Biochimica et Biophysica Acta, 688 (1982) 747–756
Elsevier Biomedical Press

BBA 71211

LOCALIZATION OF (Ca^{2+} + Mg^{2+})-ATPase, Ca^{2+} PUMP AND OTHER ATPase ACTIVITIES IN CARDIAC SARCOLEMMA

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(Received November 11th, 1981)

Key words: (Ca^{2+} + Mg^{2+})-ATPase; Ca^{2+} pump; Enzyme localization; (Cardiac sarcolemma)

N-Ethylmaleimide was employed as a surface label for sarcolemmal proteins after demonstrating that it does not penetrate to the intracellular space at concentrations below $1 \cdot 10^{-4}$ M. The sarcolemmal markers, ouabain-sensitive (Na^+ + K^+)-ATPase and Na^+/Ca^{2+}-exchange activities, were inhibited in N-ethylmaleimide perfused hearts. Intracellular activities such as creatine phosphokinase, glutamate-oxaloacetate transaminase and the internal phosphatase site of the Na^+ pump (K^+-p-nitrophosphatase) were not affected. Almost 20% of the (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} pump were inhibited indicating the localization of a portion of this activity in the sarcolemma. Sarcolemma purified by a recent method (Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27–39) from N-ethylmaleimide-perfused hearts showed loss of approx. 85% of its (Ca^{2+} + Mg^{2+}-ATPase and Ca^{2+} pump compared to control hearts. (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} pump activities showed two classes of sensitivity to vanadate ion inhibition. The high vanadate affinity class ($K_{1/2}$ for inhibition approx. 1.5 μM) may be localized in the sarcolemma and represented approx. 20% of the total inhibitable activity in agreement with estimates from N-ethylmaleimide studies. Sucrose density fractionation indicated that only a small portion of Mg^{2+}-ATPase and Ca^{2+}-ATPase may be associated with the sarcolemma. The major portion of these activities seems to be associated with high density particles.

Introduction

The necessity of regulation of the cytoplasmic Ca^{2+} pool in heart muscle and the strong concentration gradient at the plasma membrane led several authors [1,2] to propose a transport mechanism for Ca^{2+} linked to Ca^{2+}-stimulated-ATPase at the sarcolemma. Recently Ca^{2+}-transport ATPases with high affinity for Ca^{2+} had been demonstrated in many cell membranes [3]. Several investigators demonstrated some evidence of a Ca^{2+} binding and uptake system in cardiac sarcolemma and showed fragmentary evidence that the sarcolemma also contains a (Ca^{2+} + Mg^{2+})-ATPase [4–7]. Demonstration of a (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} pump in cardiac sarcolemma encountered many difficulties since procedures for isolation of high purity plasma membranes from heart were fraught with difficulties and uncertainties. More recently Morcos and Drummond [8] provided evidence for the existence of a (Ca^{2+} + Mg^{2+})-ATPase in purified canine cardiac

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

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sarcolemma. In their study, the ATPase was solubilized, purified, the preparation allowed to re-form vesicles and the capacity of these vesicles to bind and accumulate Ca^{2+} in an ATP-dependent manner demonstrated, a result that suggested the possible role of the ATPase as a Ca^{2+} pump. Later Morcos [9] demonstrated the presence of a $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump in cardiac sarcolemma from frog heart which possesses very sparse sarcoplasmic reticulum [10–12]. The low probability of contamination with sarcoplasmic reticulum (usually a major difficulty in such studies) in this preparation and the presence of significant amounts of ATPase and pump suggested that they are of sarcolemmal origin and should be a part of excitation-contraction coupling models.

Many studies which demonstrate evidence of a cardiac $(Ca^{2+}Mg^{2+})$-ATPase and Ca^{2+} pump rely heavily on extensive purification of the sarcolemma in order to free it from possible sarcoplasmic reticulum contamination or employ tissue that already possesses sparse sarcoplasmic reticulum such as the frog heart. The aim of this study was to utilize an approach which was independent of the level of membrane purity to demonstrate the presence of $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump in cardiac sarcolemma. One such approach was to employ an agent that does not cross the membrane but yet alters the activity of $(Ca^{2+} + Mg^{2+})$-ATPase at the sarcolemma and also alters other activities and pumps whose function is sensitive to chemical modification of their externally accessible protein chains. The present study describes procedures to employ the protein labelling agent, *N*-ethylmaleimide [13], for localization of $(Ca^{2+} + Mg^{2+})$-ATPase, Ca^{2+} pump and other ATPases. In this communication, a very large difference is demonstrated between $K_{1/2}$ for inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump of sarcolemma by vanadate and those previously reported for sarcoplasmic reticulum [15,16]. The difference is employed to quantitate and differentiate between the sarcolemmal ATPase and pump and the corresponding entities of sarcoplasmic reticulum.

Materials and Methods

$^{45}CaCl_2$ (24–30 mCi/mg) was obtained from ICN Radiochemicals. Dithiothreitol, enzyme grade Tris, bovine serum albumin, *N*-ethylmaleimide and Tris-ATP were purchased from Sigma Chemical Co. Sucrose was purchased from Malinckrodt Co., orthovanadate from Fisher Scientific Co., and *N*-[1-^{14}C]ethylmaleimide (24 Ci/mol) was obtained from New England Nuclear. Male New Zealand rabbits (6–7 lbs) were from Curd Caviary Animal Supplies, CA.

Preparation of hearts and isolation of sarcolemma

After administration of 10 mg of sodium heparin and 180 mg of pentobarbital to a rabbit via an ear vein, the heart was rapidly excised through a median sternotomy and placed in warm, oxygenated Krebs-Ringer bicarbonate solution. Excess fat around the aorta was removed and the aorta cannulated with a small heated polyethylene cannula secured in place with a 6–0 silk suture. No more than 2 min elapsed from the time the heart was excised to cannulation. Flow was maintained at 6 ml/min by a Sage peristaltic pump. The posterior and inferior vena cava as well as pulmonary veins were closed with slik sutures. The atrio-ventricular valve areas were intact. A small atrial orifice was made for venting. The perfusate contained (in mM): NaCl, 130; $CaCl_2$, 1.5; KCl, 4.0; $MgCl_2$, 1.0; NaH_2PO_2, 0.435; $NaHCO_3$, 12, dextrose, 5.6. The perfusate was continuously equilibrated with 98% O_2/2% CO_2, yielding a pH of 7.3–7.4 at 37°C. Stimulating electrodes from a Grass S5 Stimulator were attached to the atria and 6 mV, 10 ms stimuli were delivered at a rate of 72/min. Control hearts were perfused for 75 min, removed from the cannula, atria dissected out, ventricles cleansed of fat, blotted dry, weighed and frozen in liquid nitrogen within 2 min from the end of perfusion. Hearts treated with *N*-ethylmaleimide were first perfused with regular perfusate for 20 min, then *N*-ethylmaleimide was included in the perfusate at a final concentration of $5 \cdot 10^{-5}$ M for 8 min. The hearts were then perfused with regular perfusate for 45 min to remove excess and unbound *N*-ethylmaleimide. The ventricles were cleaned and frozen in liquid nitrogen as described above.

Frozen ventricles were thawed within 3 days of freezing and all steps of the sarcolemmal preparation were carried out at 4°C by the method described previously for dog heart [8]. Routinely the

8 to 28°C sucrose fractions ($d_{20°C}$, 1.0591–1.1192) were combined and termed purified sarcolemma. The yields and purification factors were similar to those obtained from dog heart.

Creatine phosphokinase and glutamate-oxaloacetate transaminase

These enzymes were employed as soluble cytoplasmic markers and were assayed in the supernatant resulting after centrifugation of the initial crude homogenate for 20 min at $12000 \times g$. Creatine phosphokinase assays were performed using the modified Rosalki procedure [17]. Assay packs were obtained from Calbiochem (Calbiochem-Behring Corp. American Hoechst Corp., San Diego, CA). Glutamate-oxaloacetate transaminase assay was performed according to the method of Reitman and Frankel [18] and calibrated with a kinetic ultraviolet procedure based on the method of Karmen [19]. Assay reagents were obtained from Sigma Corp.

ATPases

$(Ca^{2+} + Mg^{2+})$-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase (all in the presence of 0.1 mM ouabain and 5 mM NaN_3) and ouabain-sensitive $(Na^+ + K^+)$-ATPase activities were measured by incubating protein for 10 min at 37°C and assays performed as described previously [8]. In vanadate experiments, membrane protein was initially preincubated with specified concentrations of vanadate for 1 h at 4°C then added to the $(Ca^{2+} + Mg^{2+})$-ATPase reaction medium which contained the same concentration of vanadate and $1.65 \cdot 10^{-9}$ M of Ca^{2+} ionophore A23187. Inorganic phosphate determination was described previously [20]. K^+-stimulated *p*-nitrophenylphosphatase was assayed according to Heller and Hanahan [21].

Ca^{2+} uptake

ATP-dependent Ca^{2+} uptake was measured by incubating membrane protein at 37°C for 10 min in a medium containing 5 mM free Ca^{2+} ($^{45}CaCl_2$, $(11–28) \cdot 10^5$ cpm/nmol) and identical in composition to that used for $(Ca^{2+} + Mg^{2+})$-ATPase assay in the absence or presence of 5 mM ATP as described previously [8]. The difference between Ca^{2+}-uptake in the absence and presence of ATP was considered as ATP-dependent-Ca^{2+}-uptake or Ca^{2+} pump activity.

Na^+/Ca^{2+}-exchange was performed as previously described [9].

N-[1-^{14}C]Ethylmaleimide labelling

Estimation of *N*-ethylmaleimide bound label to membrane protein was performed by perfusing hearts as described above and including in the perfusion medium $4 \cdot 10^{-5}$ M *N*-[1-^{14}C]ethylmaleimide (29133 cpm/nmol) then washing the excess label.

Cytochrome-*c* oxidase was measured according to Cooperstein and Lazarow [22] and protein according to Lowry et al. [23] with bovine serum albumin as standard.

Results

N-Ethylmaleimide as a surface label

N-Ethylmaleimide has been used previously to form a covalently linked label with proteins as well as membrane proteins [13,8]. Hasselbach and Seraydarian [14] employed *N*-ethylmaleimide to inhibit isolated sarcoplasmic reticulum membrane activities. To employ *N*-ethylmaleimide as a surface label in the following experiments it had to satisfy

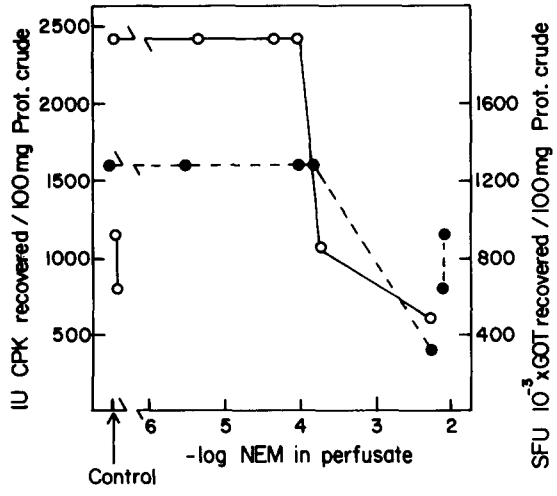

Fig. 1. Lack of interaction between intracellular markers and *N*-ethylmaleimide in the perfusate at various concentrations. Experimental conditions as in Materials an Methods. CPK, creatine phosphokinase; GOT, glutamate-oxaloacetate transaminase; NEM, *N*-ethylmaleimide.

two criteria: (a) It should not penetrate across the membrane into the intracellular space, and (b) it should have the ability to alter the activity of enzymes under study. To test the permeability of *N*-ethylmaleimide, hearts were perfused as described under Materials and Methods with various concentrations of the agent up to $5 \cdot 10^{-3}$ M. The activities of the intracellular cytoplasmic enzymes creatine phosphokinase and glutamate-oxaloacetate transaminase which are sensitive to *N*-ethylmaleimide were not affected up to an *N*-ethylmaleimide concentration of $1 \cdot 10^{-4}$ M in the perfusate (Fig. 1) indicating no penetration of label into the cytoplasm. There was however a sharp decline of activities above this concentration. Previous reports by Rao [24] and Abbott and Schachter [25] showed that the red blood cell membrane was permeable to *N*-ethylmaleimide at high concentrations (1–40 mM) and that S-H groups on both sides of the membrane were labelled. In Rao's studies the membrane-labelling reaction approached completion only after *N*-ethylmaleimide concentration was raised to 20 mM for 1 h at 37°C. At 1 mM *N*-ethylmaleimide only a very small portion (20%) of the groups were labelled in intact cells while a much larger portion (50%) was labelled in isolated membranes. These results suggest that a group of sites, probably internal, are not accessible to *N*-ethylmaleimide at lower concentations but become available at higher concentrations. This may be due to increased membrane permeability to the agent after extensive labelling and alternation of membrane properties. Indeed Knauf and Rothstein [26] showed that membrane permeability to potassium increased after labelling with sulfhydryl blocking agents. In the present communication *N*-ethylmaleimide was employed at a concentration that was 100-fold lower than those employed in experiments reported earlier and the period of contact with the agent was limited to 8 min. The critical concentrations at which *N*-ethylmaleimide starts to penetrate the membrane, under these conditions, as evidenced by loss of creatine phosphokinase and glutamate-oxaloacetate transaminase activities, were above $1 \cdot 10^{-4}$ M which includes the range of concentration employed in previous studies in which *N*-ethylmaleimide penetrated the membrane. In the labelling studies reported here, *N*-ethylmaleimide was employed at a concentration 5-fold lower ($5 \cdot 10^{-5}$) than the critical concentration for penetration. Inhibition of the cytoplasmic markers creatine phosphokinase and glutamate-oxaloacetate transaminase was chosen as an indicator of *N*-ethylmaleimide entry into the cytoplasm since they are several-fold sensitive (see below) to *N*-ethylmaleimide than other activities reported in this study and would probably become inhibited before reaction of *N*-ethylmaleimide with other less sensitive intracellular activities.

TABLE I

SENSITIVITY OF VARIOUS ACTIVITIES IN HEART CRUDE HOMOGENATE TO *N*-ETHYLMALEIMIDE

Creatine phosphokinase and glutamate-oxaloacetate transaminase were measured after pre-incubating the supernatant from a $12000 \times g$ fractionation of the crude homogenate with $5 \cdot 10^{-5}$ *N*-ethylmaleimide at 4°C for 1 h. All other measurements were performed on crude homogenate pre-incubated with $5 \cdot 10^{-3}$ M *N*-ethylmaleimide at 4°C for 1 h.

Activity	% Inhibition
$(Ca^{2+} + Mg^{2+})$-ATPase	80
$(Na^{+} + K^{+})$-ATPase	35
K^{+}-*p*-Nitrophenylphosphatase	20
Mg^{2+}-ATPase	0
Ca^{2+}-ATPase	47
Creatine phosphokinase	90
Glutamate-oxaloacetate transaminase	70

The sensitivities of activities employed in this study were measured by pre-incubating crude homogenates of heart tissue or its supernatant with $5 \cdot 10^{-3}$ M or $5 \cdot 10^{-5}$ M, respectively, at 4°C for 1 h then diluting the label and assaying. Table I shows that glutamate-oxaloacetate transaminase, and to a larger extent, creatine phosphokinase were among the most sensitive enzymes to *N*-ethylmaleimide, showing 70 and 90% inhibition, respectively, at this low *N*-ethylmaleimide concentration and low-preincubation temperature. Creatine phosphokinase and glutamate-oxaloacetate transaminase were therefore suitable intracellular cytoplasmic markers for detection of *N*-ethylmaleimide entry. Total $(Ca^{2+} + Mg^{2+})$-ATPase as well as ouabain-sensitive $(Na^{+} + K^{+})$-ATPase were susceptible to *N*-ethylmaleimide (the former being considerably more sensitive) in heart tissue

homogenates. K^+-*p*-Nitrophenylphosphatase activity which is a partial reaction of the Na^+ pump that is located at the internal side of the plasma membrane [27,28] and the Mg^{2+}-independent Ca^{2+}-ATPase were also susceptible. Mg^{2+}-ATPase activity was not sensitive in agreement with previous findings [7].

Distribution of activities on sucrose density gradient

Distribution of the sarcolemmal markers, ouabain-sensitive ($Na^+ + K^+$)-ATPase, K^+-*p*-nitrophenylphosphatase and Na^+/Ca^{2+}-exchange activity from rabbit heart on sucrose density gradient is shown in Figs. 2–4, respectively. The larger yield of these markers was obtained in the two density sucrose regions (8–28%) which were pooled and termed purified sarcolemma. Some sarcolemmal marker with low specific activity was present at the higher density fractions which may be due to aggregated particles of different origins that tend to travel down the gradient as described previously [8].

N-Ethylmaleimide-treated hearts (in all figures) show loss of Na^+/Ca^{2+}-exchange and ($Na^+ + K^+$)-ATPase activity. K^+-*p*-Nitrophenylphosphatase was not inhibited indicating absence of interaction between *N*-ethylmaleimide and this internal portion of the Na^+-pump reaction. The distribution of the surface label, *N*-ethylmaleimide

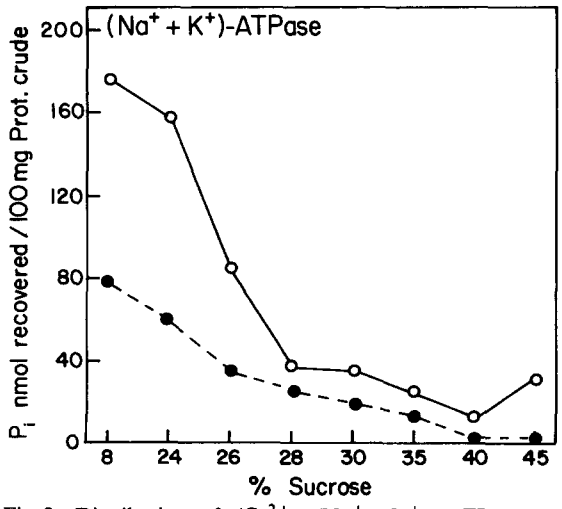

Fig. 2. Distribution of (Ca^{2+} +($Na^+ + K^+$))-ATPase from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gradient.

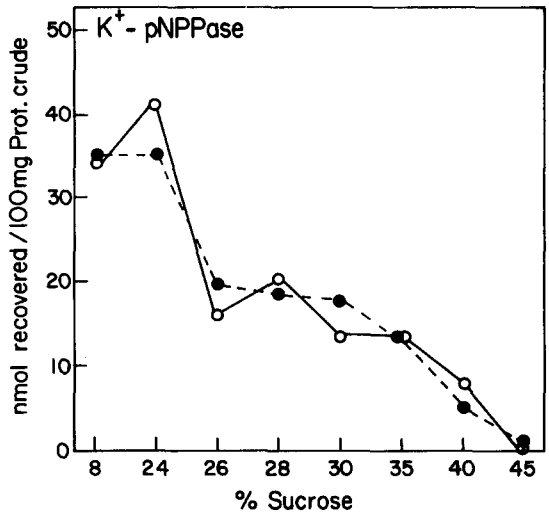

Fig. 3. Distribution of K^+-*p*-nitrophenylphosphatase from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gadient.

on the sucrose density gradient (not shown) was similar to the distribution of the three sarcolemmal markers.

Fig. 5 shows the distribution of two fractions of ($Ca^{2+} + Mg^{2+}$)-ATPase. One fraction was in the

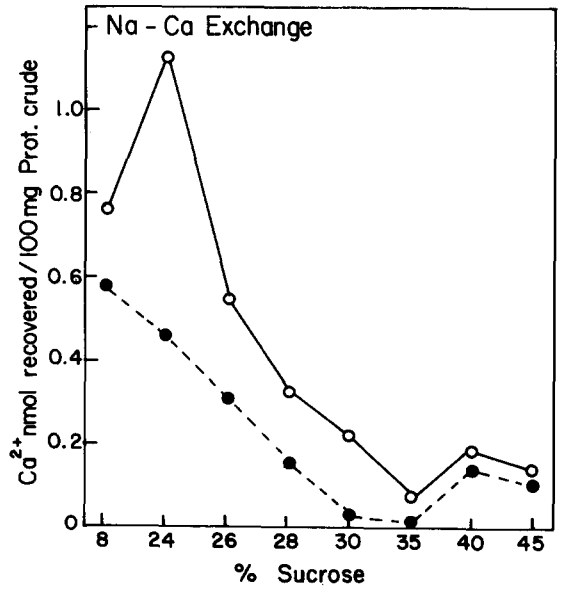

Fig. 4. Distribution of Na^+/Ca^{2+} exchange activity from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gradient.

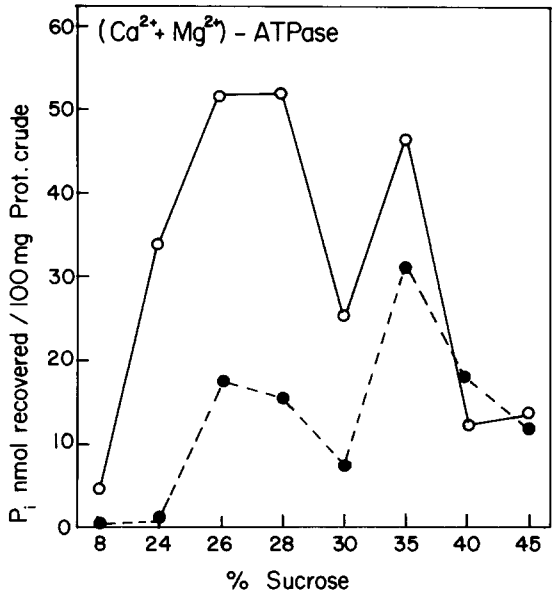

Fig. 5. Distribution of $(Ca^{2+} + Mg^{2+})$-ATPase from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gradient.

low density sucrose region where $(Na^+ + K^+)$-ATPase is concentrated and the other at high density sucrose were sarcoplasmic reticulum was reported to appear [8,29,30]. As explained previously, the low density fraction seems to be endogenous to the sarcolemma [8,9]. In *N*-ethylmaleimide-treated hearts, the $(Ca^{2+} + Mg^{2+})$-ATPase associated with the low density fraction was substantially inhibited adding evidence to its sarcolemmal origin. Some inhibition was observed in the high density fraction which may be due to loss of a portion of the enzyme contributed by sarcolemma aggregated at the higher density regions as described above.

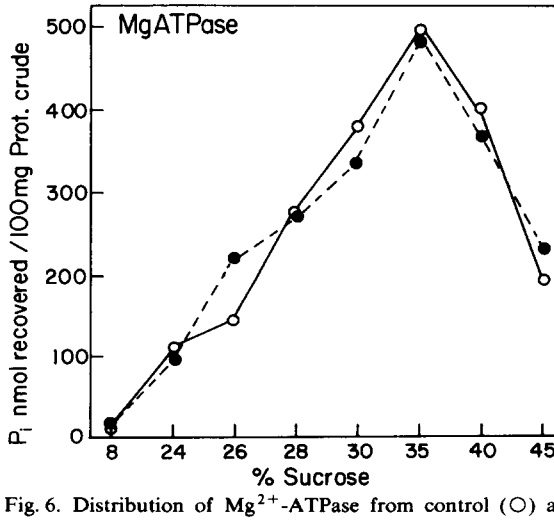

Fig. 6. Distribution of Mg^{2+}-ATPase from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gradient.

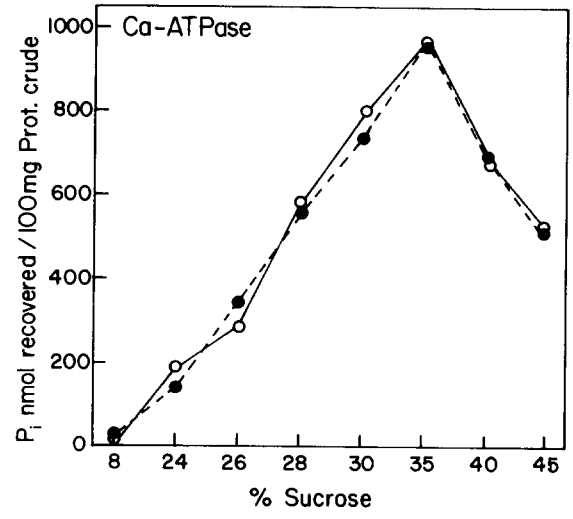

Fig. 7. Distribution of Ca^{2+}-ATPase from control (○) and *N*-ethylmaleimide-treated (●) hearts on sucrose density gradient.

Distribution of mitochondria expressed by inner mitochondrial membrane marker, cytochrome-*c* oxidase on the sucrose gradient showed that mitochondrial contamination of the low density membrane fractions is neglible. The larger yield of mitochondria is concentrated at the high-density regions (35 and 40% sucrose) as previously reported for dog heart [8].

The distribution of Mg^{2+}-ATPase and Ca^{2+}-ATPase on the sucrose density gradient (Figs. 6, 7) indicate that both enzymes show low but significant yields in the low density sucrose fractions. A small portion of the Mg^{2+}-ATPase may be understandably associated with sarcolemma since some sarcolemmal endogenous enzymes, such as $(Na^+ + K^+)$-ATPase possess basal Mg^{2+}-ATPase activities. The larger portion of both Mg^{2+}-ATPase and Ca^{2+}-ATPase activities does not seem to be associated with the sarcolemma but appears to associate with a high density fraction. It is unlikely however that both activities represent the same

TABLE II

RECOVERY OF ACTIVITIES IN CRUDE HOMOGENATE AND MEMBRANE FRACTIONS FROM CONTROL AND *N*-ETHYLMALEIMIDE-TREATED HEARTS

Control hearts were perfused with regular Krebs bicarbonate solution.
Maleimide-hearts were perfused with $5 \cdot 10^{-5}$ M *N*-ethylmaleimide for 8 min then washed.
Yields are expressed as units of activity recovered from 100 mg protein of starting crude homogenate.
(a) Units for $(Ca^{2+} + Mg^{2+})$-ATPase, $(Na^+ + K^+)$-ATPase, K^+-*p*-nitrophenylphosphatase, Mg^{2+}-ATPase and Ca^{2+}-ATPase are nmol P_i recovered.
(b) Units for ATP-Ca^{2+}-uptake and Na^+/Ca^{2+}-exchange are nmol Ca^{2+} recovered.
(c) Units for creatine phosphokinase are I.U. recovered.
(d) Units for glutamate-oxaloacetate transaminase are SFU recovered.
(e) Units for cytochrome-*c* oxidase are ΔA units recovered.

Activity	Crude homogenate			Isolated membranes			
	Control-hearts (Yield)	Maleimide-hearts		Control-hearts		Maleimide-hearts	
		Yield	%Inhibition	Yield	%Recovered	Yield	%Inhibition
$(Ca^{2+} + Mg^{2+})$-ATPase [a]	2004 ± 230	1695 ± 36	15	154.4 ± 48.8	7.7	31.7 ± 3.2	80
ATP-Ca^{2+}-uptake [b] ($\times 10^5$)	61097 ± 7356	46445 ± 617	23	3465 ± 1196	5.6	432 ± 65	88
$(Na^+ + K^+)$-ATPase [a]	2989 ± 486	1420 ± 128	53	446.7 ± 71.8	14.9	177.5 ± 37	60
Na^+/Ca^{2+} Exchange [b]	26.14 ± 2.1	18.2 ± 4.0	30	3.3 ± 0.8	12.7	2.3 ± 0.4	31
K^+-pNPPase [a]	551 ± 28	539 ± 94	2	109 ± 9	19.8	107 ± 5.4	0
Ca^{2+}-ATPase [a]	24801 ± 2855	24930 ± 2493	0	107 ± 198	0.4	1042 ± 83	0
Mg^{2+}-ATPase [a]	7922 ± 1004	7499 ± 749	5	577 ± 51	7.2	584 ± 27	0
Creatine phosphokinase [c]	2357 ± 214	2301 ± 167	0	–	–	–	–
Glutamate-oxaloacetate transaminase [d]	1217693 ± 269055	1351326 ± 189149	0	–	–	–	–
Cytochrome-*c* oxidase [e]	56127 ± 3573	–	–	187 ± 12	0.3	–	–

enzyme since they have various sensitivities to *N*-ethylmaleimide (Table I). Hearts treated with maleimide show no change in Mg^{2+}-ATPase activity distribution on the gradient (Fig. 6) which is in agreement with the lack of sensitivity of this ATPase (Table I). However, the absence of inhibition of any fraction of Ca^{2+}-ATPase on the gradient in *N*-ethylmaleimide-treated hearts suggests that the enzyme is not accessible to the maleimide from the outside and may be of intracellular origin. Morcos and Drummond [8] reported the loss of this Ca^{2+}-ATPase during sarcolemmal purification and also completely resolved and separated this activity from the $(Ca^{2+} + Mg^{2+})$-ATPase.

Yields of activities in control and N-ethylmaleimide-treated hearts

Table II summarizes the yields recovered of various activities in the initial crude homogenate and the purified membranes from both control and *N*-ethylmaleimide-treated hearts. Total $(Ca^{2+} + Mg^{2+})$-ATPase in crude homogenate (sarcolemma + sarcoplasmic reticulum) was inhibited about 15% only in maleimide-treated hearts suggesting that the portion of this ATPase accessible at the sarcolemma may represent at least 15% of the total ATPase. The remaining 85% would be of sarcoplasmic reticulum origin. Similar inhibition (23%) was observed for the ATP-dependent-Ca^{2+}-uptake of the crude. $(Na^+ + K^+)$-ATPase and Na^+/Ca^{2+} exchange activities showed 53 and 30% inhibition, respectively. It is clear that despite the higher sensitivity of the total $(Ca^{2+} + Mg^{2+})$-ATPase in relation to $(Na^+ + K^+)$-ATPase (Table I), only a small portion was inhibited in *N*-ethylmaleimide-treated hearts in relation to $(Na^+ + K^+)$-ATPase. This result is consistent with the possibility that a limited smaller portion of this enzyme is sarcolemmaly localized and accessible from the outside while the remaining larger portion is of sarcoplasmic reticulum origin and inaccessible. Internal cytoplasmic activities such as total K^+-*p*-nitrophenylphosphatase, creatine phosphokinase and glutamate-oxaloacetate transaminase were not significantly affected in *N*-ethylmaleimide treated hearts.

Table II also shows the recovery of activities in purified sarcolemma as well as the extent of *N*-ethylmaleimide inhibition of its endogenous activities. The percentage of $(Na^+ + K^+)$-ATPase and Na^+/Ca^{2+} exchange inhibited in sarcolemma from *N*-ethylmaleimide-treated hearts was only slightly changed from that observed in the total crude despite a 6-fold purification. This result is consistent with the exclusive sarcolemmal origin of these activities. In contrast $(Ca^{2+} + Mg^{2+})$-ATPase and ATP-dependent-Ca^{2+}-uptake activities in purified sarcolemma from *N*-ethylmaleimide-treated hearts were 79 and 87% inhibited, respectively. The increase in amount of inhibition of the activities over the crude homogenate upon purification of the sarcolemmal fraction further indicates that the portion of $(Ca^{2+} Mg^{2+})$-ATPase and Ca^{2+} pump accessible to *N*-ethylmaleimide is localized at the sarcolemmal membrane. Ca^{2+}-ATPase activity co-purifying with the sarcolemma showed no inhibition suggesting its intracellular origin.

Estimation of sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump by orthovanadate

Vanadate had been shown previously to inhibit both the $(Na^+ + K^+)$-ATPase [31] and the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase [32] activities at very different concentrations. One approach to demonstrating two types of $(Ca^{2+} + Mg^{2+})$-ATPase and total Ca^{2+} pump was to study their inhibition in heart crude homogenates over a wide range of vanadate concentration. The ATPase (Fig. 8) and pump (not shown) activities showed a sharp initial decrease in activity followed by a slower phase as vanadate concentration increased. When the data were plotted on a Scatchard plot (inset) two phases of inhibition were observed indicating two classes of both $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump (not shown). The high-affinity phase intercepted the *x*-axis indicating 100% inhibition at specific activities of 52 $nmol \cdot mg^{-1} \cdot min^{-1}$ and 30 $nmol \cdot mg^{-1} \cdot min^{-1} \times 10^{-4}$ representing 13 and 20% of the total ATPase and pump, respectively, with a $K_{1/2}$ for inhibition of 1.2 mM for both. The $K_{1/2}$ for inhibition of the low affinity class of ATPase and pump was 11 and 25 mM, respectively. The $K_{1/2}$ for the low affinity class was 10–30-times higher than that for the high-affinity class and is in the range reported for the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase [32].

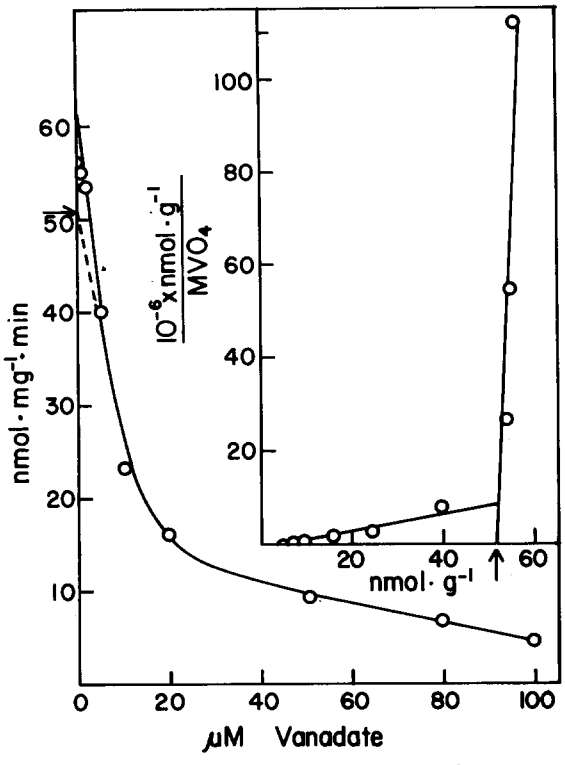

Fig. 8. Vanadate inhibition of total $(Ca^{2+} + Mg^{2+})$-ATPase in crude homogenate. Inset: data represented in a Scatchard plot (intercept on x-axis represents 100% inhibition of high-affinity phase).

Purified sarcolemmal membranes showed a much larger proportion (70%) of the high affinity class for both $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump with a $K_{1/2}$ of 1.2 mM for both. This increase in proportion of the high affinity class of both $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump activity suggests that this portion of activity may be endogenous to the sarcolemma.

Discussion

The present communication clearly demonstrates that a portion of the total $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump in the heart is localized at the sarcolemma. Early reports on the presence of these activities at the sarcolemma were cautious in view of the possibility of sarcoplasmic reticulum contamination. Recently, with the isolation of high purity membranes, the possibility of sarcoplasmic reticulum contamination was considerably minmized and more solid evidence of these activities in the sarcolemma was demonstrated [8,9,33,34]. The present study does not exclusively rely on membrane purification procedures to differentiate between those activities present in the sarcoplasmic reticulum and those of the sarcolemma. It was shown that *N*-ethylmaleimide is a useful tool in distinguishing some surface-localized activities from intracellular ones. A unique feature of this study was the demonstration of both classes of $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+}-pump activities in the same fraction by the use of either *N*-ethylmaleimide or orthovanadate without the need to separate the two membrane types. The ability to quantitate $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump of sarcolemmal origin in the purified sarcolemmal provides a means to estimate the extent of sarcoplasmic reticulum contamination.

The origin of the Mg^{2+}-ATPase and Ca^{2+}-ATPase is not clear at the present. Based on sucrose gradient distribution studies it is suggested that a small portion of these activities may be associated with the sarcolemma but that the larger portion is more likely associated with higher density cellular particles. The preservation of Ca^{2+}-ATPase activity in *N*-ethylmaleimide-treated hearts (despite its sensitivity) and its loss from purified membranes suggests that this activity may be of intracellular origin.

Recent demonstration of the existence of a $(Ca^{2+} + Mg^{2+})$-ATPase and a Ca^{2+} pump in significant amounts in frog heart which contains very sparse sarcoplasmic reticulum suggests that these activities can exist in sarcolemma and may therefore represent an important mode of Ca^{2+} sequestration and transport from inside of the cell to the outside [9]. Na^+/Ca^{2+}-exchange is presently gaining wide attention as a mechanism which may be involved in intracellular Ca^{2+} regulation [35]. The present communication demonstrates that $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+}-pump activities residing in the sarcolemmal represent approx. 20% of the total activities in the heart. The significant proportion of these activities at the sarcolemma suggest that they may play important roles in the excitation-contraction coupling mechanism.

Acknowledgement

The author wishes to express thanks to Dr. Glenn A. Langer for helpful discussions. This work was done during the tenure of a Research Fellowship and grant No. 581 F1 of the Amercain Heart Association, Greater Los Angeles Affiliate. This work was also supported by a grant from USPHS HL 11351-14-15.

References

1 Okita, G.T., Richardon, F. and Roth-Schechter, B.F. (1973) J. Pharmacol. Exp. Ther. 185, 1–11
2 Skou, J.C. (1965) Physiol. Rev. 45, 596–617
3 Schatzmann, H.J. (1975) Curr. Top. Membrane Transp. 6, 126–168
4 Hui, G.W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415–427
5 St. Louis, P.J. and Sulakke, P.V. (1976) Can. J. Biochem. 54, 946–956
6 Morcos, N.C. and Jacobson, A.L. (1978) Can. J. Biochem. 56, 1–6
7 Morcos, N.C. and Jacobson, A.L. (1979) Can. J. Physiol. Pharmacol. 57, 529–534
8 Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27–39
9 Morcos, N.C. (1981) Biochim. Biophys. Acta 643, 55–62
10 Staley, N.A. and Benson, E.S. (1968) J. Cell. Biol. 38, 99–114
11 Sommer, J.S. and Johnson, E.A. (1969) Z. Zellforsch. 98, 437–468
12 Page, S.G. and Niedergerke, R. (1972) J. Cell. Sci. 11, 179–203
13 Fahn, S., Albers, R.W. and Koval, G.J. (1964) Science 145, 283–284
14 Hasselbach, W. and Seraydarian, K. (1966) Biochem. Z. 345, 159–172
15 Simons, T.J.B. (1979) Nature 281, 337–338
16 Wong, T., Tsai, L., Solaro, R.J., Grassi de Gende, A.O. and Schwartz, A. (1979) Biochem. Biophys. Res. Commun. 91, 356–361
17 Rosalki, S.B. (1967) K. Lab. Clin. Med. 69, 696–705
18 Reitman, S. and Frankel, S. (1957) Am. J. Clin. Pathol. 28, 56–63
19 Karmen, A. (1955) J. Clin. Invest. 34, 131
20 Martin, J.B. and Doty, D.M. (1949) Anal. Chem. 21, 965–967
21 Heller, M. and Hanahan, D.J. (1972) Biochim. Biophys. Acta 255, 329–250
22 Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665–670
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Rao, A. (1979) J. Biol. Chem. 254, 3503–3511
25 Abbott, E.R. and Schachter, A.D. (1976) J. Biol. Chem. 251, 7176–7183
26 Knauf, P.A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190–210
27 Rega, A.F., Garrahan, P.J. and Pouchan, M.I. (1970) J. Membrane Biol. 3, 14–25
28 Garrahan, P.J., Pouchan, M.J. and Rega, A.F. (1970) J. Membrane Biol. 3, 26–42
29 Meisner, G. (1975) Biochim. Biophys. Acta 389, 51–68
30 Lau, Y.H., Caswell, A.H. and Brunching, J.P. (1977) J. Biol. Chem. 252, 5565–5574
31 Simons, T.J.B. (1979) Nature 281, 337–338
32 Wong, T., Tsai, L., Solaro, R.J. and Grassi de Gende, A.P. (1979) Biochem. Biophys. Res. Commun. 91, 356–361
33 Caroni, P. and Carafoli, E. (1980) Nature 283, 765–767
34 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3267–3270
35 Langer, G.A. (1982) Annu. Rev. Physiol., 44, 435–449