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AN ELECTROGENIC Na^+/Ca^{2+} ANTIPORTER IN ADDITION TO THE Ca^{2+} PUMP IN CARDIAC SARCOLEMMA

J.M.J. LAMERS and J.T. STINIS

Department of Biochemistry I, Faculty of Medicine, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

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Summary

Vesicles isolated from rat heart, particularly enriched in sarcolemma markers, were examined for their sidedness by investigation of side-specific interactions of modulators with the asymmetric ($Na^+ + K^+$)-ATPase and adenylate cyclase complex. The membrane preparation with the properties expected for inside-out vesicles showed the highest rate of ATP-driven Ca^{2+} transport. The Ca^{2+} pump was stimulated 1.7- and 2.1-fold by external Na^+ and K^+, respectively, the half-maximal activation occurring at 35 mM monovalent cation concentration. In vesicles loaded with Ca^{2+} by pump action in a medium containing 160 mM KCl, a slow spontaneous release of Ca^{2+} started after 2 min. The rate of this release could be dramatically increased by the addition of 40 mM NaCl to the external medium. In contrast, 40 mM KCl exerted no appreciable effect on vesicles loaded with Ca^{2+} in a medium containing 160 mM NaCl. Ca^{2+} movements were also studied in the absence of ATP and Mg^{2+}. Vesicles containing an outwardly directed Na^+ gradient showed the highest Ca^{2+} uptake activity. These findings suggested the operation of a Ca^{2+}/Na^+ antiporter in addition to the active Ca^{2+} pump in these sarcolemmal vesicles. A valinomycin-induced inward K^+-diffusion potential stimulated the Na^+-Ca^{2+} exchange, suggesting its electrogenic nature. If in the absence of ATP and Mg^{2+} the transmembrane Na_i^+/Na_o^+ gradient exceeded 160/15 mM concentrations, Ca^{2+} uptake could be stimulated by the addition of 5 mM oxalate, indicating Na^+ gradient-induced Ca^{2+} uptake to be a translocation of Ca^{2+} to

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Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

the lumen of the vesicle. A sarcoplasmic reticulum contamination, removed by further sucrose gradient fractionation, contained rather low Na^+-Ca^{2+} exchange activity. This result suggests that the activity can be entirely accounted for by the sarcolemmal content of the cardiac membrane preparation.

Introduction

In cardiac muscle, unlike skeletal muscle, the sarcolemmal Ca^{2+} fluxes seem to play an important role in regulating the free Ca^{2+} concentration inside the cell which is necessary for activation of contraction [1]. Ca^{2+} entering the myocardial cell with each beat during the plateau phase of the action potential has to be removed to avoid an overload of the sarcoplasmic reticulum and mitochondria. This passive Ca^{2+} influx is believed to be induced by a membrane permeability change due to excitation [2,3] and some authors have proposed that an electrogenic Ca^{2+}-K^+ exchange could occur [3,4]. Both the hypothesis that glycosides increase the force of contraction through inhibition of ($Na^+ + K^+$)-ATPase [5,6] and the compelling evidence for a link between Na^+ and Ca^{2+} movements based on isotopic measurements [7,8], mechanical response [9] and electrophysiological data [2] require that Na^+, K^+ and Ca^{2+} fluxes over the cardiac sarcolemma are indeed tightly coupled. Evidence for the existence of a distinct ATP-driven Ca^{2+} pump in cardiac sarcolemma can be found in recent literature [10—14], although others have eliminated this possibility [15,16]. By analogy with the microsomal Ca^{2+} pumps reported in other organs, one might expect the sarcolemmal pump to be regulated by Na^+ and/or K^+ [17]. The presence of an Na^+/Ca^{2+} antiporter has been shown recently [18,19] in cardiac membrane vesicles and this system in vivo may accelerate Ca^{2+} extrusion energetically furnished by the Na^+ gradient, that in turn would be maintained by the ($Na^+ + K^+$)-ATPase pump. Furthermore, it has been proposed that under certain conditions, the ($Na^+ + K^+$)-pump may couple Na^+ efflux with K^+ and Ca^{2+} influx [4]. Since it is known that ($Na^+ + K^+$)-ATPase is inhibited by Ca^{2+} [4], which competes for the intracellular Na^+ activation site of the enzyme, removal of Ca^{2+} by an ATP-driven pump might be essential to maintain high ($Na^+ + K^+$)-ATPase activity, which in turn generates the driving force for the Na^+/Ca^{2+} antiporter function. The present paper examines the effects of Na^+ and K^+ on the active as well as passive Ca^{2+} transport in vesicles mainly derived from cardiac plasma membrane.

Materials and Methods

Materials. [8-^{14}C]ATP (40—60 mCi/mmol) and $^{45}CaCl_2$ (20—40 mCi/mg) were purchased from the Radiochemical Centre (Amersham, U.K.). Cyclic AMP, creatine kinase, creatine phosphate, valinomycin and gramicidin were all products of Boehringer (Mannheim, F.R.G.). Tris-ATP and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). The antibiotic ionophore, X537A, was supplied by Hoffman La Roche (U.S.A.). All other

chemicals were obtained from Merck (Darmstadt, F.R.G.). Millipore filters (0.45 μm) were purchased from Sartorius (Göttingen, F.R.G.).

Isolation of the sarcolemmal fractions. Two different procedures were used to isolate cardiac sarcolemmal vesicles. In both techniques, hearts were removed from ether-anesthetized rats, immediately placed in homogenization buffer, cut into fine pieces and homogenized for 15 s in 5 vol. (v/v) of buffer, using a Polytron homogenizer (PT 10, setting 8). Procedure I, used to obtain particles by sucrose gradient centrifugation, is essentially the same as that described recently [13]. In some experiments sarcolemmal vesicles were prepared using procedure II, in which material was sedimented by centrifugation of a rat ventricle homogenate for 15 min at $1000 \times g$. This sediment was subsequently extracted with hypotonic LiBr and KCl media as described earlier [20,21]. The membrane pellets finally obtained were suspended in 160 mM choline chloride, KCl or NaCl medium containing 20 mM Mops (pH 7.5) if being used for Ca^{2+} transport studies, whereas the pellets were suspended in 0.25 M sucrose and 2 mM dithiothreitol if adenylate cyclase and $(Na^+ + K^+)$-ATPase activities were being assessed. Usually, membrane suspensions, to a final protein concentration of 1—2 mg/ml, were frozen in liquid N_2. Activities of the putative plasma membrane marker enzymes, $(Na^+ + K^+)$-ATPase and adenylate cyclase, were not markedly different in fractions obtained by procedures I and II (cf. Table I). The degree of contamination of either fraction by other subcellular particles (mitochondria, myofibrils and sarcoplasmic reticulum) has been evaluated earlier [13,20].

Separation of a sarcoplasmic reticulum contaminant. The method for removing sarcoplasmic reticulum contamination from the vesicles isolated by procedure I has been described in detail in an earlier report [13]. In rat heart, unlike guinea-pig heart, the ATP-dependent Ca^{2+} uptake did not survive this fractionation procedure and so the distribution pattern for this parameter could not be ascertained.

Enzyme assays. The activity of the sarcolemmal preparation with respect to $(Na^+ + K^+)$-ATPase was assayed at 37°C in a medium containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 4 mM Tris-ATP. The Mg^{2+}-ATPase was assessed in the same medium from which NaCl and KCl were omitted. The ouabain sensitivity of $(Na^+ + K^+)$-ATPase was estimated at 2 mM concentration of the drug. The ATPase reaction was started by the addition of ATP and terminated by the addition of 1 ml ice-cold 12% trichloroacetic acid to 1 ml medium. P_i was measured without delay by using the method of Taussky and Shorr [22]. The mixture for the assay of K^+-stimulated *p*-nitrophenylphosphatase was similar to that previously reported [23], however, the reaction was not continuously registered. After incubation for 20 min at 37°C, the reaction was stopped by the addition of 0.2 ml of 24% trichloroacetic acid to 1 ml of reaction mixture, the denatured protein removed by centrifugation, mixed with 2 ml of 1 M Tris-HCl (pH 10.5) and finally the absorbance registrated at 400 nm ($\epsilon = 15\ mol^{-1} \cdot cm^{-1}$). The assay of adenylate cyclase was performed by using the method of Drummond and Duncan [24], except that the ATP-regenerating system of pyruvate kinase and phospho*enol*pyruvate was replaced by 20 mM creatine phosphate and 0.5 mg creatine kinase. Protein content was determined by using the

procedure of Lowry et al. [40] with bovine serum albumin as the standard.

Measurement of ATP-dependent Ca^{2+} uptake. Choline chloride vesicles (20 μg membrane protein), isolated by procedure I, were added to 100-μl incubation mixture at 37°C containing 50 μM $^{45}CaCl_2$ (40 dpm/nmol), 5 mM $MgCl_2$, 20 mM Mops (pH 7.5) and 160 mM choline chloride, and when required choline chloride was replaced by NaCl or KCl. Aliquots of 50 μl were withdrawn after 15 s of incubation and immediately filtered through Millipore filters. The filters were washed once with 3 ml of an ice-cold solution of a composition similar to that of the incubation medium including 0.5 mM $LaCl_3$ to avoid Ca^{2+} movements during the filtration time (5 s). The radioactivity remaining on the filter was counted by the standard scintillation technique in Lumagel (Packard). For the Na^+-stimulated Ca^{2+} efflux from vesicles loaded previously with Ca^{2+} in the presence of MgATP, the experimental conditions are described in the legend to Fig. 2.

Assay of Na^+-Ca^{2+} exchange in the absence of MgATP. The basic composition of the incubation medium was 50 μM $^{45}CaCl_2$ (150 dpm/nmol) and 160 mM choline chloride, KCl or NaCl in 20 mM Mops (pH 7.5). Vesicles were preloaded with NaCl or KCl by incubating procedure I vesicles, which were originally suspended and frozen in the same medium, for 30 min at 30°C. Usually 20-μl of membrane suspension were added to 200 μl of incubation medium kept in a thermostatically controlled bath at 37°C. At different time intervals, aliquots of 50 μl were stopped by filtration. Routinely, a control incubation was carried out with no NaCl or KCl gradient from the inside to the outside of the vesicle. This level of Ca^{2+} uptake could then be subtracted from other values to estimate uptake due to Na^+-Ca^{2+} exchange.

Results and Discussion

Sidedness of the sarcolemmal vesicles

To study the sarcolemmal Ca^{2+} pump by measuring ATP-dependent Ca^{2+} accumulation, vesicles possessing an inside-out orientation are required. Although insufficient purity has been a shortcoming in work with subcellular cardiac muscle fractions prepared for enrichment of sarcolemma [14], for the purpose of our investigations on passive and active Ca^{2+} transport our attention was especially focussed on the intactness and sidedness of the membranes isolated. In our laboratory, a sarcolemma-enriched fraction from rat heart has been used [13] that can be obtained by centrifugation of a homogenate in a discontinuous sucrose gradient in which neither high salt nor high detergent concentration is present. Lüllman and Peters [25], using a similar method for isolation, could show that these vesicles entrapped extracellular fluid containing [^{3}H]inulin, indicative of their 'intactness' and inside-out orientation. Indeed, these so-called sucrose gradient particles (Table I) displayed activity of adenylate cyclase that is not stimulated by 20 μM isoproterenol, the receptor situated on the external surface of the cell. However, 5′-guanylylimidodiphosphate (100 μM), which binds to the enzyme located in the cell at the cytoplasmic face [26], is able to stimulate adenylate cyclase. As shown in Table I, both modulators of adenylate cyclase were effective in the cardiac homogenate, which probably contains an equal amount of inside-out and right-

TABLE I

INTERACTIONS OF MODULATORS WITH THE SARCOLEMMAL (Na^+, K^+)-PUMP AND THE ADENYLATE CYCLASE COMPLEX

Enzymatic activities were determined as described under Materials and Methods. Sucrose gradient and salt-extracted particles correspond to membrane fractions obtained from rat heart by the two different procedures I and II, respectively. Values represent the mean ±S.E. of four separate experiments. K^+-pNPPase, K^+-stimulated *p*-nitrophenylphosphatase; GMP-P(NH)P, 5′-guanylylimidodiphosphate.

Fractions	(Na^+, K^+)-pump			Adenylate cyclase		
	($Na^+ + K^+$)-ATPase activity (nmol · min^{-1} · mg^{-1})	Inhibition by ouabain (%)	($Na^+ + K^+$)-ATPase/ K^+-pNPPase ratio	Basal	+100 μM GMP-P(NH)P (pmol · min^{-1} · mg^{-1})	+20 μM isoproterenol
Homogenate	—	—	—	49 ± 2	72 ± 5 *	85 ± 3 *
Sucrose gradient particles	417 ± 105	17.3 ± 3.0 **	19.1 ± 2.7 **	480 ± 12	672 ± 21 *	473 ± 23
Salt-extracted particles	305 ± 33	60.0 ± 7.0	8.8 ± 0.6	343 ± 25	331 ± 10	468 ± 20 *

* Activities differ significantly from the corresponding basal adenylate cyclase activities ($P < 0.005$).

** Activities differ significantly from those found in the salt-extracted particles ($P < 0.01$).

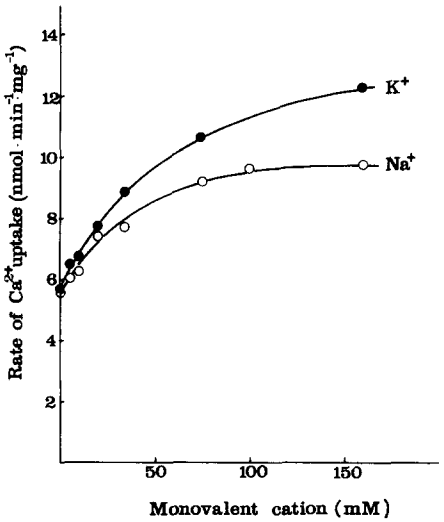

Fig. 1. The effect of monovalent cations on the sarcolemmal Ca^{2+} pump activity. The experiments were carried out as described in Materials and Methods with vesicles isolated by procedure I. Data points are the means of five experiments.

side-out vesicles. The ratio of $(Na^+ + K^+)$-ATPase to K^+-stimulated *p*-nitrophenylphosphatase activity in the sucrose gradient particles is 19 : 1 (Table I), which is considerably higher than the values of 6—10 reported in studies on solubilized $(Na^+ + K^+)$-ATPase preparations [27,28]. This indicated that *p*-nitrophenyl phosphate and K^+ are less accessible to their sites exposed in vivo to the extracellular side of the plasma membrane [27]. It is not surprising then to find only 17% inhibition of $(Na^+ + K^+)$-ATPase by ouabain, that is also believed to act at the outer surface of the sarcolemma [27]. On the other hand, in a previous report [21], we described a sarcolemmal preparation obtained by high salt extraction of an easily sedimenting membrane fraction as first described and extensively characterized by McNamara et al. [20]. Since the specific activities of putative plasma membrane markers in this preparation were not markedly different when compared to the sucrose gradient particles (Table I), it is evaluated on the sidedness too. The data presented in Table I show that the ouabain sensitivity of $(Na^+ + K^+)$-ATPase in the salt-extracted particles was considerably higher than that in the sucrose gradient particles, which would suggest more intact right-side-out or leaky vesicles. The vectorial properties of adenylate cyclase and $(Na^+ + K^+)$-ATPase indeed suggest the salt-extracted vesicles to be predominantly right-side-out. This preparation is therefore inconvenient for the investigation of the sarcolemmal Ca^{2+} pump. Indeed, it was shown earlier [21] to bind only 0.2 nmol Ca^{2+}/min per mg protein in the presence of ATP. From Fig. 1 it can be seen that the sucrose gradient particles are able to accumulate Ca^{2+} at a maximal rate of 12.2 nmol Ca^{2+}/min per mg protein.

Na^+ and K^+ effects on the ATP-dependent Ca^{2+} uptake

Fig. 1 shows that Na^+ and K^+ are able to stimulate the initial rate of Ca^{2+} uptake and that the activation is related to the concentration of the

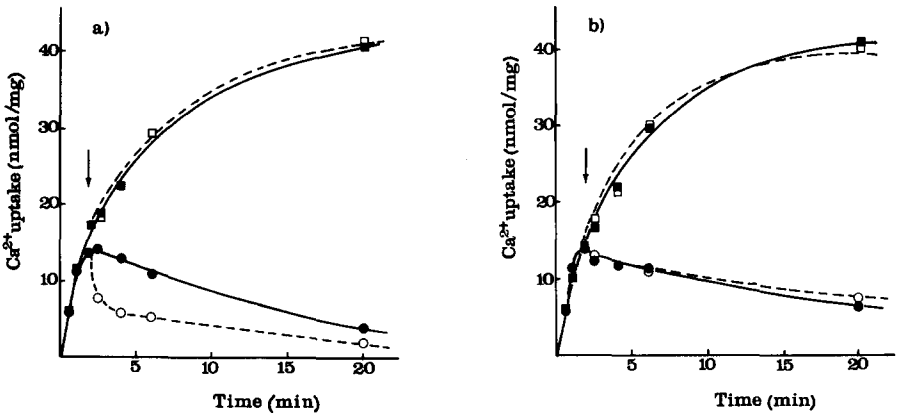

Fig. 2. Na^+-stimulated efflux of Ca^{2+} from vesicles loaded with Ca^{2+} in the presence of MgATP. At zero time, sarcolemmal vesicles (obtained by procedure I) containing intravesicular 160 mM KCl (a) or 160 mM NaCl (b) were diluted in either 600 µl of 160 mM KCl (a) or 160 mM NaCl (b) at 37°C, to which 0.05 mM $^{45}CaCl_2$, 1 mM Tris-ATP and 5 mM $MgCl_2$ had been added. Aliquots of 100 µl were withdrawn at 30 s, 1 min, 2 min and immediately after (at the arrow) 100 µl of medium, as present in the previous incubation (closed symbols), or 100 µl of medium containing 160 mM NaCl (or KCl) to produce an inward 40 mM cation gradient (open symbols, dashed lines) was added. Further aliquots were withdrawn at the times indicated. The complete set of Ca^{2+} transport measurements were carried out in the absence (●, ○) and presence of 2.5 mM Tris-oxalate (■, □).

monovalent cations. Half-maximal stimulation occurred at 35 mM added cation, however, Na^+ is less effective in increasing Ca^{2+} uptake than K^+. The stimulation is not simply due to an increase in ion strength being constant by incorporation of the choline cation. Preliminary experiments (not shown) showed the absence of an effect of choline chloride on Ca^{2+} uptake. Moreover, the sarcolemmal vesicles were routinely suspended and stored in 160 mM choline chloride. Therefore, the activating effect of monovalent cations could be attributed to an inward electrochemical diffusion potential produced by these ions. However, the ionophore (valinomycin) that was used to dissipate the K^+ gradient did not affect the stimulation of the Ca^{2+} uptake (results not shown). Our results on K^+ stimulation of Ca^{2+} uptake by cardiac sarcolemma are consistent with those of St. Louis and Sulakhe [11]. Another report, however, described an inhibition of ATP-dependent Ca^{2+} binding by Na^+ in a plasma membrane preparation purified from guinea-pig heart [29]. The Na^+ stimulation of Ca^{2+} uptake found in this study is unlikely to be due to production of an outward Na^+ gradient by $(Na^+ + K^+)$-ATPase action. This is because initial rates of Ca^{2+} uptake were estimated in the presence of an inward Na^+ gradient. Furthermore, under the conditions employed with no K^+, the $(Na^+ + K^+)$-pump activity was expected to be minimal and the Na^+ concentration of 35 mM, at which half-maximal activation of the Ca^{2+} pump was reached, differed appreciably from the K_a of 10 mM for Na^+ at 50 µM Ca^{2+} previously reported for $(Na^+ + K^+)$-ATPase [4]. That in the present report the Ca^{2+} pump rate seems to be less affected by the presence of Na^+ compared to K^+ is probably caused by an Na^+-dependent leak of Ca^{2+} from the vesicles. Indeed, as follows from Fig. 2, no difference in initial rate of Ca^{2+} movement was found when either 160 mM Na^+ or K^+ was present at

both sides of the membrane. Therefore, it was decided to determine whether an inwardly directed Na^+ gradient could drive Ca^{2+} out of the sarcolemmal vesicles. On the other hand, several investigators recently showed that the sarcoplasmic reticulum Ca^{2+} pump of heart as well as that of skeletal muscle is stimulated 3—5-fold by K^+ and Na^+ [30—32]. It is interesting to note that in these reports the order of effectiveness of Na^+ and K^+ for the stimulation of Ca^{2+} uptake was similar to that found in the present study.

The effect of an inward Na^+ gradient on Ca^{2+} accumulated by the Ca^{2+} pump

If an Na^+/Ca^{2+} antiporter were to be located in the same vesicles that contain Ca^{2+} pump activity, it could be possible to load vesicles with Ca^{2+} in the presence of MgATP and to expel Ca^{2+} by subjection to an Na^+ gradient (external > internal). For this purpose, vesicles containing 160 mM KCl at either side of the membrane were loaded with 0.05 mM Ca^{2+} in the presence of 1 mM ATP and 5 mM $MgCl_2$ for 2 min after which part (40 mM) of the external KCl was replaced by NaCl. Although a slow Ca^{2+} release occurred spontaneously, if no change in composition of the external medium was made, a large increase in the release rate was observed by applying an inward Na^+ gradient (Fig. 2a). Such an observation does not necessarily imply a molecular coupling of Na^+ and Ca^{2+} movements. A higher Ca^{2+} efflux rate in the presence of an inward Na^+ gradient could also occur if this gradient were to create an electrical diffusion potential across the membrane (vesicle inside-positive). However, in Fig. 2b it is shown that when vesicles with 160 mM NaCl at either side of the membrane were loaded with Ca^{2+}, an inward 40 mM KCl gradient was not able to mimic the effect observed with a 40 mM NaCl gradient. Because it is generally believed that the permeability of the plasma membrane is greater for K^+ than for Na^+ [27], an electrical coupling between Na^+ and Ca^{2+} flux seems unlikely as an explanation for the Na^+ gradient-induced Ca^{2+} efflux.

In the presence of oxalate (Fig. 2), no spontaneous Ca^{2+} release could be observed; instead, the Ca^{2+} accumulation continued over at least 20 min. Under this condition, the Na^+ gradient-induced Ca^{2+} release was also prevented. Even the addition of 0.5 mM EGTA only stopped the Ca^{2+} pump to start a very slow Ca^{2+} release. In sharp contrast, if 0.5 mM EGTA was added in the absence of oxalate, all Ca^{2+} loaded in 2 min was released within 1 min (results not shown). This suggests that once Ca^{2+} is precipitated as oxalate inside the lumen of the vesicle, it cannot easily be released either by an inward Na^+ gradient or by EGTA. At least, for an Na^+/Ca^{2+} antiporter to operate, a certain intravesicular free Ca^{2+} concentration might be required to saturate the carrier from the inside. The intravesicular Ca^{2+} concentration may be held below a threshold value by the sequestration due to oxalate. A remarkable finding also was the absence of an effect of oxalate on the initial rate of ATP-dependent Ca^{2+} uptake (Fig. 2), which is not compatible with recent reports [10,11] on sarcolemma from guinea-pig heart but consistent with the results of another paper [12] on canine heart sarcolemma. Therefore, in this report, no use has been made of the terms, Ca^{2+} binding and uptake, as these were estimated using assay conditions without and with oxalate, respectively, in previous reports concerning sarcolemma [10,11] and sarcoplasmic reticulum membranes [33].

At present, no suitable procedure is available to determine which part of Ca^{2+}, retained by the vesicles, is due to transport into an intravesicular space rather than binding at either side of the membrane.

The slow Ca^{2+} release spontaneously occurring after 2 min Ca^{2+} accumulation in the absence of oxalate is probably due to the inhibition of the Ca^{2+} pump by an excessive intravesicular Ca^{2+} concentration (Fig. 2). A similar binding-release cycle was observed earlier in isolated cardiac sarcoplasmic reticulum [34]. The Ca^{2+} release rate in KCl vesicles was more rapid than in NaCl vesicles (cf. Fig. 2a and b). This cannot be explained if one assumes that the Na^+/Ca^{2+} antiporter contributes to the overall release, because it would function best if Na^+ were present.

The effect of an outward Na^+ gradient on the Ca^{2+} uptake in the absence of ATP and Mg^{2+}

One could imagine that if ATP and Mg^{2+} were absent, an alternative driving force for active Ca^{2+} uptake could be provided by an outwardly directed Na^+ gradient across the vesicle membrane. As shown in Fig. 3, NaCl vesicles diluted in KCl medium accumulated, time-dependently, Ca^{2+} to a greater extent than vesicles that were added to NaCl medium. An overshooting Ca^{2+} uptake pattern was obtained which could be ascribed to dissipation of the Na^+ gradient. The results in Fig. 3 indicate that the initial rate of Ca^{2+} uptake by an outward 160/14.5 mM Na^+ gradient came well within the range of the rate of the Ca^{2+} pump (Fig. 2). Reeves and Sutko [18] recently identified Na^+-Ca^{2+} exchange activity in a vesicular preparation from cardiac muscle by using Li^+ as the alternative cation and showed a 10-fold increase in Ca^{2+} uptake under conditions of an outward 160/5 mM Na^+ gradient as compared to vesicles without an Na^+ gradient. However, in the present study, a considerable Ca^{2+} uptake occurred in the absence of an Na^+ gradient (Fig. 3). Passive binding of Ca^{2+} to sarcolemma with a large number of sites of low affinity has been described in numerous reports (recently reviewed by Langer [35]) and is thought to

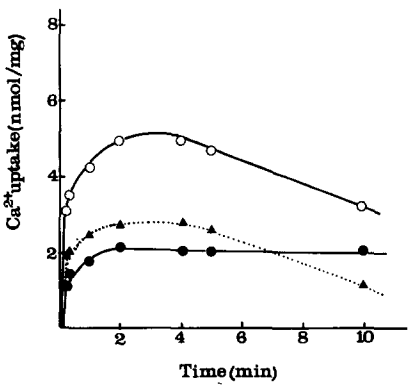

Fig. 3. Influx of Ca^{2+} into sarcolemmal vesicles in the absence of MgATP: The effect of an outwardly directed Na^+ gradient. Vesicles (obtained by procedure I) preloaded with 160 mM NaCl were diluted 11-fold into 160 mM NaCl (●) or 160 mM KCl (○) medium containing 20 mM Mops, pH 7.5, and 0.05 mM $^{45}CaCl_2$. (▲- - - - -▲) Difference between vesicles diluted in KCl minus NaCl medium. Data points are the means of three experiments.

TABLE II

THE EFFECT OF Na^+ and K^+ GRADIENTS ON THE Ca^{2+} INFLUX INTO SARCOLEMMAL VESICLES

Measurements of Ca^{2+} uptake in the absence of MgATP were made using vesicles (obtained by procedure I) preloaded with either 160 mM NaCl or KCl. Ion gradients, as indicated, were produced by 11-fold dilution of the suspension in media containing a different alkali cation under the conditions described in Materials and Methods. Values represent the means ±S.E. of five experiments.

Ion concentration (mM) at either side of the vesicle membrane				Ca^{2+} uptake (nmol/2 min per mg)
$[Na^+]_{in}$	$[Na^+]_{out}$	$[K^+]_{in}$	$[K^+]_{out}$	
160	14.5	0	145.5	5.24 ± 0.96 *
160	160	0	0	2.19 ± 0.34
0	145.5	160	14.5	1.84 ± 0.26
0	0	160	160	2.92 ± 0.23 **

* Value differs significantly from those found under the other ion conditions ($P < 0.005$).

** Value differs significantly from that found when only Na^+ was present in the same concentrations at either side of the membrane ($P < 0.05$).

represent the Ca^{2+} pool essential for the depolarization-induced Ca^{2+} entry into the cardiac cell.

When the outward Na^+ gradient was replaced by K^+, no extra Ca^{2+} uptake was observed above that measured in vesicles having NaCl in the same concentrations at either side of the membrane (Table II). Thus, a similarly directed gradient of K^+ does not produce an equal accumulation of Ca^{2+}, providing support for the operation of an Na^+/Ca^2 antiporter. An unexpected finding was that vesicles having KCl at both sides of a membrane, accumulated significantly more Ca^{2+} compared to NaCl vesicles (Table II). This observation might be related to the more rapid spontaneous Ca^{2+} release found in KCl vesicles (Fig. 2) and would then suggest that the permeability or affinity for Ca^{2+} of the membrane is dependent on the nature of the monovalent cation added.

From Table II it can be seen that an inwardly directed K^+ gradient always

TABLE III

THE EFFECT OF CATION IONOPHORES ON THE Ca^{2+} UPTAKE DRIVEN BY AN Na^+ GRADIENT

Experiments were carried out as indicated in the legend to Fig. 3, except that ethanolic ionophore solutions were added during the preloading and incubation of the sarcolemmal vesicles. In the control ethanol was added to the same concentration (0.25%). Average values (±S.E.) of five independent experiments are given.

Ionophore (5 μg/ml)	Ca^{2+} transport activity (nmol/2 min per mg)	
	Uptake in the absence of an Na^+ gradient	Uptake due to an Na^+ gradient
No addition	2.63 ± 0.21	2.40 ± 0.19
Gramicidin	2.78 ± 0.19	2.43 ± 0.11
Valinomycin	2.61 ± 0.17	3.39 ± 0.26 *
X537A	3.00 ± 0.22	0.81 ± 0.35 *

* Activities differ significantly from those found if no addition to the incubation medium was made ($P < 0.01$).

accompanied the outward Na^+ gradient as was chosen to imitate the in vivo conditions. Both gradients produce diffusion potentials that will counteract with each other. Therefore, if these membrane potentials could be selectively influenced by ionophores and if the Na^+/Ca^{2+} antiporter works electrogenically (three Na^+ for one Ca^{2+}, as shown by Pitts [19]) an effect by valinomycin might be expected. Valinomycin, shown to be a specific K^+ ionophore [36], indeed appeared to stimulate the Na^+ gradient-induced Ca^{2+} uptake, although the ethanol present in the ionophore solution slightly decreased the control activity (Table III). This would then suggest the Na^+/Ca^{2+} antiporter to work electrogenically by exchanging more than two Na^+ for one Ca^{2+}. Gramicidin, which forms channels for H^+, Na^+ and K^+ [36], did not affect the Na^+ gradient-induced Ca^{2+} uptake (Table III). Owing to the increased Na^+ permeability of the membrane, the Na^+-coupled Ca^{2+} influx should decrease rather than increase. As shown in Table III, treating the vesicles with the divalent cation ionophore X537A [34,36] reduced the Na^+ gradient-induced Ca^{2+} uptake, which presents indirect evidence supporting the Ca^{2+} accumulation against a concentration gradient. Valinomycin, gramicidin or X537A did not significantly affect the passive Ca^{2+} uptake.

Relationship between the transmembrane Na^+ gradient and the amount of Ca^{2+} accumulation

Reuter [2] has postulated that the Na^+/Ca^{2+} antiporter could be a mobile carrier that can be occupied competitively by either two Na^+ or one Ca^{2+}. Van Breemen et al. [37] recently proposed a similar carrier to be present in the plasma membrane of smooth muscle, by which three Na^+ would be exchanged for one Ca^{2+}. Independently of the affinities of the carrier for Na^+ and Ca^{2+}, according to these authors [2,37], by exchanging cations it would lead to the following distribution ratio at equilibrium:

$$\frac{[Ca^{2+}]_{in}}{[Ca^{2+}]_{out}} = \frac{[Na^+]^n_{in}}{[Na^+]^n_{out}}$$

where n is the number of Na^+ exchanging for one Ca^{2+}. Therefore, in the experiments described in Fig. 4, an attempt was made to estimate Ca^{2+} uptake at different transmembrane Na^+ gradients. The maximal overshooting occurred after 2 min (cf. Fig. 3), which was interpreted to be the equilibrium state. On a theoretical basis, going with the $[Na^+]_{in}/[Na^+]_{out}$ from 5.5 to 80, one might expect at least 210-fold ($n = 2$) more $^{45}Ca^{2+}$ to be accumulated. In fact, only a 3-fold higher Ca^{2+} uptake was found in the vesicle preparation (Fig. 4). However, it is interesting to note that addition of 5 mM oxalate to either side of the vesicle membrane could increase this factor to 10. In any case, this finding indicates Na^+ gradient-induced Ca^{2+} uptake to be a translocation of Ca^{2+} across the vesicle membrane after which it could be precipitated as oxalate. The discrepancy between these results and the theoretical considerations could be due to many uncontrollable factors such as real transport equilibrium, stability of the imposed Na^+ gradients and intactness of the vesicles. The possibility that the initial Na^+ gradient could be dissipated by the antiporter itself is very unlikely if the relative concentrations of internal Na^+

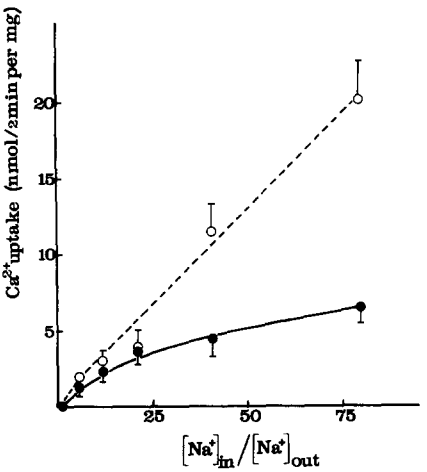

Fig. 4. Relationship between the transmembrane Na^+ gradient and the amount of Ca^{2+} accumulated. Membrane vesicles (obtained by procedure I) were loaded with 160 mM NaCl and then 6-, 11-, 21-, 41- or 81-fold diluted into either 160 mM KCl or NaCl containing 0.05 mM $^{45}CaCl_2$ and 20 mM Mops (pH 7.5). Incubation was carried out for 2 min at 37°C. The values were all corrected for the Ca^{2+} uptake found if the Na^+ gradient was absent. The experiments have been performed in the absence (•) as well as in the presence of 5 mM Tris-oxalate (○). Each point represents the mean ±S.E. of four experiments.

and external Ca^{2+} are considered. Although in the present study no measurement of intravesicular volume has been carried out, on the basis of the data on sarcolemmal vesicles reported in Ref. 18 (5 μl ^3H_2O/mg protein and three Na^+ for one Ca^{2+} exchange), maximally 7% of internal Na^+ was calculated to be released in the experiments shown in Fig. 4.

Evidence for the Na^+/Ca^{2+} antiporter being exclusively localized in sarcolemma

An Na^+-Ca^{2+} exchange carrier has been demonstrated earlier in cardiac mitochondria [38]. The rate of Na^+-induced Ca^{2+} efflux from pure mitochondria [38] is well within the range of the rate (Fig. 2) found in the present membrane preparation, while the mitochondrial contamination has previously been shown to be rather low [13]. Another possibility is the involvement of the sarcoplasmic reticulum, although it has been suggested by others [12,18, 19] that Na^+-Ca^{2+} exchange does not exist in this membrane system. To our knowledge, only one report [39] has described Na^+ gradient-induced Ca^{2+} release in cardiac microsomes, but the fraction was not investigated as to its subcellular origin. The sarcolemmal preparation used in the present report does contain sarcoplasmic reticulum contamination [13]. After removal, however, Ca^{2+} pump activities in both membrane fractions were almost completely lost [13]. From the distribution of 5'-nucleotidase, Ca^{2+}, Mg^{2+}-ATPase and $^{45}Ca^{2+}$ in the sucrose gradient, conclusions could be made concerning the nature of the fractions harvested. As shown in Table IV, the particles earlier shown to be of sarcolemmal origin displayed the highest Na^+-Ca^{2+} exchange activity. The 1.8-fold increase in activity per mg protein above the unfractionated vesicles came well within the range of increase (2.5-fold) observed earlier for the plasma membrane marker, 5'-nucleotidase [13]. On the

TABLE IV

Na^+/Ca^{2+} ANTIPORTER ACTIVITIES IN SARCOLEMMA AND SARCOPLASMIC RETICULUM

The further fractionation of the crude sarcolemma preparation obtained by procedure I has been described previously and the sucrose gradient fractions B and E [13] were shown to be of sarcolemmal and sarcoplasmic reticulum origin, respectively. The Na^+ gradient-induced Ca^{2+} uptake activity was estimated as described in Materials and Methods. Values represent the means ±S.E. of three experiments.

Fractions	Ca^{2+} transport activity (nmol/2 min per mg)	
	Uptake in the absence of an Na^+ gradient	Uptake due to an Na^+ gradient
Unfractionated	1.79 ± 0.48	2.43 ± 0.37
Sarcolemma (B)	1.33 ± 0.44	4.34 ± 0.27
Sarcoplasmic reticulum (E)	1.00 ± 0.20	0.82 ± 0.22

other hand, the sarcoplasmic reticulum particles contained rather low activity (Table IV). This result demonstrates that the Na^+/Ca^{2+} antiporter activity can be entirely accounted for the sarcolemmal content of the crude membranes used in the foregoing experiments.

Concluding remarks

The results suggest that cardiac sarcolemma, besides a Ca^{2+} pump activated by Na^+ and K^+, possesses a Na^+/Ca^{2+} antiporter. This system functions during Ca^{2+} pump action. Oxalate-dependent Ca^{2+} loading could now be demonstrated in the absence of MgATP. Furthermore, a transmembrane K^+-diffusion potential is able to stimulate the antiporter. Therefore, this acts electrogenically, while it exchanges more than two Na^+ for one Ca^{2+}. This means that at the resting membrane potential of the cardiac muscle cell, the antiporter can extrude Ca^{2+}, driven both by an inward Na^+ gradient and by a membrane potential.

Note added in proof (Received November 25th, 1980)

Three additional reports on valinomycin-induced K^+-diffusion potential, which stimulates Na^+—Ca^{2+} exchange activity in cardiac sarcolemma, have recently appeared [41—43].

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References

1 Langer, G.A. (1973) Annu. Rev. Physiol. 35, 55—86
2 Reuter, H. (1974) Circ. Res. 34, 599—605

3 Morad, M. and Goldman, Y. (1973) Prog. Biophys. Mol. Biol. 27, 257—313
4 Lindenmayer, G.E. and Schwartz, A. (1975) J. Mol. Cell. Cardiol. 7, 591—612
5 Akera, T., Larson, F.S. and Brody, T.M. (1970) J. Pharmacol. Exp. Ther. 173, 145—151
6 Besch, H.R., Allen, J.C., Glick, G. and Schwartz, A. (1970) J. Pharmacol. Exp. Ther. 171, 1—12
7 Wendt, I.R. and Langer, G.A. (1977) J. Mol. Cell. Cardiol. 9, 551—564
8 Jundt, H., Porzig, H., Reuter, H. and Stucki, J.W. (1975) J. Physiol. 246, 229—253
9 Lüttgau, H.C. and Niedergerke, R. (1958) J. Physiol. 143, 486—505
10 Hui, C.-W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415—427
11 St. Louis, P.J. and Sulakhe, P.V. (1976) Can. J. Biochem. 54, 946—956
12 Caroni, P. and Carafoli, E. (1980) Nature 283, 765—767
13 Lamers, J.M.J. and Stinis, J.T. (1980) Biochim. Biophys. Acta 624, 443—459
14 Wollenberger, A. and Will, H. (1978) Life Sci. 22, 1159—1178
15 Van Alstyne, E., Bartschat, D.K., Wellsmith, N.V., Poe, S.L., Schilling, W.P. and Lindenmayer, G.E. (1979) Biochim. Biophys. Acta 553, 388—395
16 Jones, L.R., Besch, H.R., Fleming, J.W., McConnaughey, M.M. and Watanabe, A.M. (1979) J. Biol. Chem. 254, 530—539
17 Adams, M. and Duggan, P.F. (1979) Biochem. Soc. Trans. 7, 49—50
18 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 590—594
19 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232—6235
20 McNamara, D.B., Sulakhe, P.V., Singh, J.N. and Dhalla, N.S. (1974) J. Biochem. 75, 795—803
21 Lamers, J.M.J., Panagia, V. and Dhalla, N.S. (1980) in Advances in Myocardiology (Tajuddin, M., Das, P.K., Tariq, M. and Dhalla, N.S., eds.), Vol. 5, pp. 65—75, University Park Press, Baltimore
22 Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675—685
23 Lamers, J.M.J., Stinis, J.T., Kort, W.J. and Hülsmann, W.C. (1978) J. Mol. Cell. Cardiol. 10, 235—248
24 Drummond, G.I. and Duncan, L. (1970) J. Biol. Chem. 245, 976—983
25 Lüllmann, H. and Peters, T. (1976) Recent Adv. Cardiac Struct. Metab. 9, 311—328
26 Abramowitz, J., Iyengar, R. and Birnbaumer, L. (1979) Mol. Cell. Endocrinol. 16, 129—146
27 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3—134
28 Besch, H.R., Jones, L.R. and Watanabe, A.M. (1976) Circ. Res. 39, 586—595
29 Morcos, N.C. and Jacobson, A.L. (1978) Can. J. Biochem. 56, 1—6
30 Jones, L.R., Besch, H.R. and Watanabe, A.M. (1977) J. Biol. Chem. 252, 3315—3323
31 Duggan, P.F. (1977) J. Biol. Chem. 252, 1620—1627
32 Shigekawa, M. and Pearl, L.J. (1976) J. Biol. Chem. 251, 6947—6952
33 Dhalla, N.S., Ziegelhoffer, A. and Harrow, J.A.C. (1977) Can. J. Physiol. Pharmacol. 55, 1—22
34 Entman, M.L., Gillette, P.C., Wallick, E.T., Pressman, B.C. and Schwartz, A. (1972) Biochem. Biophys. Res. Commun. 48, 847—853
35 Langer, G.A. (1980) J. Mol. Cell. Cardiol. 12, 231—239
36 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501—530
37 Van Breemen, C., Aaronson, P. and Loutzenhiser, R. (1979) Pharmacol. Rev. 30, 167—208
38 Crompton, M., Kunzi, M. and Carafoli, E. (1977) Eur. J. Biochem. 79, 549—558
39 Palmer, R.F. and Posey, V.A. (1967) J. Gen. Physiol. 50, 2085—2095
40 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
41 Reeves, J.P. and Sutko, J.L. (1980) Science 208, 1461—1464
42 Miyamoto, H. and Racker, E. (1980) J. Biol. Chem. 255, 2656—2658
43 Bers, D.M., Philipson, K.D. and Nishimoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358—371