Behaviour of Cardiac Microsomal Ca^{2+} Pump Under Conditions That May Simulate Pathological Situations

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Abstract. The behaviour of Ca^{2+} ATPase activity in relation to Ca^{2+} transport process was studied under different experimental conditions in canine cardiac microsomal fraction predominantly containing sarcoplasmic reticulum. The total Ca^{2+} concentration required for half maximal activation (Ka) of microsomal Ca^{2+} ATPase and Ca^{2+} uptake did not differ significantly, unless 0.1 mmol/l EGTA was present in the incubation media. Pretreatment of cardiac microsomes with membrane disruptive agents like phospholipase A, trypsin as well as deoxycholate strongly increased (2—3 fold) Ca^{2+} ATPase activity but uptake rate of Ca^{2+} declined. Only in phospholipase C and β-glucuronidase pretreatment, a parallel decrease of Ca^{2+} ATPase and uptake was observed. In presence of excess (free)Ca^{2+} (>10 μmol/l) both Ca^{2+} ATPase as well as Ca^{2+} uptake were inhibited, however, Ca^{2+} binding process remained unaltered. Likewise, low pH completely altered the relation between Ca^{2+} binding and ATPase activity; whereas Ca^{2+} ATPase was inhibited, Ca^{2+} binding did not change. Our present data provide evidence for some cellular factors that may be involved in producing uncoupling of microsomal Ca^{2+} ATPase from Ca^{2+} accumulation process that was previously observed in various pathological situations.

Key words: Ca^{2+}-stimulated ATPase — Ca^{2+} uptake — Ca^{2+} binding — Membrane disruptive agents — Dog heart microsomes

Introduction

Sarcoplasmic reticulum (microsomal fraction) from the cardiac muscle is shown to possess a remarkable ability to transport Ca^{2+} in an energy dependent manner and this capability has been considered to be intimately associated with the relaxation of the myocardium (Ebashi et al. 1971; Martonosi 1972; Weber and Murray 1973; Van Winkle and Entman 1979; Dhalla et al. 1982). In addition, it may serve as an intracellular source of Ca^{2+} the release of which stimulates contractile protein

upon depolarization of cardiac cell membrane. Therefore, it is well expected that changes in the capacity of microsomes to handle Ca^{2+} efficiently would have a strong impact on the performance of the cardiac contractile machinery. Several investigators have reported varying degrees of defects in cardiac microsomal Ca^{2+} accumulating properties in different types of failing hearts (Dhalla et al. 1978, 1982; Bing 1983). Although an abnormality in microsomal Ca^{2+} uptake in failing heart is usually found to be associated with a depression in microsomal Ca^{2+}-stimulated Mg^{2+} dependent ATPase activity (Gertz et al. 1967; Suko et al. 1970; Lamers and Stinis 1979), the exact mechanisms for such a change are far from being clearly understood. Furthermore, normal (Dhalla et al. 1979) or even high (Muir et al. 1970; Limas and Cohn 1977) Ca^{2+}-stimulated ATPase activity has also been observed in microsomes exhibiting reduced Ca^{2+} uptake activity under certain models of heart failure. Since changes in the function of microsomal Ca^{2+} transport system are generally considered to be due to alteration in the phospholipid-protein interaction (Owens et al. 1973; Wang et al. 1979), it was thought of interest to examine the behaviour of Ca^{2+} ATPase and Ca^{2+} uptake activities in isolated microsomal membrane preparation upon treatment with various membrane disruptive agents. In addition, the present report also deals with the effects of high Ca^{2+} and H^+ concentrations on microsomal Ca^{2+} transport process, since a large body of evidence indicates that both Ca^{2+} and H^+ which are increased in situation such as ischaemic heart disease (Serur et al. 1976; Dhalla et al. 1978, 1982), activate various degradative enzymes (Pieterson et al. 1974; Ignarro 1975; Dayton et al.1976) and participate in the generation of subcellular damage.

Materials and Methods

Cardiac microsomes enriched in sarcoplasmic reticulum were isolated from mongrel dogs in a manner similar to that previously reported by Harigaya and Schwartz (1969) with slight modification (Dhalla et al. 1970, 1983). In brief, the hearts were removed quickly, thoroughly washed in 0.25 mol/l sucrose containing 1 mmol/l EDTA, pH 7.0 and homogenized in 10 volumes of a medium containing 10 mmol/l $NaHCO_3$, 5 mmol/l sodium azide and 10 mmol/l Tris-HCl, pH 6.8 in a waring blender at 0 – 5 °C for 45 s. The homogenate was filtered through four layers of gauze and centrifuged at $10,000 \times g$ for 20 min to remove cell debris, myofibrils, nuclei and mitochondria. The $10,000 \times g$ supernatant fraction was then centrifuged at $40,000 \times g$ for 45 min. The pellet thus obtained was thoroughly washed and resuspended in 8 volumes of 0.6 mol/l KCl and centrifuged at $40,000 \times g$ for 45 min. The sediment was suspended in a solution containing 0.3 mol/l sucrose, 10 mmol/l Tris-maleate, pH 7.0. The data concerning biochemical marker enzyme activities, sensitivity of ATP dependent Ca^{2+} uptake to inhibitors of mitochondrial Ca^{2+} transport and electronmicroscopic studies revealed negligible mitochondrial or myofibrillar contamination (Dhalla et al. 1970, 1983). The purified Ca^{2+} ATPase from microsomes was separated by a method previously described by MacLennan (1970) through the use of deoxycholate and salt fractionation.

Microsomal vesicles were digested by incubation with various membrane disruptive agents like phospholipase A, phospholipase C, trypsin, deoxycholate and β-glucuronidase for the different time

periods as indicated in the results. The exact experimental conditions are given in the legends to the figures.

Ca^{2+} uptake and Ca^{2+} binding activities of microsomes were determined using Millipore filtration technique. It is understood that Ca^{2+} binding refers to an ATP-dependent Ca^{2+} accumulation by subcellular particles in the absence of a permeant anion, whereas Ca^{2+} uptake refers to Ca^{2+} accumulation in the presence of a permeant anion-like oxalate. The most frequently used conditions for the measurement of Ca^{2+} uptake were as follows: 100 mmol/l KCl, 20 mmol/l Tris HCl; pH 6.8,5 mmol/l $MgCl_2$, 5 mmol/l ATP and 5 mmol/l K-oxalate. The microsomal protein concentration was 0.15 mg per ml; total volume was 1 ml. The reactions were initiated by the addition of either $^{45}CaCl_2$/ethylene glycol-bis (β-aminoethylether)-N, N'-tetra-acetate (EGTA) buffer (Katz et al. 1970) or $^{45}CaCl_2$ alone. Incubation was carried out at 37 °C for 3 min and was terminated by filtration through Millipore filters (0.45 μm). The amount of bound Ca^{2+} was calculated from the difference between the radioactivity of the original solution and the filtrate, based on the known concentration of Ca^{2+} in the system. For Ca^{2+} binding experiments, the microsomes (0.4 mg/ml) were incubated at 30 °C for 1 min in a similar medium used for Ca^{2+} uptake assay except K-oxalate was omitted from the incubation mixture.

Total (0.05 mmol/l $CaCl_2$, 5 mmol/l $MgCl_2$) and basal (0.1 mmol/l EGTA, 5 mmol/l $MgCl_2$) ATPase activities were determined in an incubation medium similar to that used for Ca^{2+} uptake assay, except that the reaction was initiated by the addition of 5 mmol/l ATP. The reaction was terminated after 5 min by 1 ml of 12% (w/v) cold trichloroacetic acid. Inorganic phosphate liberated into the protein-free filtrate was assayed by the procedure of Taussky and Shorr (1953). The Ca^{2+}-stimulated Mg^{2+} dependent ATPase reported here is the difference between the total and basal ATPase activities.

Protein concentrations were determined by the method of Lowry et al. (1951). All experiments were repeated at least two to four times. The values presented here are representative from single experiment carried out on the same day.

Results

Fig. 1 shows the Lineweaver-Burke plots of microsomal Ca^{2+} uptake and ATPase activity over a range of total Ca^{2+} concentrations (1 – 50 μmol/l). The average Ka of the Ca^{2+} uptake rate of the microsomal preparation (9 ± 1.8 μmol/l) was not statistically different ($P > 0.05$) from that of Ca^{2+} ATPase (7 ± 1.4 μmol/l). Thus, the dependence of rate of Ca^{2+} uptake and Ca^{2+} ATPase on total Ca^{2+} were same. However, when 0.1 mmol/l EGTA was used in the incubation medium the Ka for Ca^{2+} uptake (1.3 ± 0.2 μmol/l) was significantly different ($P < 0.05$) from Ca^{2+} ATPase activity (0.7 ± 0.1 μmol/l) indicating that EGTA itself might have some membrane effect and supports the findings as reported earlier by Berman (1982).

In order to understand the effect of more membrane disruptive agents on Ca^{2+} pump activity, the microsomes were treated with phospholipase C, phospholipase A, trypsin, deoxycholate and with a lysosomal enzyme — β glucuronidase. The membranes were washed and then Ca^{2+} ATPase and Ca^{2+} uptake were determined. Fig. 2 shows the treatment of phospholipase C on cardiac microsomal Ca^{2+} uptake and ATPase activity. Pretreatment of microsomes with phospholipase C led to a time dependent inhibition of both Ca^{2+} uptake and Ca^{2+} ATPase. The similar pattern with skeletal muscle microsomes has been previously shown by Martonosi

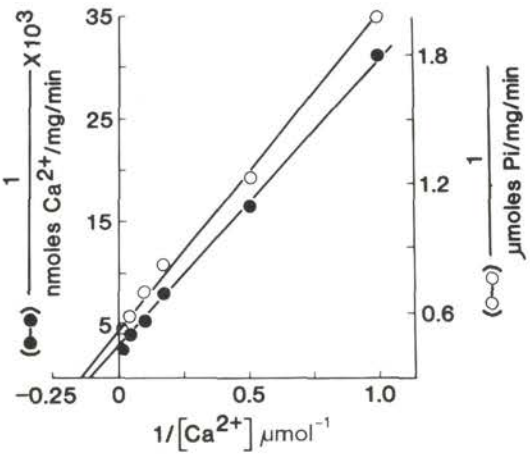

Fig. 1. Lineweaver-Burke plots of microsomal Ca^{2+} uptake(●—●) and Ca^{2+} ATPase (○—○) activity over a range of total Ca^{2+} concentrations (1—50 μmol/l). Reactions were carried out as described under "Methods". In a separate set of experiments, the Ka of both uptake and ATPase were determined (not shown) using $CaCl_2$/EGTA buffers and the free Ca^{2+} concentration was calculated according to Katz et al. (1970).

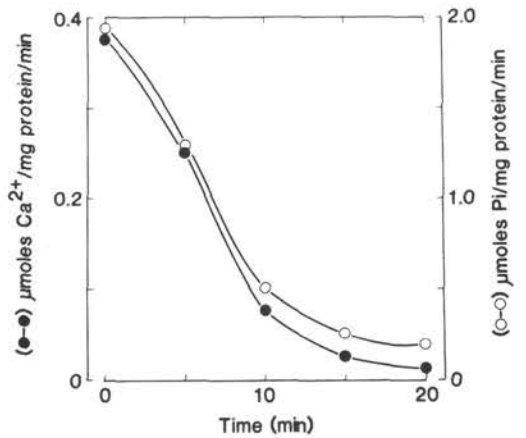

Fig. 2. The effect of treatment of phospholipase C on cardiac microsomal Ca^{2+} uptake (●—●) and Ca^{2+} ATPase (○—○) activity. Microsomal vesicles (5 mg/ml) were digested with phospholipase C (0.50 mg/mg protein) in a medium containing 100 mmol/l KCl, 20 mmol/l Tris-HCl; pH 7 in the presence of 3 mmol/l Ca^{2+} at room temperature for the different time periods as indicated. The treated vesicles were separated by centrifugation at 40,000 *g* for 45 min, resuspended in 50 mmol/l KCl, 20 mmol/l Tris-HCl; pH 6.8 and were then used for Ca^{2+} uptake and ATPase assay. Ca^{2+} uptake was measured as described under "Methods" in a medium containing 100 mmol/l KCl, 20 mmol/l Tris-HCl; pH 6.8, 5 mmol/l $MgCl_2$, 5 mmol/l ATP, 5 mmol/l K-oxalate, 0.05 mmol/l total $^{45}CaCl_2$ and 0.15 mg/ml membrane protein. ATPase assay was carried out with 0.05 mmol/l non radioactive $CaCl_2$ to the otherwise complete assay medium.

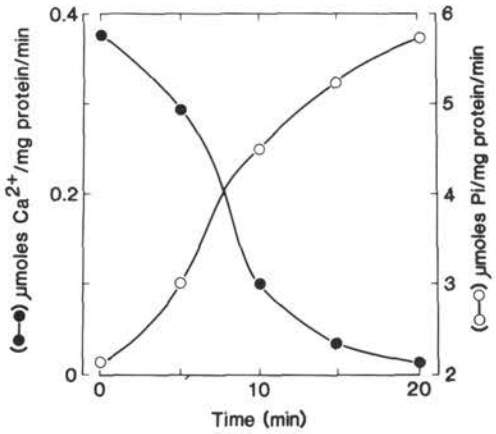

Fig. 3. The effect of treatment of phospholipase A on cardiac microsomal Ca^{2+} uptake (●—●) and Ca^{2+} ATPase (○—○) activity. Microsomal vesicles (5 mg/ml) were digested with phospholipase A (0.25 mg/mg protein) in a similar medium as described in Fig. 2. The treated vesicles were then used similarly for Ca^{2+} uptake and ATPase assay.

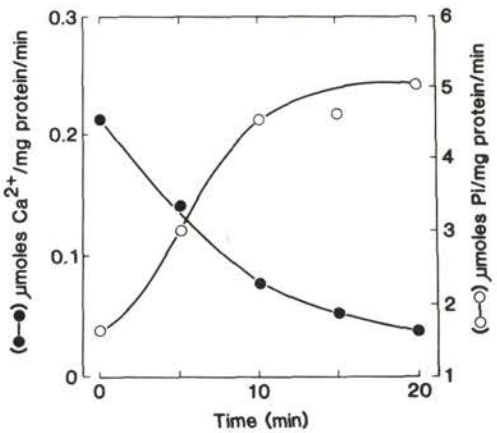

Fig. 4. The effect of treatment of trypsin on cardiac microsomal Ca^{2+} uptake (●—●) and Ca^{2+} ATPase (○—○) activity. Microsomal vesicles (2 mg/ml) were incubated with trypsin (0.1 mg/mg protein) in a similar medium described in Fig. 2. Aliquots were taken at intervals and the reaction stopped with the addition of 4 fold excess trypsin inhibitor. Ca^{2+} uptake and ATPase measurements were carried out as described previously.

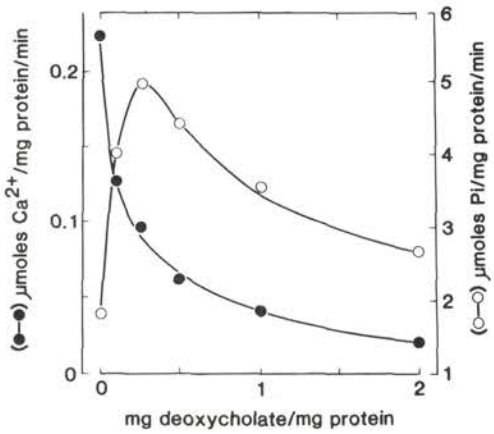

Fig. 5. The effect of treatment of deoxycholate on cardiac microsomal Ca^{2+} uptake (●—●) and Ca^{2+} ATPase (○—○) activity. Microsomal vesicles (5 mg/ml) were incubated with different concentrations of deoxycholate as indicated, for 20 min in a similar medium described in Fig. 2. The vesicles were then separated by centrifugation and the Ca^{2+} uptake and ATPase experiments were performed.

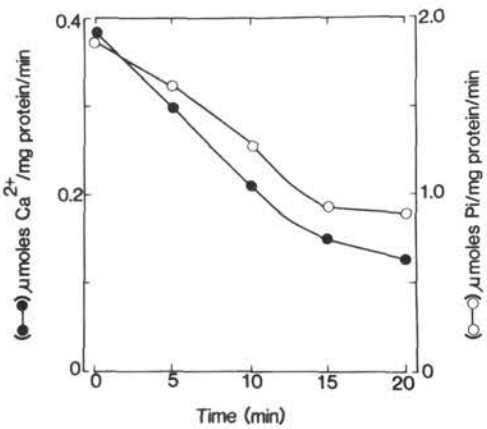

Fig. 6. The effect of treatment of β-glucuronidase on cardiac microsomal Ca^{2+} uptake (●—●) and Ca^{2+} ATPase (○—○) activity. Microsomal vesicles (2 mg/ml) were incubated with β-glucuronidase (0.05 mg/mg protein) at 37 °C and the treated vesicles were used for Ca^{2+} uptake and ATPase assay similarly as described in Fig. 2.

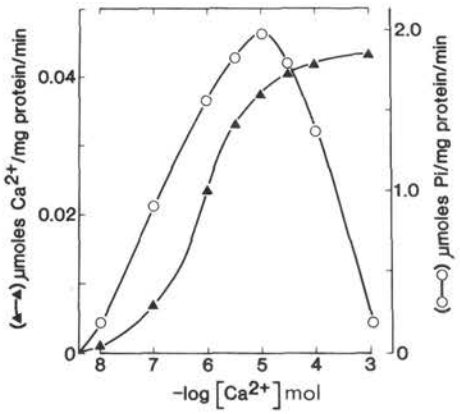

Fig. 7. Kinetic patterns of microsomal Ca^{2+} binding (▲—▲) and Ca^{2+} ATPase (○—○) over a range of free Ca^{2+} concentrations (10^{-8} to 10^{-3} mol/l). For Ca^{2+} ATPase assay, 0.15 mg/ml membrane protein was incubated in a medium containing 20 mmol/l Tris-HCl (pH 6.8), 100 mmol/l KCl, 5 mmol/l ATP, 5 mmol/l $MgCl_2$ and different concentrations of $^{45}CaCl_2$ using $CaCl_2$/EGTA buffers. Ca^{2+} binding was determined in the same assay medium except the membrane protein was 0.4 mg/ml.

et al. (1968). In sharp contrast to the phospholipase C action, an apparent dissociation of Ca^{2+} translocation process and ATPase was seen by phospholipase A. After 20 minutes of preincubation, the Ca^{2+} ATPase was increased to approximately 3 fold and Ca^{2+} uptake was decreased maximally (Fig. 3). However, Fiehn and Hasselbach (1970) did not find any change in the activity of Ca^{2+} ATPase under similar situation. The reason for this discrepancy in results is unclear at present but may entail methodological differences in experimental protocol.

In another series of experiments, microsomes were incubated with trypsin (Fig. 4). Quantitatively, the same results were obtained after 20 minutes of incubation. Ca^{2+} ATPase activity was found to increase in spite of the decrease of Ca^{2+} uptake process. When microsomes were treated with different concentrations (0.2—2 mg/mg protein) of deoxycholate, a similar non parellelism was observed but only with low concentration of deoxycholate. At higher concentrations, however, both Ca^{2+} ATPase and Ca^{2+} uptake were reduced simultaneously (Fig. 5). Since functional and structural disintegration of the cardiac microsomes by lysosomal enzymes have been suggested earlier (Dhalla et al. 1978, 1982), the effect of one of the enzymes from the lysosomal population, β-glucuronidase, was studied on the behaviour of the microsomal Ca^{2+} ATPase and Ca^{2+} uptake activity. Pretreatment of microsomes with β-glucuronidase resulted in inhibition of both Ca^{2+} ATPase and Ca^{2+} uptake in a time dependent manner (Fig. 6).

In order to investigate the ability of the microsomal Ca^{2+} transport system to handle Ca^{2+} and H^+, microsomal ATP dependent Ca^{2+} binding — a more

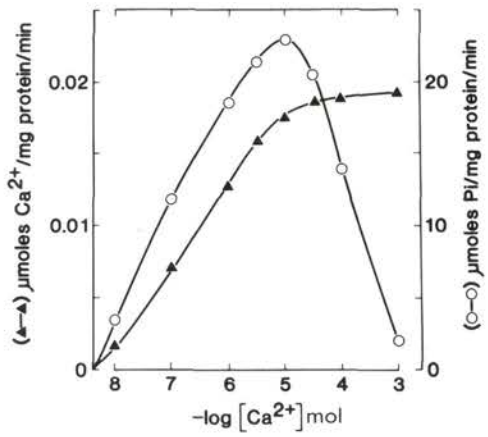

Fig. 8. Kinetic patterns of Ca^{2+} binding (▲—▲) and Ca^{2+} ATPase (○—○) over a range of free Ca^{2+} concentrations (10^{-8} to 10^{-3} mol/l) in purified Ca^{2+} ATPase preparation. Assay conditions were same as described in Fig. 7 except 0.03—0.06 mg/ml membrane protein was used for Ca^{2+} ATPase assay.

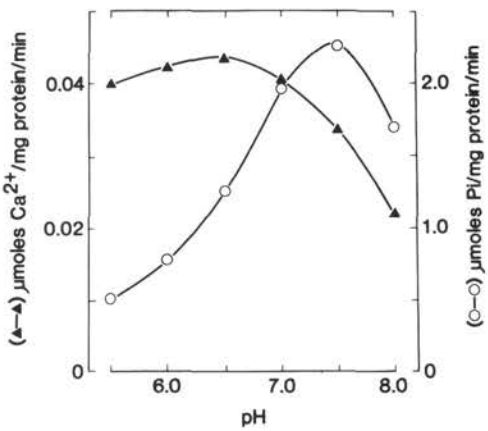

Fig. 9. The effect of pH on microsomal Ca^{2+} binding (▲—▲) and Ca^{2+} ATPase (○—○) activity. The reaction medium was same as described in Fig. 7 except for pH 5.5—6.5, 20 mmol/l histidine — KOH and for pH 7.0—8.0, 20 mmol/l Tris-HCl were used. 0.05 mmol/l total $^{45}CaCl_2$ was present in all assay media and 0.15 mg/ml membrane protein was incubated for Ca^{2+} ATPase assay.

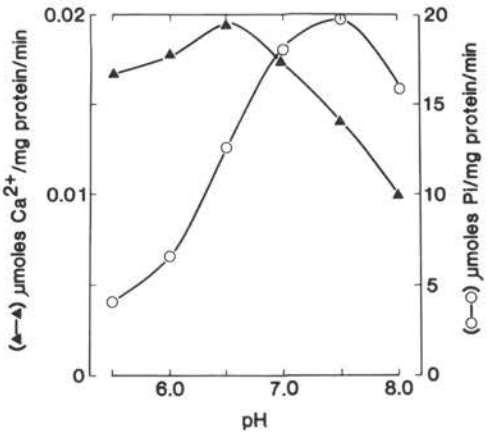

Fig. 10. The effect of pH on Ca^{2+} binding (▲—▲) and Ca^{2+} ATPase (○—○) activity in purified Ca^{2+} ATPase preparation. The reaction medium was same as described under Fig. 9 except 0.03—0.06 mg/ml membrane protein was used for Ca^{2+} ATPase assay.

physiological process (Martonosi 1972), Ca^{2+} uptake and Ca^{2+} ATPase activity were studied in presence of excess Ca^{2+} and H^+. Furthermore, the studies were also carried out in purified microsomal Ca^{2+} ATPase preparation to substantiate the observations obtained from crude microsomal membrane. Figs. 7 and 8 show the effects of varying concentrations of Ca^{2+} on Ca^{2+} ATPase and Ca^{2+} binding activity in microsomes and in purified Ca^{2+} ATPase preparation, respectively. A linear increase of the activity of ATPase and Ca^{2+} binding was observed by increasing the free Ca^{2+} concentrations in either of these two preparations. However, at free Ca^{2+} concentrations beyond 10 μmol/l, a decrease of the ATPase activity was evident: the maximal effect was found at 1 mmol/l Ca^{2+}. Microsomal Ca^{2+} accumulation in presence of oxalate also showed a parallel inhibition with higher Ca^{2+} concentration (not shown). On the other hand, a different kinetic pattern was observed with Ca^{2+} binding process. The maximum saturation of Ca^{2+} binding was reached with 50—100 μmol/l Ca^{2+} and it did not decline even in presence of higher Ca^{2+} concentration.

Similarly, Figs. 9 and 10 show the effects of pH on Ca^{2+} ATPase and Ca^{2+} binding activity in microsomes and in purified Ca^{2+} ATPase preparation, respectively. Although the pH optimum for Ca^{2+} binding was 6.5 and that for Ca^{2+} ATPase was 7.5, at lower pH Ca^{2+} binding was almost unaffected while Ca^{2+} ATPase was inhibited to a great extent in either of these preparations. Moreover, Ca^{2+} accumulation when studied in presence of oxalate showed pH optimum at 6.8 and the activity was reduced by lowering the pH in the incubation media (not shown).

Discussion

It has been shown earlier that a pronounced Ca^{2+} activation of Ca^{2+} ATPase occurs during the transport of Ca^{2+} across cardiac microsomal membrane and a tight coupling between ATP hydrolysis and Ca^{2+} transport exists in this membrane (Tada et al. 1978). Since there are many reports to define the fluidity of bilayer lipid of microsomes (Eletr and Inesi 1972; Nakamura and Ohnishi 1975) and relating these findings to lipid-ATPase interactions as well as to the ATPase and Ca^{2+} transport activities (Madeira et al. 1974; Hidalgo et al. 1976), it is conceivable that the coupling between Ca^{2+} ATPase activity and Ca^{2+} transport could be affected as a result of some structural changes in these membranes. In fact, a defective Ca^{2+} transport by microsomes in different types of failing hearts (Gertz et al. 1967; Suko et al. 1970; Muir et al. 1970; Dhalla et al. 1979; Lamers and Stinis 1979) has been demonstrated and a functional or a structural disintegration of the microsomes by activation of lysosomes and other disruptive mechanisms leading to abnormalities in the Ca^{2+} transport system has been suggested recently (Dhalla et al. 1979, 1982, 1983). It may be noted that the hydrolysis of membrane lipids could play an important role in producing the abnormalities in cardiac function that accompany many pathological situations, both by depleting the membranes of their natural lipid content and by releasing potentially detrimental products of these hydrolytic fractions (Katz and Messineo 1981). The present study shows that cardiac microsomes treated with phospholipase A, trypsin and deoxycholate had enhanced Ca^{2+} ATPase activity while the Ca^{2+} uptake process declined. These data indicate uncoupling of Ca^{2+} uptake from ATPase activity. Similar effects under different experimental conditions were also observed by Martonosi et al. (1968). It seems that the membrane functions are dependent on the protein and phospholipid components which are integrated into the organized structure of biological membranes. The selective removal of a membrane component, therefore, alters membrane integrity and subsequently changes the enzymatic and transport functions. On the other hand, a parallel loss of Ca^{2+} transport and ATPase activity resulted after phospholipase C and β -glucuronidase treatment favouring coupling between these two phenomena. Therefore, a specific interactions of the membrane components are needed to guide the assembly of stable and functional biological membranes. Alterations in the membrane protein or phospholipid composition of microsomes have been reported earlier in different pathological situations (Samaha and Gergely 1965; Owens et al. 1973). Such alterations may provide different hydrophobic environment particularly at the enzyme site. The defective protein-lipid interaction, thus, may enhance conformational change of the enzyme molecule and may induce uncoupling from Ca^{2+} accumulation in cardiac microsomes. In the present work it is

very unlikely that an increase in Ca^{2+} permeability of the vesicles is responsible for uncoupling of Ca^{2+} uptake from Ca^{2+} ATPase, because if the permeability to Ca^{2+} would have changed in microsomes by lipase or protease pretreatment, one could speculate a decrease in Ca^{2+} uptake with no change in Ca^{2+} ATPase activity.

Our present data indicate that Ca^{2+} transport of cardiac microsomes may be dependent on intracellular Ca^{2+} concentration. In fact, Ca^{2+} ATPase, Ca^{2+} uptake and Ca^{2+}binding were all stimulatory so long as the free Ca^{2+} concentration was maintained approximately upto 10 μmol/l in the incubation media. However, in presence of higher Ca^{2+}, the microsomal Ca^{2+} pump activity was inhibited, but not Ca^{2+} binding. Therefore, though an uncoupling of Ca^{2+} binding from ATP hydrolysis during cellular Ca^{2+} overload may be suggested at present, our results also may provide some evidence for the depressed microsomal function in conditions where intracellular Ca^{2+} concentrations were increased. It is of particular interest to know that the production of Ca^{2+} overload in the myocardial cell during prolonged hypoxic and ischaemic conditions is well documented (Shen and Jennings 1972; Nayler et al. 1979). A decreased Ca^{2+} transport in cardiac microsomes has been reported earlier in these situations (Feher et al. 1980).

While there are reports to demonstrate that a lowering of both the extracellular and the intracellular pH may be associated with a loss of function in myocardial ischaemia, the identity of the cellular site at which the interaction between Ca^{2+} and H^+ may influence myocardial contraction has been the subject of much speculation (Serur et al. 1976; Gevers 1977). It may be possible that the acidosis produced in ischaemic conditions alters the Ca^{2+} affinity of microsomal Ca^{2+} binding or Ca^{2+} ATPase activity. Using both purified Ca^{2+} ATPase as well as crude microsomal preparations we have demonstrated that there was a clear dissociation of Ca^{2+} ATPase from Ca^{2+} binding activity following the decrease of pH in the incubation media. Furthermore, both Ca^{2+} ATPase and oxalate-supported Ca^{2+} accumulation were decreased. It is more likely, therefore, that excess H^+ ions compete for the active uptake process of Ca^{2+} and thereby reduce the microsomal Ca^{2+} transport activity. It may be pointed out that although altered microsomal function may influence the myocardial contractility that is usually associated with the various pathological conditions like myocardial ischaemia (Dhalla et al. 1982), one should be cautious in extrapolating the above results to the situation *in vivo* since other factors modifying the expression of overall myocardial function may play an important and, as yet, undefined role.

Acknowledgements. The work reported in this paper was supported from the Manitoba Heart Foundation. Dr. Lamers was partly supported by the Dutch Heart Foundation.

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Received April 13, 1984 / Accepted July 23, 1984