 16	100	0	244 ± 19
20	256 ± 12	95 ± 6	0	229 ± 18
25	244 ± 13	76 ± 12*	8 ± 5	150 ± 16*
30	251 ± 13	15 ± 9*	35 ± 8*	121 ± 18*
35	258 ± 6	0*	50 ± 6*	106 ± 11*
37	247 ± 18	0*	59 ± 8*	89 ± 10*

Results are expressed as mean ± SE of 5 experiments. CF: contractile force, dP: developed tension during the equilibration period, RT: resting tension.

* Significantly different from control ($P < 0.05$).

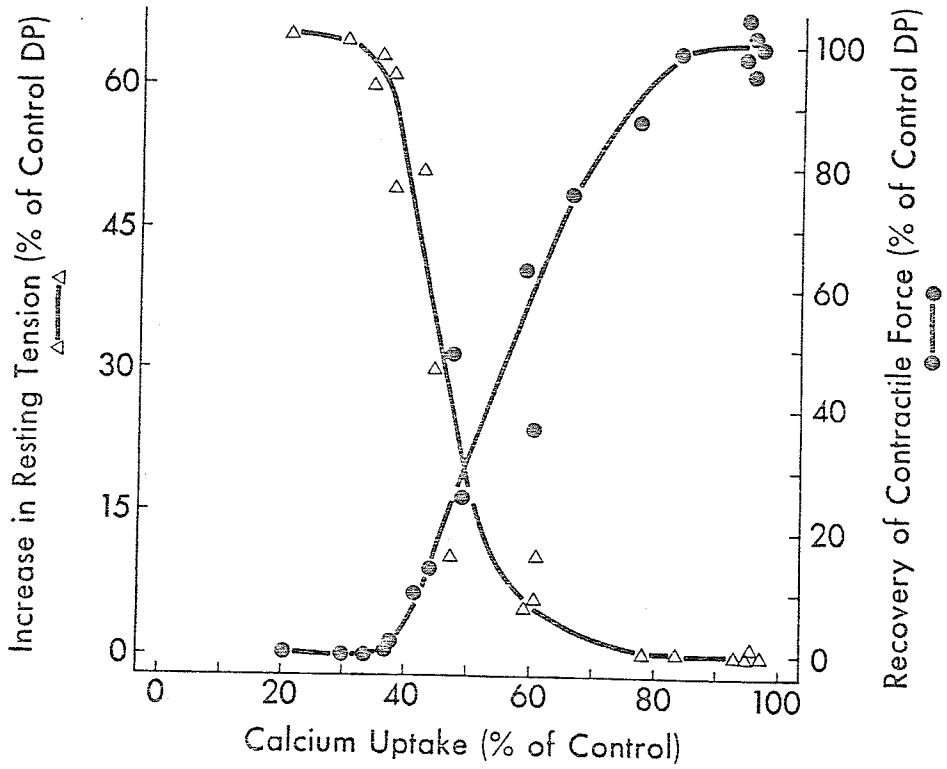

FIGURE 5. Relationship of changes in microsomal calcium uptake with the ability of heart to recover its contractile force as well as the increase in resting tension. The data for these plots are taken from Tables 1,2, and 5.

with reperfusion can be prevented. In the present study (Table 6) low Na^+: Ca^{2+}-free perfusion for 5 min had no effect ($P > 0.05$) on microsomal Ca^{2+} transport in spite of the fact that as usual the hearts were incapable of generating any active tension. After 1 min of reperfusion with normonatremic (145 mM) normocalcemic (1.25 mM) medium however, contractile force had recovered completely to equilibration period levels. This was associated with unaltered Ca^{2+}-uptake abilities of the isolated sarcoplasmic reticular membranes. With longer periods of reperfusion control contractile and Ca^{2+} accumulating activities were maintained ($P < 0.05$).

B. Effects on Sarcolemmal Mg^{2+} and Na^+/K^+ ATPases

In earlier studies (6) we have demonstrated that the calcium paradox is associated with changes in myocardial cations like K^+ and Na^+. Therefore, we hoped to analyze the existence of a relationship between the Na^+/K^+ pump, expressed as Na^+/K^+ ATPase activity, and the alterations in myocardial Na^+ and K^+ content seen during the course of the calcium paradox. The activity of Mg^{2+} ATPase has also been reported because its determination is essential in the measurement of Na^+/K ATPase. Furthermore, the magnitude of change in Mg^{2+} ATPase as compared to Na^+/K^+ ATPase activity may shed light on the specificity or lack of specificity of calcium paradoxic sarcolemmal alterations.

In the first group, Table 7, isolated hearts were perfused with different Ca^{2+} concentrations for 5 min and then reperfused with normal Ca^{2+} containing medium for 5 additional min. It should be pointed out that preperfusion per se had no effect on either enzyme activity (data not reported) and, as long as 0.1 mM Ca^{2+} was present during

TABLE 6 Effects of Reperfusion for Different Intervals with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused for 5 Minutes with Ca^{2+}-free Medium Containing 35 mM Sodium

Duration of Reperfusion (min)	Ca^{2+}-uptake activity (nmol Ca^{2+}/mg protein/5 min)	CF after reperfusion (% of control dP)
Control	253 ± 14	100
0	246 ± 7	0
1	248 ± 16	97 ± 2
2	251 ± 3	98 ± 3
5	245 ± 6	101 ± 4
10	239 ± 10	100 ± 2

Results are expressed as mean ± SE of 4 experiments. CF: contractile force, dP: developed tension during the equilibration period.

TABLE 7 Effects of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused with Medium Containing Different Concentrations of Calcium for 5 Minutes

Calcium Concentration (mM)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^+/K^+ ATPase
1.25 (control)	39.2 ± 3.3	12.4 ± 1.6
0.50	37.3 ± 3.9	12.7 ± 0.9
0.25	39.2 ± 1.7	12.9 ± 1.3
0.10	36.3 ± 2.2	13.6 ± 1.1
0.05	29.9 ± 3.9*	9.0 ± 1.1*
0.025	23.6 ± 2.1*	4.2 ± 0.7*
0	21.6 ± 1.6*	3.3 ± 0.2*

Results are expressed as mean ± SE of 5 experiments.

* Significantly different from control ($P < 0.05$).

the initial 5 min perfusion, reperfusion induced no change ($P > 0.05$) in Mg^{2+} or Na^+/K^+ ATPase activities. Reperfusion after 5 min of perfusion with media containing 0.05 mM or 0.025 mM Ca^{2+} was associated with significant ($P < 0.05$) depression in both ATPases but Na^+/K^+ ATPase activity was more markedly inhibited. In those reperfused with Ca^{2+} after 5 min of Ca^{2+}-free exposure, sarcolemmal Na^+/K^+ ATPase and Mg^{2+} ATPase activities were 27% and 55% of control values ($P < 0.05$) respectively.

The effects of reperfusion after different durations of Ca^{2+}-free perfusion were then investigated. Once again even 10 min of Ca^{2+}-free perfusion had no effect on sarcolemmal enzyme activity (results not given). As can be seen from Table 8 the reperfusion-induced alterations were found to be dependent on the duration of Ca^{2+} depletion. After 1 min of Ca^{2+}-free perfusion, reperfusion produced no effect ($P > 0.05$) on either Mg^{2+} or Na^+/K^+ ATPase activity. With increasing periods of Ca^{2+}-free perfusion, reperfusion induced progressive decreases in enzyme activity with the most severe inhibition being present in hearts reperfused for 5 min after 10 min of Ca^{2+} depletion; Mg^{2+} ATPase activity was 40% and Na^+/K^+ ATPase activity was 23% of control values. While Na^+/K^+ ATPase was more affected than Mg^{2+} ATPase activity, significant ($P < 0.05$) depression was first observed simultaneously in both enzymes of sarcolemma isolated from hearts reperfused after 2 min of Ca^{2+}-free perfusion.

The dependence of ATPase inhibition on the Ca^{2+} concentration of the reperfusion medium was then determined in hearts perfused for 5 min with Ca^{2+}-free medium. Under these conditions a separation between changes in Mg^{2+} ATPase and Na^+/K^+ ATPase activities was first

TABLE 8 Effects of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused with Ca^{2+}-free Medium for Different Intervals

Duration of Ca^{2+}-free perfusion (min)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^{+}/K^{+} ATPase
0 (control)	37.6 ± 2.3	13.3 ± 1.7
1	34.7 ± 3.8	11.6 ± 1.2
2	24.6 ± 2.7*	8.2 ± 1.4*
3	19.9 ± 4.1*	4.1 ± 0.5*
4	17.0 ± 5.2*	4.1 ± 0.7*
5	18.7 ± 1.3*	3.9 ± 0.8*
10	14.9 ± 2.1*	3.0 ± 0.2*

Results are expressed as mean ± SE of 5 experiments.

*Significantly different from control ($P < 0.05$).

appreciated (Table 9). While reperfusion with medium containing 0.05 mM or less Ca^{2+} was without effect ($P > 0.05$), 0.10 mM Ca^{2+} reperfusion produced a significant ($P < 0.05$) effect on Na^{+}/K^{+} ATPase activity but Mg^{2+} ATPase though slightly decreased, was not significantly ($P > 0.05$) changed from control values. Increasing the reperfusion Ca^{2+} concentration to 0.25 mM resulted in significant inhibition of Mg^{2+} and Na^{+}/K^{+} ATPase activities. The degree of depression in both enzyme activities then increased with progressively larger reperfusion Ca^{2+} concentrations such that hearts reperfused with 4.0 mM Ca^{2+} had sarcolemmal Mg^{2+} and Na^{+}/K^{+} ATPase activities decreased by approximately 59% and 89% from control respectively; re-emphasizing the marked sensitivity of Na^{+}/K^{+} ATPase to calcium paradoxic changes.

The observation that Na^{+}/K^{+} ATPase is more susceptible than Mg^{2+} ATPase to calcium paradoxic influences can be further appreciated from the results reported on Table 10. In this series of experiments hearts were perfused for 5 min with Ca^{2+}-free medium and then reperfused for 0-10 min with normocalcemic solution. Reperfusion for 1 min was found to significantly ($P < 0.05$) depress Mg^{2+} ATPase some 26% but Na^{+}/K^{+} ATPase was decreased 53% from control. To achieve 50% inhibition of sarcolemmal Mg^{2+} ATPase the isolated hearts required approximately 5 min of reperfusion; this same perfusion sequence resulted in 76% inhibition of sarcolemmal Na^{+}/K^{+} ATPase.

In the next couple of experiments (Tables 11 and 12) two conditions known to prevent the occurrence of the calcium paradox (3,4,6) were employed in order to determine if sarcolemmal enzyme activity would also be preserved. As can be appreciated from Table 11, regardless of the duration of reperfusion (0-10 min), preperfusion for 5

TABLE 9 Effects of Reperfusion for 5 Minutes with Medium Containing Different Concentrations of Calcium on Isolated Rat Hearts Perfused for 5 Minutes with Ca^{2+}-free Medium

Calcium Concentration (mM)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^{+}/K^{+} ATPase
Control	38.8 ± 3.1	13.8 ± 2.2
0	35.4 ± 2.1	12.5 ± 1.0
0.025	33.8 ± 5.2	12.5 ± 1.2
0.05	35.1 ± 2.9	11.9 ± 1.1
0.10	32.0 ± 2.6	9.4 ± 0.2*
0.25	30.4 ± 0.9*	6.8 ± 0.7*
0.5	24.7 ± 4.1*	5.1 ± 1.1*
1.0	22.2 ± 3.4*	4.0 ± 0.1*
1.25	18.7 ± 1.2*	3.2 ± 0.9*
2.5	17.1 ± 2.2*	2.8 ± 0.3*
4.0	16.0 ± 0.5*	1.5 ± 0.2*

Results are expressed as mean ± SE of 4 experiments. Control hearts were perfused with 1.25 mM Ca^{2+} for 10 minutes.

* Significantly different from control ($P < 0.05$).

TABLE 10 Effects of Reperfusion for Different Intervals with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused for 5 Minutes with Ca^{2+}-free Medium

Duration of Reperfusion (min)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^{+}/K^{+} ATPase
Control	39.2 ± 2.4	12.7 ± 1.6
0	36.2 ± 2.7	11.5 ± 0.8
1	28.9 ± 2.1*	6.0 ± 0.4*
2	22.0 ± 1.5*	4.2 ± 0.7*
3	21.9 ± 2.2*	3.6 ± 0.4*
5	19.1 ± 1.4*	3.1 ± 0.9*
10	17.0 ± 0.4*	2.2 ± 0.4*

Results are expressed as mean ± SE of 5 experiments.

* Significantly different from control ($P < 0.05$).

TABLE 11 Effects of Reperfusion for Different Intervals with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused for 5 Minutes with Ca^{2+}-free Medium Containing 35 mM Sodium

Duration of Reperfusion (min)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^{+}/K^{+} ATPase
Control	38.2 ± 3.1	12.9 ± 2.0
0	39.6 ± 2.3	13.2 ± 1.1
1	40.3 ± 1.8	13.6 ± 1.8
2	40.2 ± 2.7	12.0 ± 0.3
3	38.7 ± 2.5	12.6 ± 0.9
5	35.8 ± 3.6	9.7 ± 0.8
10	32.0 ± 1.7*	10.0 ± 0.3

Results are expressed as mean ± SE of 4 experiments.

* Significantly different from control ($P < 0.05$).

min with Ca^{2+}-free medium in which the sodium concentration had been lowered from 145 mM to 35 mM, was able to abolish the reperfusion-induced depression in Mg^{2+} and Na^{+}/K^{+} ATPase activities except for a small though statistically significant ($P < 0.05$) decrease in Mg^{2+} ATPase from hearts reperfused for 10 min. This later observation has admittedly complicated our estimation of sarcolemmal enzyme susceptibility in spite of the fact that this appeared to be an isolated case.

In Table 12 the effects of reperfusing hearts perfused for 5 min with hypothermic Ca^{2+}-free media, on sarcolemmal Mg^{2+} and Na^{+}/K^{+} ATPase activities are reported. Perfusion with Ca^{2+}-free medium cooled to room temperature was sufficient to prevent ($P > 0.05$) normothermic reperfusion-induced enzyme depression and as described earlier, these hearts recovered contractile force completely. As the temperature of the Ca^{2+}-free medium was increased reperfusion resulted in progressively greater degrees of enzyme depression ($P < 0.05$) in both Mg^{2+} and Na^{+}/K^{+} ATPase activities; the later still being more sensitive to the effects of reperfusion.

In order to more fully characterize and compare the reperfusion-induced inhibition of Mg^{2+} ATPase and Na^{+}/K^{+} ATPase activities, kinetic analysis for calculating Km and Vmax was undertaken for each enzyme under control conditions and under those situations where enzyme activity had been reduced approximately 50% from control. In the case of Na^{+}/K^{+} ATPase, control hearts were equilibrated for 15 min followed by 6 min of perfusion with normal K-H solution. Reperfused hearts represent those hearts equilibrated for 15 min and then perfused for 5 min with Ca^{2+}-free medium followed by 1 min of reperfusion

TABLE 12 Effects of Reperfusion for 5 Minutes with Medium Containing 1.25 mM Calcium on Isolated Rat Hearts Perfused for 5 Minutes with Ca^{2+}-free Medium at Different Temperatures

Temperature of Ca^{2+}-free medium (°C)	ATPase Activities (μmol Pi/mg protein/hr)	
	Mg^{2+} ATPase	Na^{+}/K^{+} ATPase
Control	38.9 ± 4.1	13.1 ± 1.2
21	37.4 ± 2.6	11.9 ± 1.1
25	30.8 ± 2.0*	8.0 ± 0.5*
30	26.4 ± 2.7*	6.3 ± 0.7*
35	22.1 ± 3.8*	5.0 ± 0.1*
37	20.9 ± 0.9*	3.7 ± 0.6*

Results are expressed as mean ± SE of 4 experiments.

* Significantly different from control ($P < 0.05$).

with normal K-H solution. For experiments on Mg^{2+}-ATPase, control hearts were equilibrated as above followed by 10 min of normal K-H perfusion while reperfused sarcolemma came from equilibrated hearts perfused for 5 min with Ca^{2+}-free medium and subsequently reperfused for 5 min with normal Ca^{2+} containing medium. Sarcolemmal Na^+/K^+ and Mg^{2+} ATPase activities from control and reperfused hearts were studied by varying the ATP concentration of the incubation medium and the results are reported in Fig. 6 and Fig. 7 for Na^+/K^+ and Mg^{2+} ATPases respectively. Lineweaver-Burk analysis of the data can also be seen for each enzyme in Fig. 6 and Fig. 7. As depicted in Fig. 6 reperfusion did not change the K_m (1.21 mM ATP) of Na^+/K^+ ATPase but the Vmax was reduced in reperfused sarcolemma from 20 to 9 μ moles/mg protein/hour. As for Mg^{2+} ATPase, Fig. 7, there was no change in K_m (0.25 mM ATP) but in reperfused hearts the Vmax decreased from 47 to 22 μ moles Pi/mg protein/hour. (non-competitive)

One of the characteristics of cardiac Na^+/K^+ ATPase is its sensitivity to the inhibitory effects of ouabain (145). In the next series of experiments sarcolemmal membranes were isolated from control and reperfused hearts; the perfusion protocols being identical to those described for Fig. 6. Na^+/K^+ ATPase activity from the two sarcolemmal preparations was then studied in the presence of varying concentrations of ouabain. As can be appreciated from Fig. 8, reperfused heart sarcolemmal Na^+/K^+ ATPase activity was more sensitive to the effects of ouabain then that of control heart sarcolemma. The ID_{50} for reperfused Na^+/K^+ ATPase was approximately 0.03 mM while that for control enzyme was approximately 0.10 mM. It should be pointed out however, that in both cases 2.0 mM ouabain effected a

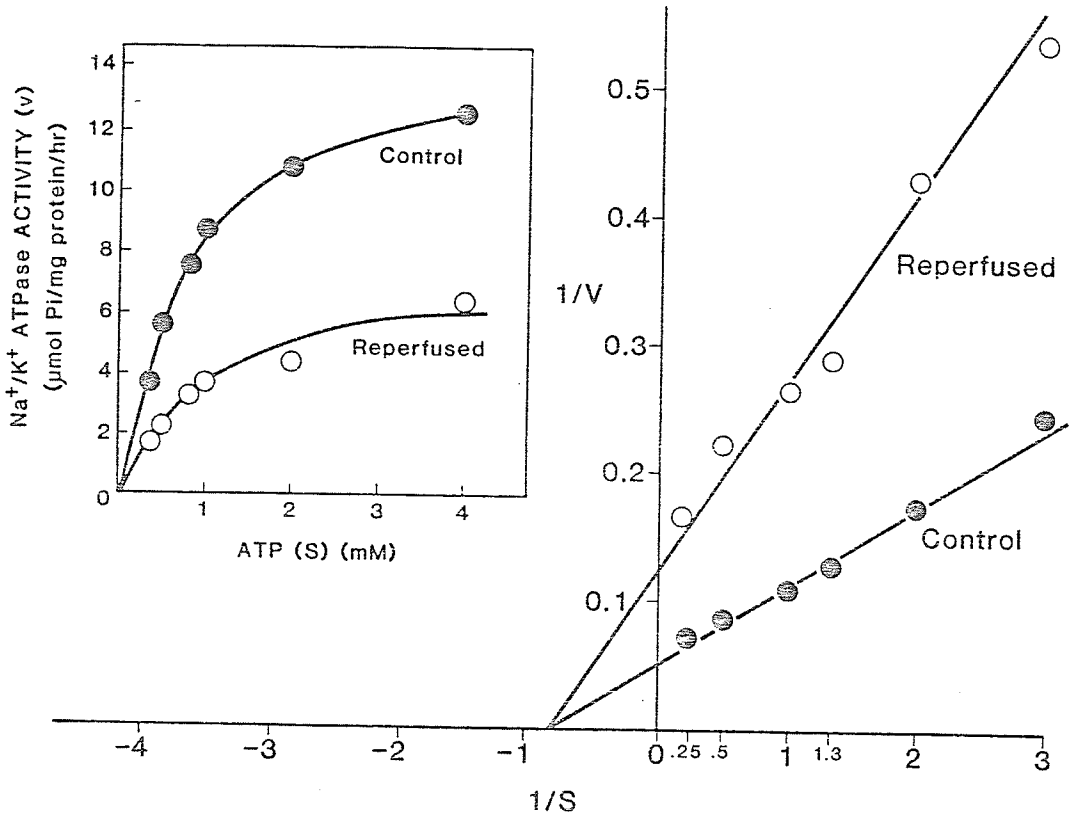

FIGURE 6. Sarcolemmal Na^+/K^+ ATPase activities from control and reperfused rat hearts at different ATP concentrations. Results are expressed as the mean of 3 experiments in which after 15 minutes of equilibration, control hearts were perfused for 6 minutes with normal (1.25 mM Ca^{2+}) medium and reperfused hearts were perfused for 1 minute with calcium containing medium after 5 minutes of Ca^{2+}-free perfusion.

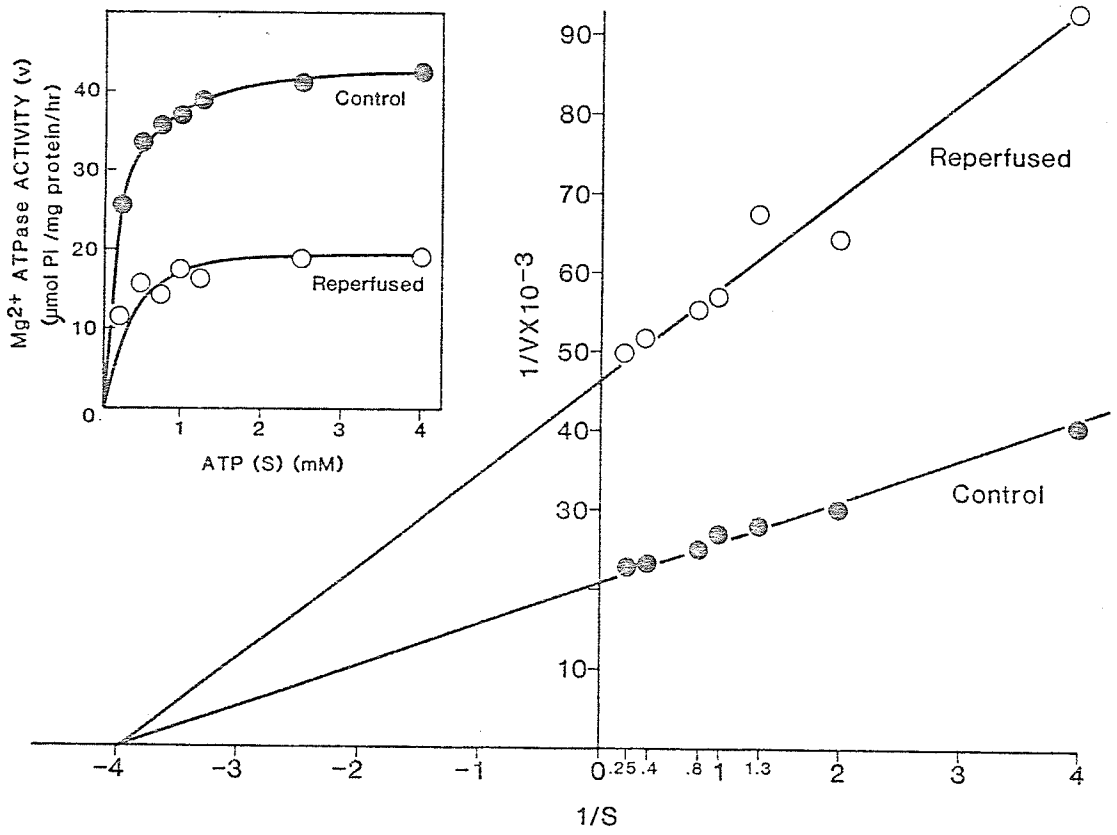

FIGURE 7. Sarcolemmal Mg^{2+} ATPase activities from control and reperfused rat hearts at different ATP concentrations. Results are expressed as a mean of 3 experiments in which after 15 minutes of equilibration, control hearts were perfused for 10 minutes with normal medium and reperfused hearts were perfused for 5 minutes with calcium containing medium after 5 minutes of Ca^{2+}-free perfusion.

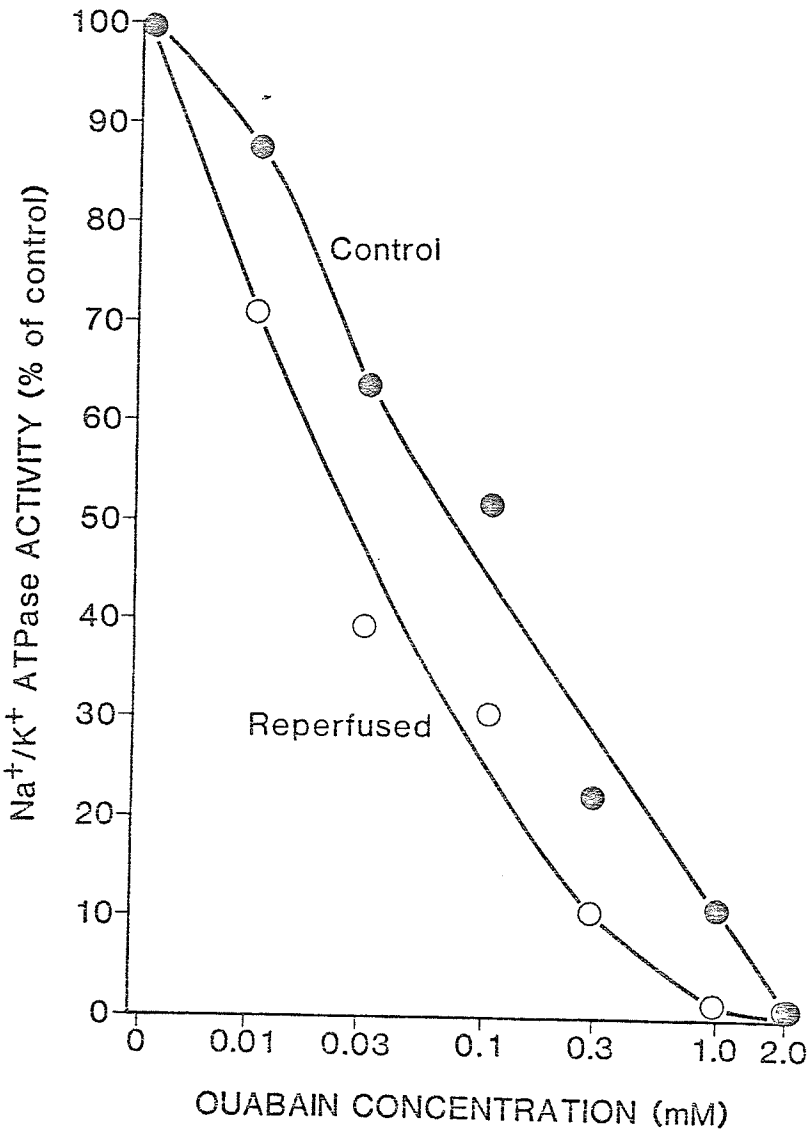

FIGURE 8. Effect of different concentrations of ouabain on Na^+/K^+ ATPase activity of sarcolemmal membranes isolated from control and reperfused rat hearts. Conditions are the same as those described in figure 6. Values are expressed as the mean of 3 experiments. The control values for Na^+/K^+ ATPase from control and reperfused heart sarcolemma were 13.2 ± 0.9 and 6.0 ± 0.8 μmol Pi/mg protein/hr respectively.

98% inhibition of enzyme activity.

Next we attempted to investigate the existence of a relationship between changes in Na^+/K^+ ATPase activity and alterations in myocardial Na^+ and K^+ content under identical conditions. For this purpose we empolyed data from Tables 7, 8 and 12 in the present study and data regarding changes in cation contents under identical conditions from an earlier report published by us (6). The reason for choosing these conditions is that they represent the spectrum of reversible, partly reversible and irreversible changes in contractile recovery during the occurrence of the calcium paradox. In this way we hoped not to prejudice our interpretation to any one extreme. As can be seen in Fig. 9 a 30% decrease in Na^+/K^+ ATPase activity is not associated with changes in intracellular Na^+ or K^+. However, if ATPase activity is depressed to approximately 35-40% of control values or lower, a rapid increase in Na^+ and decrease in cellular K^+ occurs and from our earlier (6) and present investigations this point marks exactly the transition from completely reversible to completely irreversible contractile, ultrastructural and biochemical damage.

C. Effects on Sarcolemmal Ca^{2+} ATPase

In the last major series of experiments the effects of the calcium paradox on another prominent sarcolemmal enzyme were investigated. Sarcolemmal Ca^{2+}-dependent ATPase is believed to have a role in governing the entry of Ca^{2+} into the cell (146). This enzyme is viewed as a gating mechanism whereby channels are opened in the membrane at the expense of ATP hydrolysis so as to permit the passive flux of extracellular Ca^{2+} down its concentration gradient

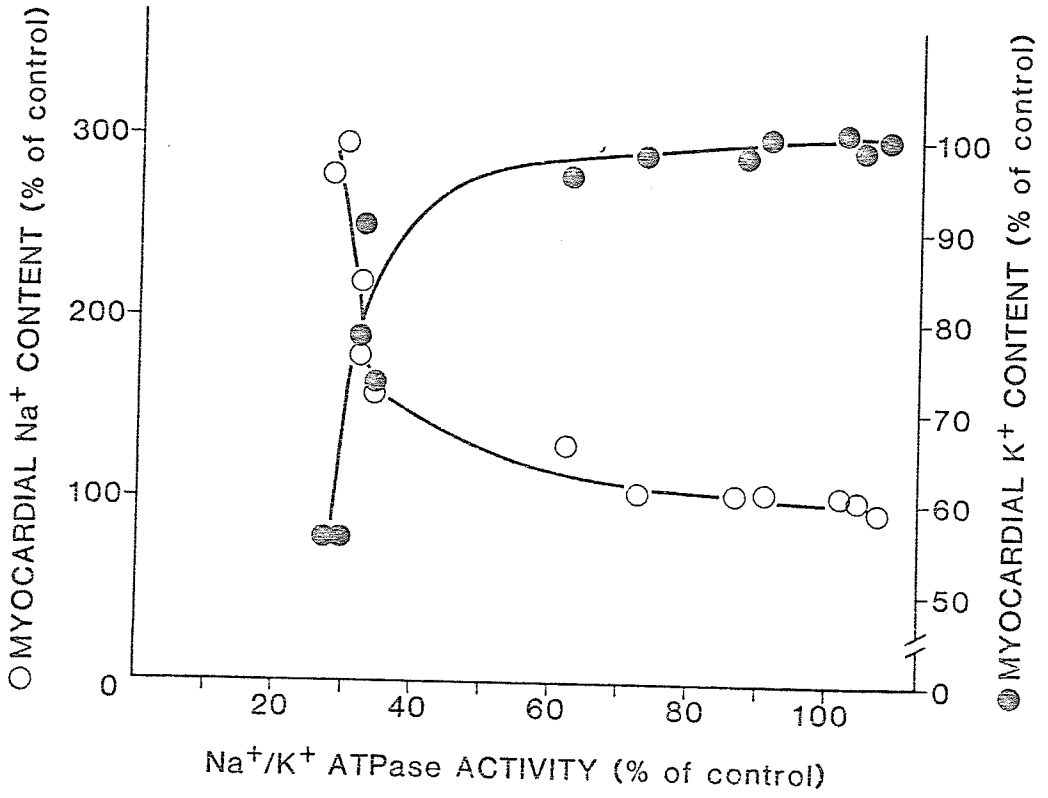

FIGURE 9. Relationship of changes in sarcolemmal Na^+/K^+ ATPase activity with myocardial Na^+ and K^+ contents under identical conditions. The data regarding enzyme activity were taken from Tables 7, 8, and 12. Cation contents were taken from our earlier studies (6).

to intracellular sites. Furthermore, this enzyme activity has been found to be directly related to the ability of the heart to generate contractile force (146). Therefore in so much as the calcium paradox represents a model of altered contractile performance, we decided to investigate the possible role played by sarcolemmal Ca^{2+}-dependent ATPase in the development of the calcium paradox under conditions which represent reversible, partly reversible and irreversible losses of mechanical activity. It should be pointed out that Ca^{2+}-dependent ATPase is quite different from Ca^{2+}-stimulated Mg^{2+}-dependent ATPase; an enzyme thought to be involved in the efflux of Ca^{2+} from the myocardial cell (12,147-150).

Our first goal was to characterize our sarcolemmal membrane preparations with respect to purity and orientation of the membrane vessicles. Control hearts were perfused with normal K-H solution for a total of 25 min. Ca^{2+}-free hearts were first equilibrated for 15 min with normal solution followed by 5 min of Ca^{2+} deprivation while reperfused hearts had a subsequent re-introduction of Ca^{2+} for 5 additional min. Various characteristics of the isolated membranes are reported on Table 13. Sarcolemmal protein yield was not influenced by Ca^{2+}-free perfusion per se but was significantly ($P < 0.05$) elevated in the case of reperfused hearts. The hypotonic shock-LiBr procedure for sarcolemmal isolation (131,132) depends for its success on the disruption of myocardial cells so that undesireable cellular constituents such as mitochondria, sarcoplasmic reticulum and myofibrils can be separated out by differential centrifugation and/or extracted by high salt solutions. The calcium paradox is known to effect marked cellular disruption (2,3,4,14,21), therefore

TABLE 13 Various Characteristics of Sarcolemmal Membranes Isolated from Control, Ca^{2+}-free and Reperfused Rat Hearts

Activity	Control	Ca^{2+}-free	Reperfused
Protein yield (mg protein/gm ventricular wt)	3.8 ± 0.2	3.9 ± 0.3	5.1 ± 0.4*
Endogenous calcium (nmol/mg protein)	2.6 ± 0.5	2.2 ± 0.5	2.4 ± 0.2
Adenylate cyclase activity (pmol cAMP/mg protein/min)	398 ± 22	326 ± 14*	266 ± 23*
Ouabain sensitive Na^+/K^+ ATPase (μmol Pi/mg protein/hr)	13.4 ± 1.1	12.7 ± 1.1	3.9 ± 0.8*
Ca^{2+}-stimulated Mg^{2+}-dependent ATPase (μmol Pi/mg protein/hr)	ND	ND	ND
ATP-stimulated Ca^{2+} binding (nmol Ca^{2+}/mg protein/5 min)	ND	ND	ND

Values are means ± SE of 4 experiments. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes each. ND: not detectable

* Significantly different from control ($P < 0.05$).

the increased protein yield from reperfused hearts most likely represents enhanced cellular breakdown and the freeing of more cell membrane such that further hypotonic treatment and salt extraction merely allows for an improved yield rather than the explanation being made on the basis of subcellular contamination. Electron microscopic examination of control, Ca^{2+}-free and reperfused heart sarcolemmal membrane revealed that the vesicular appearance of the membranes was similar in all preparations and there was no obvious contamination by other subcellular fractions (micrographs not included). Furthering our contention of minimal contamination by other subcellular constituents, were the measurements of marker activities. The absence of detectable Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities precludes the presence of significant microsomal or myofibrillar contamination. Similarily the lack of ATP-dependent Ca^{2+} binding activity indicates negligable microsomal or mitochondrial components within the sarcolemmal isolates. Furthermore, in all three final membrane preparations, adenylate cyclase and ouabain sensitive Na^{+}/K^{+} ATPase activities (characteristic sarcolemmal enzymes) represented an 11-13 fold increase over those activities present in crude homogenates of hearts subjected to the corresponding perfusion protocols. With regards to the orientation of our vesicular membrane preparations we believe that the vast majority are right-sided out. This stems from earlier studies on this isolation procedure (136) and the present observations of undetectable Ca^{2+}-stimulated Mg-dependent ATPase and ATP-dependent Ca^{2+} binding activities; both of these are considered to reside on the internal side of the sarcolemmal bilayer (12,147). The right-sided out nature of our preparations is further

suggested by the virtually complete (98%) inhibition of Na^+/K^+ ATPase activity in all three sarcolemmal preparations, by the additions of 2.0 mM ouabain.

We also investigated the effects of the calcium paradox on the composition of our sarcolemmal preparations. Hearts were perfused under conditions identical to those described for Table 13 and the sarcolemmal membranes isolated. These preparations were then processed for either the determination of membrane protein constituents by SDS-polyacrylamide gel electrophoresis or for phospholipid analysis by two directional thin layer chromatography. On Fig. 10 are representative protein profiles of control, Ca^{2+}-free and reperfused heart sarcolemma. Ca^{2+}-free perfusion per se did not alter the electrophoretic pattern from that obtained in control sarcolemmal preparations. Reperfusion however, while not influencing the major polypeptide peaks, did appear to reduce the magnitude of only the mid range (29,000-41,000 daltons) peaks. Since a complete characterization of the nature of each control sarcolemmal peak has not as yet been achieved, the significance of this observation was not assessed any further.

The phospholipid analysis is reported in Table 14. In hearts merely perfused for 5 min with Ca^{2+}-free medium the phospholipid composition of the isolated sarcolemma was not appreciably different ($P > 0.05$) from control heart sarcolemma. Reperfusion on the other hand, while not affecting the total amount of phospholipid extracted, did cause a small but significant ($P < 0.05$) decrease in the amount of phosphatidylserine (PS) as compared to control. However, reperfused heart sarcolemmal PS was not significantly different from the

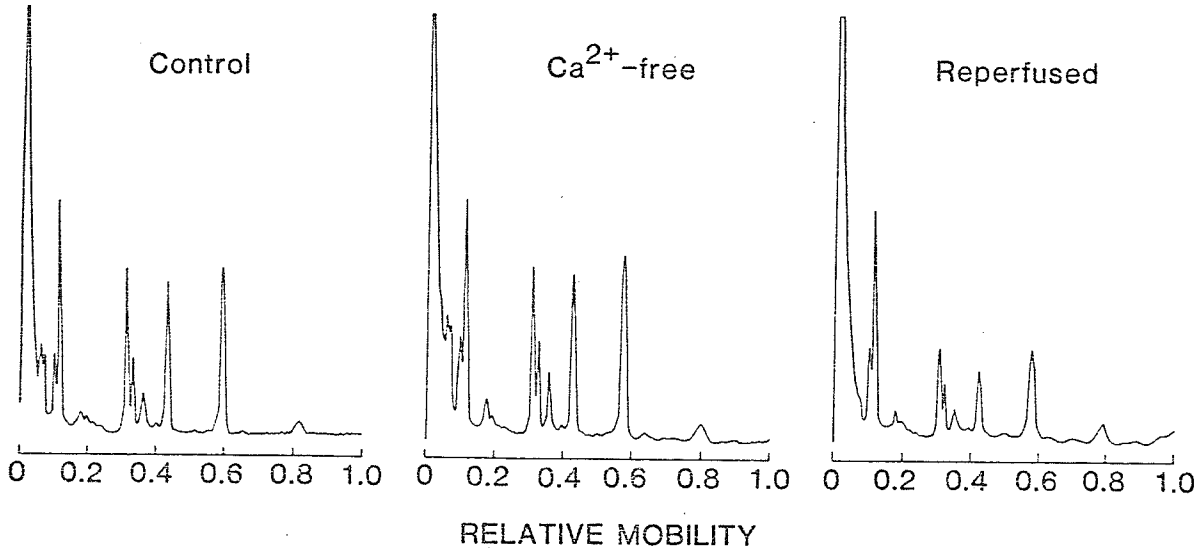

FIGURE 10. SDS-polyacrylamide gel electrophoretic patterns of sarcolemmal membranes isolated from control, Ca^{2+}-free and reperfused rat hearts. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM calcium were carried out for 5 minutes each. The tracings are typical for at least 3 experiments in each case.

TABLE 14 Phospholipid Composition of Sarcolemmal Membranes Isolated from Control, Ca^{2+}-free and Reperfused Rat Hearts

Phospholipid	Amount of Phospholipid (nmoles Pi/mg protein)		
	Control	Ca^{2+}-free	Reperfused
Phosphatidylcholine	82.1 ± 8.9	78.8 ± 7.0	82.1 ± 6.2
L-phosphatidylcholine	2.9 ± 1.2	3.4 ± 0.8	2.9 ± 1.5
Phosphatidylethanolamine	79.8 ± 9.4	81.0 ± 9.5	74.5 ± 5.4
Sphingomyelin	15.3 ± 1.3	14.1 ± 1.5	13.9 ± 1.4
Phosphatidylserine	16.1 ± 0.8	14.6 ± 1.8	10.5 ± 1.8*
Phosphatidylinositol	8.3 ± 1.5	8.1 ± 0.5	8.5 ± 2.1
Diphosphatidylglycerol	18.8 ± 1.7	19.5 ± 1.5	16.6 ± 2.6
Phosphatidic acid	4.6 ± 1.6	3.8 ± 1.3	4.0 ± 1.3
Unidentified phospholipid	4.4 ± 0.5	4.1 ± 0.6	4.7 ± 0.6

Results are expressed as the mean ± SE of 4 experiments. Perfusion with Ca^{2+}-free medium and reperfusion with medium containing 1.25 mM Ca^{2+} were carried out for 5 minutes each. * Significantly different from control ($P < 0.05$).

PS of Ca^{2+}-free sarcolemma which in turn was also not different from control. In reperfused sarcolemmal all other phospholipid moities were unaltered.

It is important to emphasize that while the results reported on Table 14 and Fig. 10 fail to shed any light on the mechanism whereby reperfusion induces such marked changes in sarcolemmal morphology (2,3,4,14,21) and enzyme activity, they do support our other data concerning the purity and structural similarities of the three different sarcolemmal preparations.

The presence of 0.025 mM or greater concentrations of calcium during the 5 min preperfusion period is known to be sufficient to allow for varying degrees of recovery in contractile force when normal (1.25 mM) calcium is reintroduced (5,6). In Fig. 11 are reported the contractile force generation and sarcolemmal Ca^{2+} ATPase activities of hearts reperfused for 5 min with normal K-H solution after 5 min of perfusion with media containing varying amounts of Ca^{2+}. As has been the case with all other biochemical activities discussed thus far, preperfusion per se even with Ca^{2+}-free media, did not significantly alter Ca^{2+} ATPase activity in isolated sarcolemma in spite of the fact that these hearts generate no or very little contractile force; the latter easily explained on the basis of insufficient extracellular calcium to maintain an intact excitation-contraction coupling axis. In hearts perfused with 0.1 mM or greater concentrations of Ca^{2+}, contractile force recovered completely and sarcolemmal Ca^{2+} ATPase was unaltered ($P > 0.05$) when Ca^{2+} was reintroduced. When the preperfusion medium contained 0.05 mM Ca^{2+}, reperfusion was associated with only partial recovery of contractile

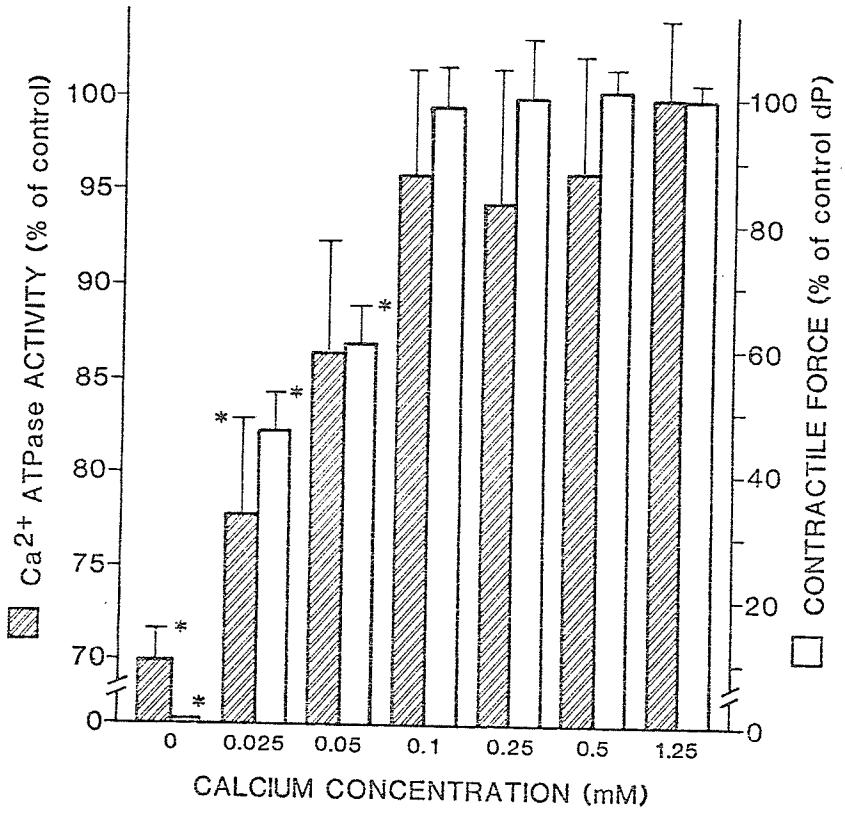

FIGURE 11. Effect of reperfusion for 5 minutes with medium containing 1.25 mM calcium on sarcolemmal Ca^{2+} ATPase activity and contractile force in hearts perfused for 5 minutes with medium containing different concentrations of calcium. Contractile force is expressed as a percent of the force generated by the heart (dP) during the equilibration period. Results are expressed as a mean ± SE of experiments. The control value for Ca^{2+} ATPase was 44.1 ± 4.3 μmol Pi/mg protein/hr. *Significantly different from control ($P < 0.05$).

force ($P < 0.05$) but Ca^{2+} ATPase though somewhat reduced (17.5%), was not significantly different from control ($P > 0.05$). A further reduction in the preperfusion Ca^{2+} concentration was found to be associated with significant ($P < 0.05$) depression in ATPase activity and contractile force generation; reperfusion was associated with a 30% inhibition of Ca^{2+} ATPase activity.

The possible existence of a relationship between contractile force and Ca^{2+} ATPase activity was tested further using hearts perfused with Ca^{2+}-free medium for different durations. In earlier reports (3,6) varying degrees in contractile force recovery can be achieved during reperfusion as long as the Ca^{2+}-free period is less than 5 min. In Fig 12 these perfusion protocols were employed and Ca^{2+} ATPase and contractile force measured. Reperfusion after one min of Ca^{2+} deprivation allowed for complete return of control force generation and sarcolemmal Ca^{2+} ATPase activity was unaffected. However, in hearts perfused with Ca^{2+}-free medium for 2 or 3 min an incomplete ($P < 0.05$) recovery of contractileforce was noted (84% and 29% respectively) upon reperfusion, but sarcolemmal membranes isolated from these hearts demonstrated only slightly less than ($P > 0.05$) control values for Ca^{2+} ATPase. This was in contrast to the effects of reperfusing hearts preperfused with Ca^{2+}-free solution for more than 3 min. In this case significant ($P < 0.05$) depression was observed in both parameters with complete loss of force associated initially with a 40% reduction in Ca^{2+} ATPase activity even though an even greater depression in ATPase activity could be obtained by reperfusion after 10 min of Ca^{2+}-free exposure.

In addition to inducing an inability to generate active tension,

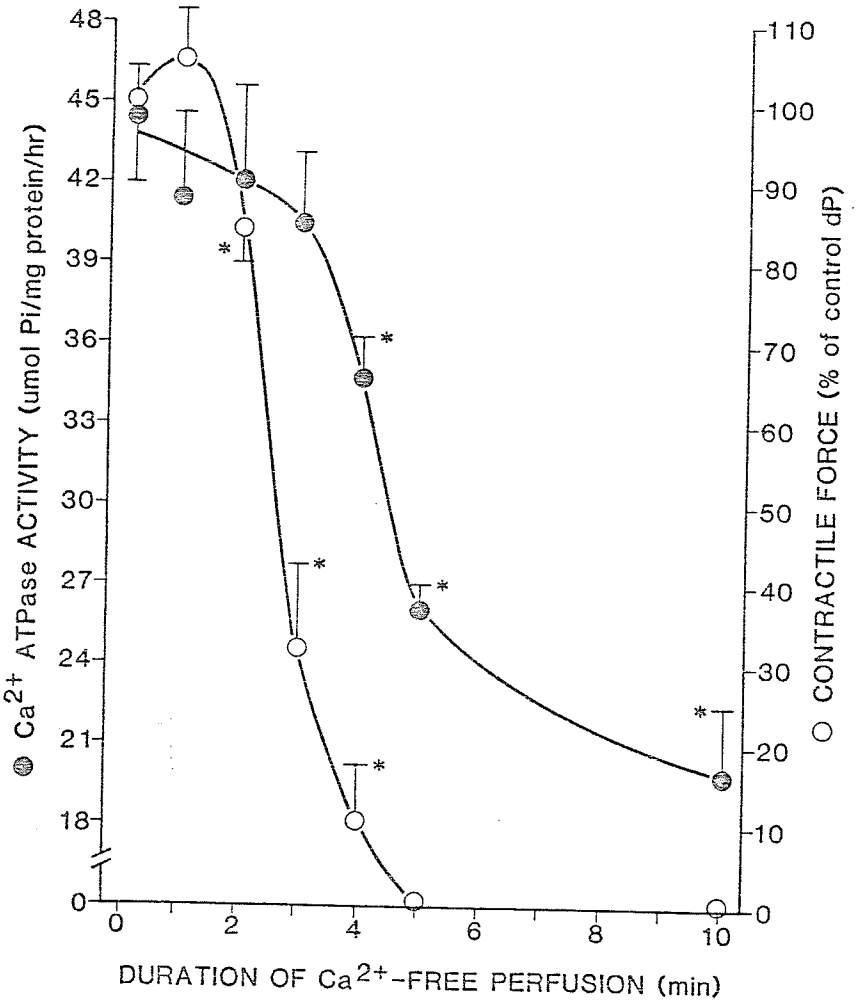

FIGURE 12. Effect of reperfusion for 5 minutes with medium containing 1.25 mM calcium on sarcolemmal Ca^{2+} ATPase activity and contractile force in hearts perfused for different intervals with Ca^{2+}-free medium. Contractile force is expressed as a percent of the force generated by the heart (dP) during the equilibration period. Results are expressed as a mean ± SE of 5 experiments for each point. *Significantly different from control ($P < 0.05$).

reperfusion of Ca^{2+} deprived hearts results in a marked rise in resting tension which is known to be dependent on the reperfusion (extracellular) Ca^{2+} concentration which in turn governs the degree of intracellular calcium overload (6). The next series of experiments investigated the effects of reperfusing 5 min Ca^{2+}-depleted hearts, with different concentrations of Ca^{2+}, on sarcolemmal Ca^{2+} ATPase activity and whole heart resting tension. The results are reported on Fig. 13. As long as the reperfusion medium contained at least 0.1 mM Ca^{2+} a significant ($P < 0.05$) rise in resting tension was observed. Five times more Ca^{2+} (0.5 mM) was required in order for 5 min of reperfusion to induce a significant depression in Ca^{2+} ATPase activity indicating once again that changes in mechanical performance are more sensitive than and preceed that of enzyme activity.

Previous studies (3,4,6,76) have demonstrated that hypothermia or hyponatremia during Ca^{2+}-free perfusion, prevent the reperfusion-induced changes seen in the calcium paradox. To see if the integrity of Ca^{2+} ATPase is also preserved hearts were perfused for 5 min with Ca^{2+}-free media under 3 different conditions and the effects of varying durations of reperfusion studied (Fig 14). The open squares represent hearts perfused with Ca^{2+}-free medium at 21°C. The open triangles represent hearts perfused with Ca^{2+}-free K-H solution in which the Na^{+} concentration was reduced to 35 mM while the solid circles refer to normothermic, normonatremic Ca^{2+}-free perfusion. Regardless of the duration of reperfusion hypothermia and hyponatremia prevented ($P > 0.05$) the loss in Ca^{2+} ATPase activity and in all cases contractile force recovered fully (data not reported). In hearts

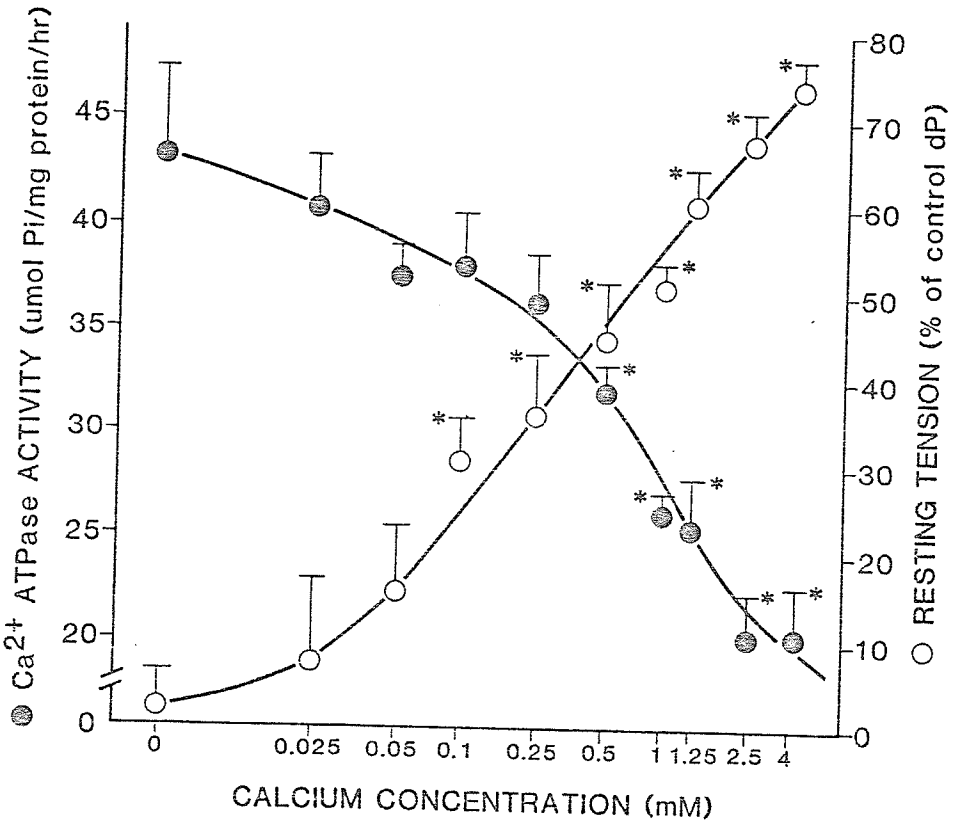

FIGURE 13. Effect of reperfusion for 5 minutes with medium containing different concentrations of calcium on sarcolemmal Ca^{2+} ATPase activity and resting tension in hearts perfused with Ca^{2+}-free medium for 5 minutes. Resting tension is expressed as a percent of the active tension generated by the heart (dP) during the equilibration period. Results are expressed as a mean ± SE of 4 experiments for each point. *Significantly different from control ($P < 0.05$).

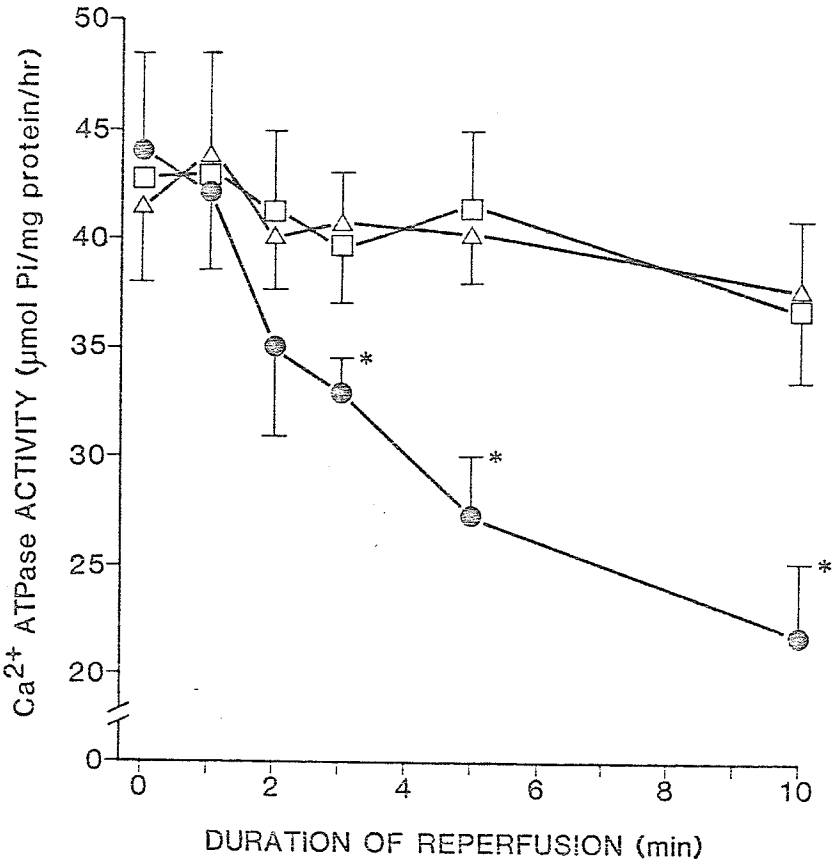

FIGURE 14. Effect of reperfusion for different intervals with medium containing 1.25 mM calcium on sarcolemmal Ca^{2+} ATPase activity of hearts perfused for 5 minutes with Ca^{2+}-free medium at 21°C (□), with Ca^{2+}-free medium containing 35 mM Na^{+} (△) or with normothermic (37.5°C) normonatremic (145 mM Na^{+}) Ca^{2+}-free medium(●). Results are expressed as a mean ± SE of 5 experiments for each point. *Significantly different from control ($P < 0.05$).

perfused with Ca^{2+}-free medium under standard conditions, the depression in Ca^{2+} ATPase activity was found to be dependent on the duration of reperfusion; the longer the period of Ca^{2+} reintroduction the more marked the loss of enzyme activity. Significant ($P < 0.05$) depression however was first seen in those hearts reperfused for 3 min. If the duration of reperfusion was shorter than 3 min, small though insignificant ($P > 0.05$) depression was apparent but as has been reported earlier, these hearts demonstrate the full range of calcium paradoxic changes including the absence of contractile force and increased resting tension.

In the last experiment, control and reperfused (5 min Ca^{2+}-free with 5 min reperfusion) heart sarcolemmal Ca^{2+} ATPase was analyzed kinetically and the Km and Vmax values were determined for each preparation by varying the ATP concentration of the incubation medium. These data and the Lineweaver-Burk plots are given in Fig 15. The control Km was found to be 0.31 mM ATP and this did not change in reperfused heart sarcolemmal. Vmax however was approximately 64 μmoles Pi/mg prot/hr for control and 37 μmoles Pi/mg prot/hr for reperfused sarcolemmal Ca^{2+} ATPase.

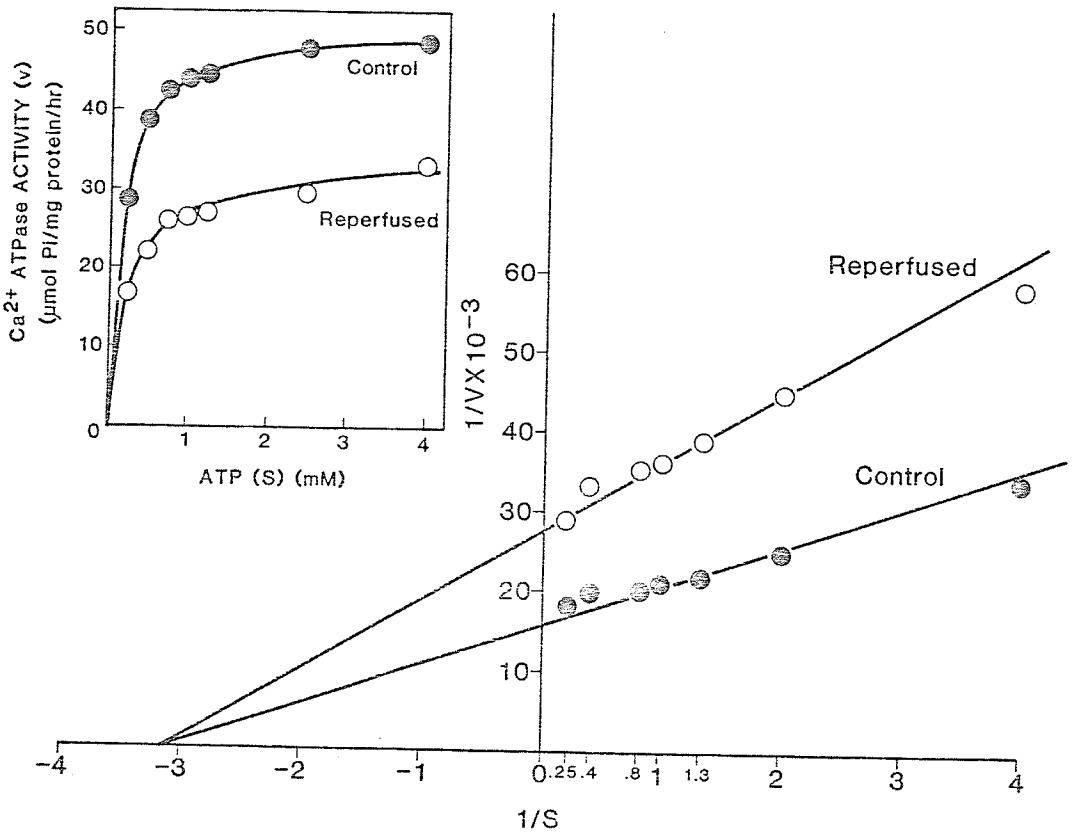

FIGURE 15. Sarcolemmal Ca^{2+} ATPase activities from control and reperfused rat hearts at different ATP concentrations. Conditions are the same as those described in Figure 7. Results are expressed as a mean of 3 experiments.

V. DISCUSSION

A. Microsomal Ca^{2+} Transport in the Calcium Paradox

The inability of the isolated rat heart to recover contractile function when Ca^{2+} is reintroduced after a brief period of Ca^{2+}-free perfusion, has intrigued investigators since the observation was first made by Zimmerman and Hulsmann (1,2). The reperfusion-induced intracellular calcium overload which results (6) is believed to be due to an alteration in the ability of the myocardium to regulate the level of intracellular calcium. Such an alteration may be partly due to a defect in the cardiac sarcoplasmic reticulum, which is considered to be intimately involved in intracellular Ca^{2+} homeostasis and contractile function (12,13). This viewpoint is supported by the results of the present study as well as earlier observations (34) indicating that reperfusion of Ca^{2+}-deprived hearts produces a defect in microsomal Ca^{2+}-uptake and binding. These microsomal changes were not seen in hearts perfused with Ca^{2+}-free media alone, and seemingly not apparent due to major contamination by other subcellular structures. Furthermore, the results are not likely to be due to artifacts of the isolation procedure or differences in assay conditions. The depression in Ca^{2+} accumulation cannot be attributed to gross changes in the protein pattern or phospholipid composition of reperfused heart microsomes. While the possibility exists that subtle changes in membrane composition may contribute to the depressed activity, an analysis of this was limited by the techniques employed in the present study. The ultrastructural appearance of microsomal membranes which demonstrated impaired Ca^{2+} transport, was also not different from control in spite of the swelling seen in the sarcoplasmic

reticulum when reperfused hearts were examined under the electron microscope (3,4). Depressed Ca^{2+}-uptake is most likely due to the decreased activity of the sarcoplasmic reticular Ca^{2+}-pump ATPase though it should be noted that the 100,000 dalton protein peak, considered to represent Ca^{2+}-stimulated ATPase (144), was essentially unaltered in reperfused heart microsomes. The degree of depressed ATPase activity however, was greater than the loss of Ca^{2+} accumulation. Since this study was not designed to investigate the stoicheometic relationship, due to low Ca^{2+}-stimulated and high basal ATPase activities of rat heart microsomes, further interpretation must await future studies employing other experimental models. In so much as marked changes in reperfused heart microsomal membranes could not be demonstrated the results suggest that conformational alterations in the sarcoplasmic reticulum, in addition to inactivation of Ca^{2+} ATPase or its uncoupling upon reperfusion, may be responsible for the impairment of active accumulation by the isolated microsomal fractions.

Defective sarcoplasmic reticular function may account for impaired contractile force under conditions where the loss of contractile force was partially reversible due to reperfusion. According to the current state of the excitation-contraction coupling hypothesis, depolarization of the sarcolemma induces either directly or indirectly via trigger Ca^{2+} (52,52), the sarcoplasmic reticulum to release its Ca^{2+} stores and along with transsarcolemmal Ca^{2+}-influx, raise the cytosolic Ca^{2+} concentration from approximately 10^{-7} to 10^{-5}M and activate the contractile proteins. Accordingly, it would seem likely that a defect in sarcoplasmic reticular Ca^{2+} uptake would decrease

the stores making less Ca^{2+} available for release upon excitation of the myocardium and this would be translated into an impairment of contractile force development. Some information regarding this point can be gained from a relationship between changes in contractile force and microsomal calcium uptake under the experimental conditions employed in this study, by plotting data from Tables 1,2 and 5. It can be seen from Fig. 5 that the recovery of contractile force begins to decline when a decrease of about 20% in microsomal calcium uptake is evident whereas a complete loss of contractile force recovery is seen when caclium uptake decreases by about 60%. Although the relationship between these parameters is sigmoidal in nature, the linear portion of the curve had a regression coefficient of 0.87. These data thus point out the major role played by the sarcoplasmic reticulum in the reperfusion phenomenon and the reversibility of calcium paradoxic hearts. A plot of data from Table 1,2 and 5 also shows a sigmoidal relationship between increases in resting tension and changes in microsomal calcium uptake (Fig. 5). It is evident that resting tension begins to rise when the calcium uptake is depressed by about 35% whereas resting tension reaches its maximum when calcium uptake is decreased by about 60%. Although an inverse relationship between microsomal calcium uptake and resting tension was also evident when the Ca^{2+} depleted hearts were reperfused with medium containing different concentrations of calcium (Fig. 4), changes in resting tension in this situation were found to preceed changes in calcium uptake. Such a conclusion can also be drawn from experiments in which Ca^{2+} depleted hearts were reperfused for different time intervals (Fig. 3). Thus the role of impraired sarcoplasmic reticular calcium transport

in resetting myocardial resting tension is not straight forward in light of data obtained in this study. Several factors including increased sarcolemmal calcium permeability and subsequent intracellular calcium overload (6), increased mitochondrial calcium uptake (34), depletion of ATP (35,74,99,105) and possibly altered state of contractile and regulatory proteins may also be contributing to changing the myocardial resting tension due to Ca^{2+} paradox phenomenon. When the data in this study was examined in the light of our previous results on changes in myocardial calcium contents (6), it became apparent that the observed changes in microsomal calcium transport occurred secondary to Ca^{2+} overload and thus it was not unusual to observe an increase in resting tension before detecting changes in microsomal calcium uptake in some experiments, Thus these alterations seem to play a crucial role in the inability of the heart to recover its contractile force and may contribute in increasing resting tension upon reperfusion of Ca^{2+}-deprived hearts. This view is consistent with the involvement of intracellular calcium overload in the genesis of heart failure in certain types of experimental models (7,8,9,103).

B. Sarcolemmal Na^+/K^+ ATPase in the Calcium Paradox

Major alterations in myocardial Na^+ and K^+ contents have been reported to occur under conditions of the calcium paradox (6). During Ca^{2+}-free perfusion, K^+ has been shown to leak out of the isolated heart (15) and thereby depleting intracellular levels (6), apparently due to an increase in K^+ permeability caused by a specific Ca^{2+}-dependent alteration in the lipid bilayer and not in the glycocalyx, which is believed to regulate Ca^{2+} permeability (5). Myocardial Na^+

contents on the other hand, do not change appreciably during the period of Ca^{2+}-depletion but a tendency towards a mild increase, with longer durations (10 min) of Ca^{2+}-free perfusion, have been reported (6). In an earlier report (151), 20 min of Ca^{2+}-free perfusion was necessary before a significant depression in Na^+/K^+ ATPase could be observed. Kramer et al (83) found no change in Na^+/K^+ ATPase activity after 9 min of Ca^{2+}-free exposure. In the present study, 10 min or less Ca^{2+} deprivation had virtually no effect on Na^+/K^+ ATPase in spite of the fact that this perfusion protocol results in significant depression in myocardial K^+ contents (6). It seems therefore, that K^+ loss during short periods of Ca^{2+}-free perfusion, occurs independently of the Na^+/K^+ pump. Subsequent alterations in K^+ and possibly Na^+ contents may be augmented by the development of a faulty pump mechanism as the duration of Ca^+ depletion lengthens.

When Ca^{2+} is reintroduced to Ca^{2+} deprived hearts the calcium paradox (1,2) results and this is accompanied by a further and more marked loss of cellular K^+ and increase in myocardial Na^+ contents (6). These reperfusion-induced cation alterations were originally suggested to be due to sarcolemmal disruption which allowed both K^+ and Na^+ fluxes to occur passively according to their concentration gradients. In view of the severe ultrastructural damage to the sarcolemma during the calcium paradox, this concept has not been challenged and consequently a role for the Na^+/K^+ pump has been largely overlooked. In this study we have characterized the changes that occur in Na^+/K^+ ATPase and Mg^{2+} ATPase activities under conditions of reversible, partly reversible and irreversible stages of the calcium paradox

phenomenon.

Sarcolemmal membranes isolated from hearts reperfused for 5 min with normal Ca^{2+}-containing medium after 5 min of Ca^{2+}-free perfusion, were found to have decreased Na^{+}/K^{+} ATPase and Mg^{2+} ATPase activities. This perfusion protocol results in full expression of the calcium paradox. These findings support earlier observations (83,99) of depressed sarcolemmal enzyme activity in reperfused hearts. In addition we have demonstrated significant differences in the sensitivites of these membrane bound enzymes to the effects of reperfusion. Under similar conditions, Na^{+}/K^{+} ATPase activity was consistently more depressed than Mg^{2+} ATPase. Within 1 min of reperfusion after 5 min of Ca^{2+}-free exposure, Na^{+}/K^{+} ATPase activity was depressed to less than 50% of control values while Mg^{2+} ATPase though significantly affected, still retained approximately 74% of control activity. Except for calcium overload (twice control (6)) and depleted high energy phosphates (half of control (35)), no other characteristic of the calcium paradox in its early phases, matches the suddeness and extent of Na^{+}/K^{+} ATPase inhibition. As with other features of the calcium paradox, alterations in sarcolemmal Na^{+}/K^{+} ATPase and Mg^{2+} ATPase activities were dependent upon the duration and calcium concentration of the initial and reperfusion periods. In both enzymes, the mechanism of depression was related to changes in the Vmax without changing Km. While the exact nature of the change in Vmax in unknown, the observation that reperfused heart sarcolemmal Na^{+}/K^{+} ATPase is more sensitive to the inhibitory action of ouabain suggests that an alteration in protein configuration or protein-phospholipid interaction may have occurred and in so doing have altered

the activity of the enzyme.

Hypothermia or reducing the Na^+ concentration during the Ca^{2+}-free perfusion period are known to prevent the development of reperfusion-induced paradoxical calcium necrosis and cardiac contractile failure (4,6,76). These conditions were also found to protect sarcolemmal Na^+/K^+ ATPase and Mg^{2+} ATPase from the deleterious effects of the calcium paradox.

We have also analyzed the possiblity that reperfusion-induced increases in myocardial Na^+ content and decreased K^+ content may be related to impaired Na^+/K^+ ATPase activity, since under normal conditions this enzyme is responsible for maintaining the chemical gradients of Na^+ and K^+. Data from our earlier work concerning myocardial cation contents (6) were plotted against the activities of Na^+/K^+ ATPase measured under identical conditions to those of our previous investigation. What emerged was a rather striking relationship between increased Na^+-decreased K^+ contents and impaired Na^+/K^+ ATPase activity. It appears that as long as 60-70% of Na^+/K^+ ATPase activity is present the Na^+/K^+ pump can maintain normal intracellular levels of Na^+ and K^+. A further loss in enzyme activity begins to manifest itself through changes in myocardial Na^+ and K^+ such that when ATPase activity has fallen to 35-40% of control, rapid increases in Na^+ and decreases in intracellular K^+ levels results. It is interesting to note that the perfusion conditions which relate to this transition zone also correspond to the point where partially reversible contractile failure becomes completely irreversible under the experimental conditions of the calcium paradox.

A great deal of attention has been paid to assessing the status

of Na^+/K^+ ATPase in different types of heart failure and its role in heart function. Increased Na^+/K^+ ATPase activity has been observed in heart failure due to mitral valve insufficiency (152) and in moderate stages of failure in the B10 14.6 strain of cardiomyopathic hamsters (153). Prolonged treatment with digitoxin and potassium-deficient diet (154) or with thyroid hormone (155) also increases Na^+/K^+ ATPase activity. Decreased enzyme activity has been documented in failure due to aortic constriction (156), bacterial endocarditis (156), cardiomyopathy (UM-X7.1) (134) and myocardial ischemia (158). It is evident therefore that heart failure can be associated with increased or decreased enzyme activity, making a clear relationship between heart function and Na^+/K^+ ATPase activity impossible. Such a complex relationship may relate to the type and degree of heart failure so that increased pump activity may serve as an adaptive mechanism whereas decreased Na^+/K^+ ATPase may be associated with depressed contractile function under pathophysiological conditions.

There is evidence however that changes in Na^+/K^+ ATPase may relate to calcium movements which in turn would alter the contractile state. The positive inotropic effect of cardiac glycosides is believed to be due to inhibition of Na^+/K^+ ATPase which leads to an increase in intracellular Na^+ content and an augmentation of Ca^{2+} influx via the Na-Ca exchange mechanism (8,159). As has been suggested previously (6,33), there is growing evidence that one of the routes of calcium entry responsible for reperfusion activated calcium overload may be via Na-Ca exchange. The present study supports this contention by implicating a mechanism whereby one of the prerequisites

for Na-Ca exchange is satisfied. An early and rapid depression in the Na^+-K^+ pump upon reperfusion, can be conceived to cause an increase in intracellular Na^+ which would favor Na-Ca exchange in the direction of Ca^{2+} influx (67,68,160-162). This might explain why the eventual increase in intracellular Na^+ lags behind calcium overload in the calcium paradox (6). That Na-Ca exchange is not the only route of massive calcium influx during reperfusion of Ca^{2+}-depleted hearts (33)is re-emphasized, but it does appear that alterations in sarcolemmal Na^+/K^+ ATPase may contribute directly to the observed changes in cellular Na^+ and K^+ and indirectly to the development of intracellular calcium overload during the calcium paradox phenomenon.

C. Sarcolemmal Ca^{2+} ATPase in the Calcium Paradox

In 1976, Ruigrok et al (105) demonstrated the energy dependence of the calcium paradox. These investigators found an inability to induce the calcium paradox in hearts depleted of their high energy phosphate reserves and unable to carry out oxidative phosphorylation. The suggestion was made that the presence of ATP or electron transport is a prerequisite for the occurrence of Ca^{2+}- induced cell damage. Hearse (10) has also suggested that the damaging calcium influxes in the calcium paradox are energy dependent. The observation that reperfusion at 4^oC does not prevent the calcium paradox has been taken to mean that energy is not required (21) but the fact that the effects of reperfusion are slightly reduced upon reintroduction of Ca^{2+} at 4^oC suggests that some energy dependent component is involved.

In the normal heart the mechanisms by which Ca^{2+} enters the myocardium during the slow inward current is thought to involve membrane

channels or "gates". This gating mechanism is believed to be a metabolically controlled process (12,163,164) in spite of the fact that calcium influx occurs down its concentration gradient. Sarcolemmal Ca^{2+}-dependent ATPase has recently been implicated in the gating mechanism whereby channels are opened in the membrane at the expense of ATP hydrolysis so as to allow Ca^{2+} influx to occur passively down its concentration gradient. Support for the role of Ca^{2+} ATPase in regulating Ca^{2+} influx has come from studies on the effects of various interventions, known to modify Ca^{2+} fluxes, on sarcolemmal Ca^{2+} ATPase activity. Electrical stimulation (12,165) and membrane phosphorylation via cAMP-protein kinase (165,166) have been shown to increase Ca^{2+} ATPase activity. Ca^{2+} ATPase is maximumly stimulated by millimolar concentrations of Ca^{2+} (167) which characterize the extracellular environment. Furthermore, heavy metals like Mn^{2+}, Co^{2+}, Ni^{2+} and La^{3+} which depress Ca^{2+} influx also inhibit Ca^{2+} ATPase activity (136,168). It is apparent then that fluxes of calcium across the sarcolemma under normal conditions and during the calcium paradox, are energy dependent mechanisms which may be related to sarcolemmal Ca^{2+} ATPase activity.

Recently it has been demonstrated that cardiac contractile force and sarcolemmal Ca^{2+} ATPase activity closely parallel each other in the presence of varying Ca^{2+} concentrations (146). As can be appreciated from Fig. 11, the ability of isolated perfused rat hearts to recover contractile force under reversible and irreversible conditions of the calcium paradox, was related to Ca^{2+} ATPase activity. Further analysis of this relationship under conditions described for Fig. 12, demonstrate that depressed recovery of contractile force preceeds

changes in Ca^{2+} ATPase activity in the early phases of the calcium paradox. However the possibility exists that in later stages, depressed Ca^{2+} ATPase may contribute to impaired contractile performance and the eventual irreversibility of heart function. This suggestion is supported by the present results comparing enzyme activity to the degree of contracture (increased resting tension) in hearts where active tension generation has been irreversibly lost. A close inverse relationship between depressed Ca^{2+} ATPase activity and increased resting tension can be seen in Fig. 13. Furthermore, it should be pointed out that depression in sarcolemmal Ca^{2+} ATPase activity has been demonstrated to occur only in the late stages of heart failure due to a wide variety of interventions (9,146).

In the present study the immediate effects of reperfusion of calcium deprived hearts were not associated with major changes in Ca^{2+} ATPase activity (Fig. 14). This is in spite of the fact that 1-2 min of reperfusion with Ca^{2+} after 5 min of Ca^{2+}-free perfusion, is associated with marked intracellular calcium overload (6) and ultrastructural damage (2,3). It appears therefore, that in the initial phases of reperfusion, Ca^{2+} ATPase retains its function and so will continue to favour the influx of calcium and the development of calcium overload with resulting impairment in mechanical performance. Subsequent impairment in Ca^{2+} ATPase activity may then reflect a consequence of moderate calcium overload. This can be conceived to result from the calcium-dependent activation of phospholipases (169) and proteases (170). Indeed, a decrease in the magnitude of several sarcolemmal protein peaks was observed but the present study has demonstrated only minor changes in sarcolemmal phosphipid composition.

Why these destructiye processes do not appear to affect Ca^{2+} ATPase initially, can be explained on the basis of earlier studies on the effects of phospholipases (171) and trypsin (172) on sarcolemmal Ca^{2+} ATPase activity. Ca^{2+} ATPase was found to be resistant to the effects of various phospholipases while incubation of sarcolemmal membranes in the presence of trypsin initially increased and then with time caused a decrease in Ca^{2+} ATPase activity. This secondary effect of calcium overload on ATPase activity is further supported by the present observations that interventions like hypothermia and low Na^{+} during the Ca^{2+}-free period, which prevent calcium overload also prevent the reperfusion-induced changes in Ca^{2+} ATPase activity.

Decreased Ca^{2+} ATPase activity was related to changes in Vmax and not Km of the enzyme. This depressed activity was not related to contamination of our sarcolemmal membrane preparation by other subcellular constituents nor was it related to major changes in membrane phospholipid composition, though a significant decrease in phosphatidyl serine was noted. The significance of this observation is unclear at present. Changes in Ca^{2+} ATPase activity may reflect generalized changes in the sarcolemmal membrane since other membrane bound enzymes such as Mg^{2+} ATPase, Na^{+}/K^{+} ATPase and adenylate cyclase are also decreased in reperfused heart sarcolemma.

D. Sequence of Events in the Calcium Paradox

Using observations reported in the literature and the results of the present investigation a sequence of events during the calcium paradox can be constructed. After a critical period of time and in the presence of less than 25 μmoles of Ca^{2+}, perfusion of the isolated heart results in the loss of a significant proportion of sarcolemmal

associated Ca^{2+} such that the normal permeability characteristics of the plasma membrane are altered. When Ca^{2+} is reintroduced, a sudden increase in energy dependent Ca^{2+} influx occurs which is partially supported by an intact Ca^{2+} ATPase as well as an enhanced Na-Ca exchange mechanism; the later augmented by a depressed Na^{+}/K^{+} pump. The sites involved in these activated Ca^{2+} movements are the glycocalyx-lipid bilar complex as well as the intercalated disc region. This initial calcium overloading overwhelms mitochondrial and sarcoplasmic reticular Ca^{2+} accumulating mechanisms resulting in impaired contractility and a degree of cardiac contracture. Simultaneous to these mechanical effects, calcium overload sets into motion a series of deleterious structural and biochemical processes. High energy phosphates are depleted due to the activation of various Ca^{2+} stimulated ATPases as well as the inhibitory effect of mitochondrial calcium overloading on oxidative phosphorylation. Ca^{2+} activated proteases and phospholipases along with inadequate energy reserves then augment further membrane damage and intracellular contents appear in the extracellular space. Subsequent to this, impaired sarcoplasmic reticular calcium uptake and membrane bound enzyme activity,as well as sarcolemmal disruption,result making it impossible for the myocardial cell to regulate intracellular cations. The degree of contracture is enhanced and membrane systems become so compromised that normal function is lost. As the duration of reperfusion continues the changes progress, eventually becoming so marked that all the manifestations of the calcium paradox become readily apparent.

In conclusion, the present study has demonstrated that alterations in sarcoplasmic reticular and sarcolemmal function are involved in

the development of intracellular calcium overload and contractile failure. Furthermore it is obvious that the subcellular events which typify the calcium paradox are extremely complex and further research will hopefully clarify the exact nature of the paradoxical effects of calcium.

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