Biochimica et Biophysica Acta, 728 (1983) 239–253
Elsevier Biomedical Press

BBA 71544

MECHANISM OF PASSIVE Ca^{2+} PERMEABILITY OF VESICULAR SARCOLEMMAL PREPARATIONS FROM RAT HEARTS

VALERY V. KUPRIYANOV, ANDREY, N. PREOBRAZHENSKY and VALDUR A. SAKS

Laboratory of Cardiac Bioenergetics, USSR Research Center of Cardiology, Academy of Medical Sciences, Petroverigsky Lane, 10, Moscow (U.S.S.R.)

(Received November 4th, 1982)

Key words: Ca^{2+} channel; Sarcolemma vesicle; Ion permeability; (Rat heart)

Vesicular sarcolemmal preparations isolated from rat hearts were characterized by high total ATPase (4.32 ± 0.57 μmol/min per mg), adenylate cyclase (121 ± 11 pmol/min per mg) and creatine kinase (1.73 ± 0.35 μmol/min per mg) activities as well as Na-Ca exchange specific to sodium. ATPase activity was inhibited with digitoxigenin by 50–70% and was not changed by ouabain, ionophore A23187 or oligomycin. Sarcolemmal vesicles bound [^{3}H]digitoxigenin and [^{3}H]ouabain in isotonic medium in the presence of P_i and Mg^{2+}. The number of binding sites for hydrophobic digitoxigenin ($N = 237$ pmol/mg) was several-times higher than that for hydrophilic ouabain ($N = 32.7$ pmol/mg). These data show that sarcolemmal preparations were not significantly contaminated by mitochondria and sarcoplasmic reticulum and consisted mostly of inside-out vesicles. Incubation of these vesicles with $^{45}Ca^{2+}$ (0.5–10 mM) led to penetration of the latter into the vesicles with the following binding characteristics: number of binding sites ($N = 20.5 \pm 4.6$ nmol/mg, $K_d \approx 2.0$ mM). Ca^{2+} binding to the inner surface of vesicles was proved by the following facts: (1) Ca^{2+} ionophore A23187 increased slightly total intravesicular Ca^{2+} content but markedly accelerated Ca^{2+} efflux along its concentration gradient; (2) gramicidin and osmotic shock showed a similar accelerating effect. Ca^{2+} efflux from the vesicles along its concentration gradient ($[Ca^{2+}]_i/[Ca^{2+}]_e = 2.0$ mM/0.1 μM) was inhibited by Mn^{2+}, Co^{2+}, and verapamil when they acted inside the vesicles. The rate of Ca^{2+} efflux was hyperbolically dependent on intravesicular Ca^{2+} concentration ($K_m \approx 2.9$ mM). These data reveal that Ca^{2+} efflux from sarcolemmal vesicles is controlled by Ca^{2+} binding to the sarcolemmal membrane. Ca^{2+} efflux from the vesicles was stimulated 1.7-times after incubation of vesicles with 0.2 mM MgATP or MgADP and 15-times after treatment with 0.2 mM adenylyl β,γ-imidodiphosphate. Enhancement in the rate of Ca^{2+} efflux correlated with the increase in the intravesicular Ca^{2+} content. ATP-stimulated Ca^{2+} efflux was suppressed by verapamil and was nonmonotonically dependent upon the transmembrane potential created by the K^+ concentration gradient in the presence of valinomycin, Ca^{2+} efflux being slower at extreme values of membrane potential (± 80 mV).

Introduction

The sarcolemma of cardiac cells plays an important role in the regulation of the intracellular concentration of free calcium ions [1–5]. There are at least three ways of Ca^{2+} influx and efflux across the sarcolemma of myocardial cells [1–10]: electrogenic slow inward calcium current along Ca^{2+} concentration gradient through slow calcium channels dependent on membrane potential [1–5], Ca^{2+} movement across the sarcolemma via Na-Ca

Abbreviation: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetate.

exchange [2,6,7], and, finally, Ca^{2+}-dependent ATPase in cardiac sarcolemma catalyzing ATP-dependent Ca^{2+} extrusion from the cytoplasm of myocardial cells against the Ca^{2+} concentration gradient [8–11]. Molecular mechanisms of the regulation of sarcolemmal Ca^{2+} permeability remain unclear. Their elucidation requires a more simple system than cardiac cells, and sarcolemmal vesicle preparations have proved to be successful tools in these investigations [6–11]. With these vesicles, Ca^{2+}-dependent ATPase and Na-Ca exchange have been identified to be associated with cardiac sarcolemma [6–11]. However, an open question is that of the passive permeability of the membrane of sarcolemmal vesicles to Ca^{2+}.

Therefore, the main goal of this work was to study the mechanisms of passive Ca^{2+} influx and Ca^{2+} efflux from inside-out vesicles from cardiac ventricle. It is shown in this work that the rate of passive Ca^{2+} efflux from inside-out sarcolemmal vesicles is controlled by the process of Ca^{2+} binding with the intravesicular membrane surface.

Materials and Methods

Isolation of cardiac sarcolemmal and sarcoplasmic reticulum preparations. Preparations of sarcolemma from rat and guinea-pig hearts were isolated as described in Ref. 12. Isolation was carried out by a method first described by Kidwai et al. [13,14] and modified in our laboratory. The rat cardiac tissue was homogenized for 1 min in a Virtis-45 (U.S.A.) homogenizer at 25000 rev./min in 10 vol. (with respect to tissue weight) of buffer I, comprising 20 mM imidazole-HCl (pH 7.3)/0.25 M sucrose. The homogenate was centrifuged for 45 min at $236000 \times g_{max}$. The pellet was resuspended in buffer I and layered in a ratio 4:3 on the sucrose solution (37%, w/v) containing also 20 mM imidazole-HCl (pH 7.3). This discontinuous sucrose gradient was centrifuged for 45 min at $236000 \times g_{max}$. An upper layer supernatant was recentrifuged for 45 min at $236000 \times g_{max}$. A pellet obtained was resuspended in buffer I and layered on a linear sucrose gradient (5 ml:30 ml). Linear sucrose gradients were formed in 'Density Gradient Former' (Beckman, U.S.A.) from 8% (w/v) and 40% (w/v) sucrose solutions in 20 mM imidazole-HCl buffer (pH 7.3). After centrifugation for 14–16 h at $132000 \times g_{max}$ in an SW-27 rotor (Beckman) 30 ml of the upper layer were withdrawn (to a layer of 28–30% sucrose) and centrifuged for 60 min at $236000 \times g_{max}$. The supernatant was discarded and the pellet was resuspended in 3–4 ml of a buffer comprising 20 mM imidazole-HCl (pH 7.3)/0.3 M sucrose/0.3 mM dithiothreitol and centrifuged for 20 min at $5000 \times g_{max}$. The supernatant obtained contained sarcolemmal membrane vesicles and was used in experiments. Sarcoplasmic reticulum was isolated from guinea-pig myocardium according to Levitsky et al. [15].

Determination of enzymatic activities. Creatine kinase activity was measured as previously described [12] at 30°C in 20 mM imidazole-HCl (pH 7.3)/0.3 M sucrose. Adenylate cyclase activity was detected according to Steiner et al. [16] at 37°C as in our previous work [12]. ATPase activity was recorded spectrophotometrically at 30°C in a medium containing 20 mM imidazole-HCl (pH 7.3), 0.3 M sucrose, 5 mM $MgCl_2$, 3 mM ATP, 1 mM phospho*enol*pyruvate, 10 mM KCl, 100 mM NaCl, 0.16 mM NADH and 2–3 IU/ml of both pyruvate kinase and lactate dehydrogenase. The reaction was started by addition of membrane preparation to assay medium and the decrease in absorbance at 340 nm was recorded.

[^{3}H]Ouabain and [^{3}H]digitoxigenin binding with sarcolemma from rat heart. Specific cardenolid binding was measured according to the methods described in Refs. 17 and 18 with some modifications. Sarcolemmal vesicles (1–2 mg protein per ml) were incubated for 10 min at 37°C with [^{3}H]ouabain or [^{3}H]digitoxigenin taken in a concentration range of 0.3 to 100 μM in a medium comprising 20 mM imidazole-HCl (pH 7.3)/0.3 M sucrose/3–4 mM $MgCl_2$/2–3 mM sodium phosphate (pH 7.3). Vesicles incubated under the same conditions but with 5 mM unlabelled ouabain instead of sodium phosphate were used as a blank for nonspecific ouabain binding. After 10 min incubation, samples (50 μl) were withdrawn from the mixture, diluted 10-times with the ice-cold buffer (0.45 ml) containing 20 mM imidazole-HCl and 5 mM unlabelled ouabain just on the filters and sucked immediately. Then filters were washed twice with 0.5 ml cold 20 mM imidazole buffer (pH 7.3). The filters were placed in Bray's scintillator and their radioactivity was assayed. The con-

tent of glycosides specifically bound to the vesicles was determined by substracting blank radioactivity values from the experimental ones. Each measurement of cardiac glycoside binding for every particular concentration was repeated from three to nine times for a given sarcolemmal preparation. The specific radioactivity of [^{3}H]ouabain and [^{3}H]digitoxigenin in the incubation medium was 0.5–50 Ci/mmol and 0.020–6.0 Ci/mmol, respectively.

Determination of Ca^{2+} content in sarcolemmal vesicles and the rate of passive Ca^{2+} efflux from these vesicles. The equilibrium Ca^{2+} content in the vesicles was assayed after preincubation of the membranes (3–5 mg/ml) with various $^{45}Ca^{2+}$ concentrations for several hours at 0°C with subsequent prewarming at 37°C for 10 min. Equilibration was carried out at 0°C to avoid heat denaturation of sarcolemma. Short-term prewarming at 37°C was necessary to achieve the equilibrium of Ca^{2+} distribution specific for this temperature. The incubation medium contained 20 mM imidazole-HCl (pH 7.3), 0.3 M sucrose, 10–20 mM KCl, 0.3 mM dithiothreitol, 2 μg/ml valinomycin, 2 mM $MgCl_2$ and other components indicated in the figure legends. Then 20–25 μl (60–100 μg protein) of incubation mixture were rapidly diluted 20-tiumes with cold (0°C) incubation buffer directly on the filter (12 mm Millipore, 0.45 μm pore) and immediately filtered. Filters were washed twice with 0.4–0.5 ml medium comprising 20 mM imidazole-HCl (pH 7.3)/0.3 M sucrose/10 mM KCl/2 mM $MgCl_2$/1 mM EGTA, or 20 mM imidazole-HCl (pH 7.3)/0.3 M sucrose/10 mM $CoCl_2$. After that, the filters were dissolved in scintillation cocktail (Bray) and radioactivity was counted. Obviously, the radioactivity of a filter is determined by the ^{45}Ca in the vesicles and ^{45}Ca adsorbed on the impregnated filter. For determination of background filter radioactivity, vesicles loaded with ^{45}Ca in the concentrations required were diluted 20-times with the incubation medium containing 1–2 μM ionophore A23187 and EGTA at a concentration bringing the free Ca^{2+} level under 0.1 μM to release Ca^{2+} from vesicles. After 0.5 h incubation at 37°C, 0.4–0.5 ml aliquots were withdrawn, placed on the filters, sucked immediately and washed twice as described above. Vesicular Ca^{2+} content was determined as the difference between total filter radioactivity and background. Usually, the background level was not higher than 10% of total filter radioactivity. For the measurements of initial Ca^{2+} content when Ca^{2+} efflux kinetics were studied, the same approach was used.

Ca^{2+} efflux from sarcolemmal vesicles was triggered by 20-fold dilution of the particle suspension in the incubation buffer ($t = 37$°C) containing EGTA at a concentration maintaining free Ca^{2+} concentration outside the vesicles around 0.1 μM, corresponding to that for relaxed cardiac muscle. In addition, the dilution medium contained choline chloride, the concentration of which was equal to double the $CaCl_2$ concentration inside the vesicles. At certain times, 0.4–0.5 ml of the incubation mixture was applied to a Millipore filter (0.45 μm), rapidly filtered and washed twice by cold buffer as described above. The radioactivity of Ca^{2+} in the vesicles was calculated as the difference between filter radioactivity and blank radioactivity as mentioned above. The vesicular content of Ca^{2+} at zero time was detected by the same procedure as used for the assay of equilibrium Ca^{2+} binding. Ca^{2+} efflux from the vesicles was relatively slow at 0°C with EGTA or at 37°C without EGTA in the dilution medium in comparison to that at 37°C in the presence of EGTA. These data show the absence of any essential loss of Ca^{2+} during washing of filter-bound vesicles with cold solution containing EGTA.

Free Ca^{2+} concentration in the medium was calculated according to the formulae:

$$[Ca^{2+}]_{free} = \frac{[Ca^{2+}]_{tot}}{[EGTA]_{tot} - [Ca^{2+}]_{tot}} \cdot K_d^{CaEGTA}$$

since $[EGTA]_{tot} - [Ca^{2+}]_{tot} \gg K_d^{CaEGTA}$, where $[Ca^{2+}]_{free}$ is the concentration of free Ca^{2+}; $[Ca^{2+}]_{tot}$ and $[EGTA]_{tot}$ are total concentrations of Ca^{2+} and EGTA, respectively; K_d^{CaEGTA} is the effective dissociation constant for CaEGTA complex at pH 7.3, equal to 0.15 μM [19].

Assay of Na-Ca exchange. For the measurements of Na^+-dependent Ca^{2+} accumulation in the sarcolemmal vesicles, the vesicles (3–5 mg protein per ml) were equilibrated for several hours at 0°C with 100–120 μM $CaCl_2$ and 100–120 mM NaCl in the same medium as used for Ca^{2+} bind-

ing and efflux measurements. Then the incubation mixture was prewarmed at 37°C for 5–10 min. Ca^{2+} accumulation was initiated by dilution of the incubation mixture indicated 20–25 times with the same incubation buffer containing 95 mM choline chloride or LiCl instead of NaCl, and 100–120 μM $^{45}CaCl_2$ with the same specific radioactivity. At fixed time intervals, 0.4–0.5 ml of the incubation mixture was applied onto the filter and treated further according to the procedure described above. For detection of Na^+-dependent Ca^{2+} efflux from the vesicles, the latter were equilibrated with 100–120 μM $CaCl_2$ and 100 mM LiCl at 0°C in the medium indicated above, then prewarmed at 37°C for 5–10 min and diluted 20-times with the incubation buffer containing 95 mM NaCl and 100–120 μM $^{45}CaCl_2$ with the same specific radioactivity as the ^{45}Ca in the concentrated suspension of the membranes. Subsequent manipulations with the samples were similar to that described above.

In all cases, 0.45 μm Millipore filters of 12 mm diameter were rinsed in 20 mM imidazole-HCl buffer (pH 7.3) and washed after the installation of the filter in a holder with the same buffer in order to remove traces of radioactivity remaining after previous filtrations on the holder.

Protein determination. This was carried out according to Lowry et al. as modified by Hartree [20].

Reagents. All nucleotides, enzymes, sucrose, trisodium phospho*enol*pyruvate, disodium phosphocreatine, EGTA, glucose, $MgCl_2$, oligomycin, valinomycin, dithiothreitol, bovine serum albumin were purchased from Sigma (U.S.A.). Digitonin and rotenone were from Calbiochem (U.S.A.). Ouabain was purchased from Serva (F.R.G.), and [^{3}H]ouabain from Amersham International (U.K.). Imidazole and salts were from BDH (U.K.). Digitoxigenin and [^{3}H]digitoxigenin kindly donated by Dr. R. Grosse, Central Institute of Molecular Biology, Berlin-Buch, G.D.R. Ionophore A23187 was from Eli Lilly (U.S.A.).

Spectrophotometric measurements were carried out in Aminco DW-2UV-VIS spectrophotometer (U.S.A.). Radioactivity was detected in scintillation counter Mark III (U.S.A.).

Results

(1) Characterization of sarcolemmal preparations

Enzymatic activities. The properties of sarcolemmal preparations used in our experiments are summarized in Table I. It can be seen from this table that prepatations from rat heart possessed

TABLE I

COMPARISON OF ATPase ACTIVITIES OF SARCOLEMMAL PREPARATIONS ISOLATED FROM RAT AND GUINEA-PIG HEARTS WITH THAT OF SARCOPLASMIC RETICULUM FROM GUINEA-PIG HEART

ATPase activities were measured in the assay medium for $(Na^+ + K^+)$-ATPase described in Materials and Methods at 30°C and pH 7.3. Values are given as mean ± S.E. Numbers (n) in brackets represent number of preparations used for activity determinations. n.d., not determined.

	Additions	ATPase activities (μmol/min per mg)			
		Sarcolemma preparations		Reticulum preparations	
		Rat	Guinea pig	No. 1	No. 2
1	Control, no additions	4.32 ± 0.57 (n = 21)	1.85 (n = 3)	0.60	0.83
2	0.3 mM digitoxigenin	1.18 ± 0.40 (n = 21)	0.85 (n = 3)	0.40	0.53
3	0.5 mM ouabain	4.16 ± 0.58 (n = 21)	1.78 (n = 3)	0.61	0.86
4	1 mM EGTA	4.62 ± 0.59 (n = 9)	1.81	0.17	0.45
5	0.3 mM digitoxigenin + 1 mM EGTA	n.d.	n.d.	0.13	0.27
6	2 μg/ml oligomycin	3.94 ± 0.55 (n = 8)	n.d.	n.d.	n.d.
7	1 μM A23187	4.65 ± 0.82 (n = 5)	n.d.	1.11	1.69
8	1 μM A23187 + 1 mM EGTA	n.d.	n.d.	0.15	0.30

high total Mg^{2+}-ATPase activity (4.32 ± 0.57 μmol/mg per min), 50–70% of which was inhibited by 0.3 mM digitoxigenin (to 1.18 ± 0.40 μmol/min per mg), whereas 0.5 mM ouabain did not affect the ATPase activity. The inhibitory action of digitoxigenin developed with time: practically complete inhibition (50–70%) was observed after 5–6 min of addition of the inhibitor to an assay mixture. In addition, these preparations had high adenylate cyclase (121 ± 11 pmol/min per mg) and creatine kinase (1.74 ± 0.35 μmol/min per mg) activities. Digitonin, a detergent interacting specifically with cholesterol-containing membranes, caused stimulation of total ATPase (10–15%) and creatine kinase (approx. 20%) activities. These data may mean that sarcolemma preparations are closed vesicles impermeable to ATP (ADP) and ouabain; the major part of the vesicles (approx. 80%) is inside-out and the minor part of it (approx. 20%) is right-side-out. The closed vesicular structure of the sarcolemmal preparation was proved by electron microscopic data (Fig. 1). The low level of reticular contaminations in our preparations is confirmed by comparative inhibitory analysis of ATPase activities of sarcolemma from rat and guinea-pig myocardium on the one hand, and sarcoplasmic reticulum from guinea-pig heart on the other, as shown in Table I. As can be seen from Table I, the total ATPase activity of the sarcoplasmic reticulum was inhibited with 1 mM EGTA by 50–70% and activated 2-fold by Ca^{2+}-specific ionophore A23187, whereas sarcolemmal ATPase activity was not changed by these substances at all. First, this means that sarcolemmal preparations did not actually contain visible reticular contaminations. Second, it implies that the Ca^{2+}-ATPase activity of the sarcoplasmic reticulum was stimulated by residual Ca^{2+} from water and reagents, the removal of which by EGTA results in significant suppression of this ATPase. Oligomycin, an inhibitor of mitochondrial ATPase, taken at a concentration of 2 μg/ml did not influence the ATPase activity of sarcolemmal preparations, demonstrating the absence of any detectable mitochondrial contamination. Rotenone (2–4 μM), an inhibitor of mitochondrial NADH-oxidase, did not inhibit NADH oxidation in a coupled pyruvate kinase-lactate dehydrogenase ATPase assay, demonstrating absence of NADH-oxidase contamination in our preparations.

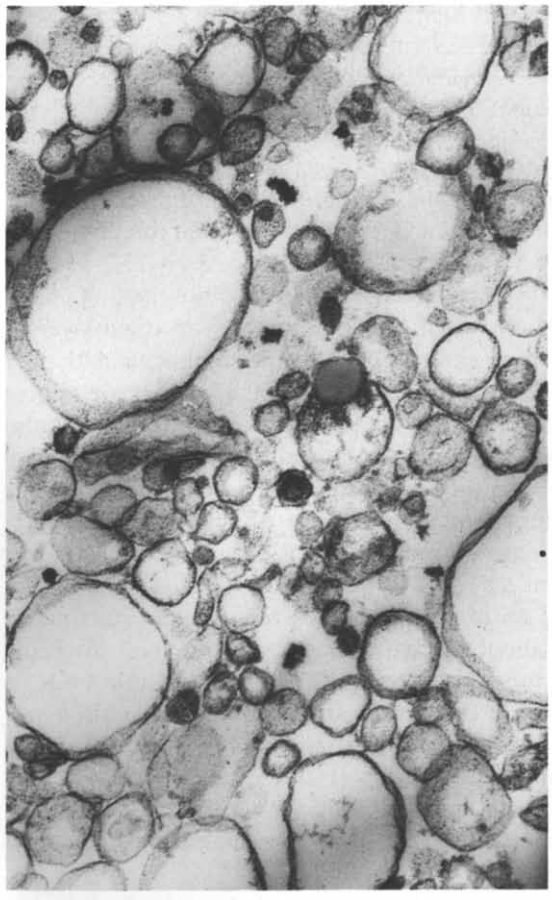

Fig. 1. Electron micrograph of sarcolemmal preparation from rat heart. Fixation by glutaric aldehyde and osmium tetroxide. Magnification, ×40000.

[^{3}H]Ouabain and [^{3}H]digitoxigenin binding to sarcolemmal vesicles. It is known that either purified or cell membrane bound ($Na^+ + K^+$)-ATPase is capable to specific and rather fast binding of cardiac glycosides in the presence of ligands such as ATP + Na^+ + Mg^{2+} or phosphate + Mg^{2+} due to phosphoenzyme formation [17,18,21,22]. In this work we used mixture of P_i and Mg^{2+}, since one can suppose that phosphate ion is able to penetrate across the vesicular membrane, in contrast to ATP. When phosphate ($+Mg^{2+}$) penetrating into the vesicles is used, ouabain can bind to right-side-out vesicles, and digitoxigenin to both types of vesicle. Thus, the amount of digitoxigenin-binding sites determined in the presence of $P_i + Mg^{2+}$ gives us

the total content of $(Na^+ + K^+)$-ATPase in preparations: the number of ouabain-binding sites under the same conditions allows us to estimate the proportion of open and right-side-out vesicles. Our sarcolemmal preparations had the following digitoxigenin- and ouabain-binding parameters under isotonic conditions, determined from the concentration dependence of their binding: digitoxigenin: $N = 234$ pmol/mg, $K_d = 11.2$ μM ($r^2 = 0.97$); ouabain: $N = 32.7$ pmol/mg, $K_d = 9.8$ μM ($r^2 = 0.79$). The number of digitoxigenin-binding sites is several times higher than that of ouabain, but the dissociation constants are almost the same. The ratio N(ouabain)/N(digitoxigenin) shows a proportion of right-side-out vesicles equal to 14%, in good agreement with our enzymic estimations. Comparison of the number of digitoxigenin-binding sites (234 pmol/mg) with the $(Na^+ + K^+)$-ATPase activity (approx. 180 μmol/mg per h at 30°C) gives a ratio of these parameters of about 1.3 pmol per one activity unit, this being in the range determined for purified $(Na^+ + K^+)$-ATPase (between 1 and 3) [21,22]. Also, the number of digitoxigenin-binding sites is close to that obtained by Jones et al. [23] (275 pmol/mg) for ouabain binding with their right-side-out sarcolemmal preparations in the presence of alamethycin.

Thus, the data presented above imply that our sarcolemmal preparations contain 80% of closed inside-out vesicles and do not contain detectable mitochondrial and reticulum contaminations.

Na-Ca exchange. Cardiac sarcolemma, as well as the plasma membrane of other cells, contains carrier catalyzing transmembrane Na-Ca exchange [6,7,10,11,24]. Therefore, Na-Ca exchange may serve as a sarcolemmal marker and, additionally, as an indicator of membrane permeability to Ca^{2+} and Na^+, since high Ca^{2+} or Na^+ permeability should result in rapid dissipation of their gradients and Na-Ca exchange cannot be detected under these conditions. As follows from Fig. 2A 20-fold dilution of sarcolemmal vesicle suspension equilibrated with 110 μM $CaCl_2$ and 100 mM NaCl in the medium containing the same Ca^{2+} concentration, 95 mM choline Cl and no Na^+ added ($[Na^+]_i/[Na^+]_e = 100$ mM/5 mM) led to 4-fold enhancement of total vesicular Ca^{2+} content. In the presence of ionophore A23187, Ca^{2+} accumulation was essentially lower and the initial Ca^{2+} uptake was followed by its efflux. This Ca^{2+} accumulation was not observed when Na^+ was substituted by K^+ (see Fig. 2A), nor when Na^+ was substituted by Li^+ (Fig. 2B), nor when intravesicular and extravesicular Na^+ concentrations were equal (absence of gradient). Ca^{2+} accumulation curves were well linearized in the semilogarithmic co-ordinates log ($Ca_\infty - Ca$) vs. time, giving pseudo-first-order rate constants between 1.9 and 3.7 min^{-1} (Fig. 2B). When the Na^+ gradient was directed inside the vesicles, a decrease in sarcolemmal Ca^{2+} content was observed; the Li^+ gradient did not cause Ca^{2+} extrusion (Fig. 2B). These data can be taken to show that the sodium-dependent Ca^{2+} movement across the sarcolemma observed in our experiments is actually Na-Ca exchange, giving rise to a transmembrane Ca^{2+} gradient at the expense of energy of the sodium gradient.

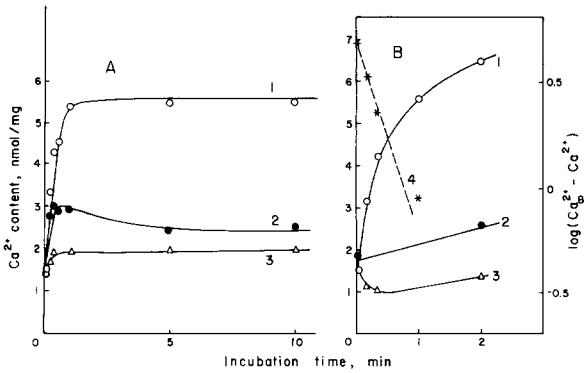

Fig. 2. Na-Ca exchange in sarcolemmal vesicles. A. Influence of ionophore A23187 on Ca^{2+} accumulation induced by an outwardly directed Na^+ gradient. (1) 100 mM NaCl inside the vesicles and 5 mM NaCl + 95 mM choline chloride outside; (2) the same as (1) plus 1 μM A23187; (3) 120 mM KCl inside and 25 mM KCl + 95 choline chloride outside the vesicles. B. Na^+-induced Ca^{2+} uptake and extrusion. (1) 100 mM NaCl inside and 5 mM NaCl + 95 mM choline chloride outside the vesicles; (2) 100 mM LiCl inside and 5 mM LiCl + 95 mM choline chloride outside the vesicles; (3) 100 mM LiCl inside and 5 mM LiCl + 95 mM NaCl outside the vesicles; (4) semilogarithmic plot of curve 1.

(2) $^{45}Ca^{2+}$ binding with sarcolemmal vesicles

We may suppose that incubation of sarcolemmal vesicles with Ca^{2+} leads to its binding with the external membrane surface, followed by its penetration into the vesicles where it fills the in-

travesicular water volume and binds to the inner membrane surface. In this case, the total Ca^{2+} content in sarcolemmal preparations consists of three Ca^{2+} pools: (1) Ca^{2+} bound to the external vesicular surface; (2) Ca^{2+} located inside the vesicles in the free soluble state (in the intravesicular water space) and (3) Ca^{2+} bound to the inner membrane surface. Since the purpose of this work was to study the transmembrane Ca^{2+} fluxes, we have focused our attention on changes in intravesicular Ca^{2+} content connected with its influx into and efflux from the vesicles. Therefore, when the vesicles were separated from reaction mixture by rapid filtration on Millipore filters (0.45 μm), filters were washed twice with cold isotonic buffer containing 1 mM EGTA or 10 mM $CoCl_2$. Such a treatment would completely remove Ca^{2+} bound to the external membrane surface since, according to the data of Philipson et al. [25,26], even washing with buffer solution causes removal of the greater part of the Ca^{2+} bound to the external vesicular surface.

Primarily, we investigated the kinetics of passive Ca^{2+} accumulation in sarcolemmal vesicles. As shown in Fig. 3A, Ca^{2+} accumulation is rather slow, even at 37°C, and is completed practically after 20 min of incubation. Addition of the Ca^{2+}-specific ionophore, A23187 (1–2 μM), to the incubation medium accelerated Ca^{2+} influx insignificantly and only slightly increased maximal intravesicular Ca^{2+} content (Ca^{2+}_{∞}). The latter fact shows that the observable Ca^{2+} accumulation is at least partially transmembrane Ca^{2+} movement into the vesicles. In fact, if Ca^{2+} binding occurred only to the external membrane surface, then the ionophore should markedly increase the vesicular Ca^{2+} content owing to ionophore-induced Ca^{2+} transfer inside the vesicles, as was the case, for example, for the sarcolemmal preparations used by Philipson et al. [25,26]. Analysis of accumulation curves in coordinates log ($Ca^{2+}_{\infty} - Ca^{2+}$) vs. time (Fig. 3B) shows that the initial parts of the curves in semilogarithmic plots approximate to straight lines, but later the dependence deviates from linearity. This means that for initial moments of time, Ca^{2+} accumulation in the vesicles is described by pseudo-first-order kinetics, as could be expected for passive diffusion across the membrane. Observable pseudo-first-order rate constants varied

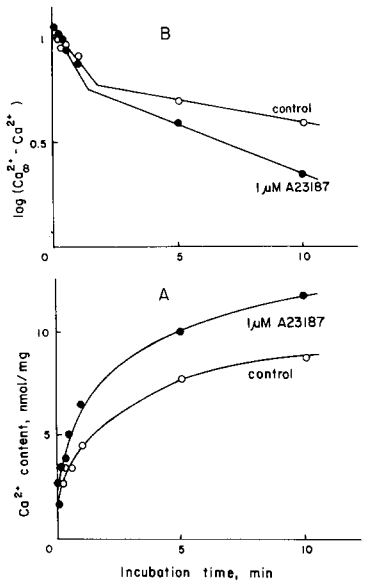

Fig. 3. A. Kinetics of passive Ca^{2+} accumulation in sarcolemmal vesicles from rat myocardium. B. Data shown in as dependences of log ($Ca^{2+}_{\infty} - Ca^{2+}$) vs. time. The incubation medium contained 1.86 mM $^{45}CaCl_2$ with specific radioactivity 1.0 μCi/mmol and 5.03 mg sarcolemmal protein per ml. Temperature, 37°C; pH 7.3.

from 0.26 to 0.42 min^{-1}. In the presence of 2 μM ionophore A23187, this value was enhanced approx. 1.5-times.

Further, we studied equilibrium Ca^{2+} binding with sarcolemmal vesicles over the concentration range corresponding to physiological extracellular concentrations of calcium. Sarcolemmal vesicles were preincubated at 0°C for several hours at various concentrations of Ca^{2+} and prewarmed at 37°C for 10 min, after which vesicular Ca^{2+} content was determined. Fig. 4A shows that dependences of vesicular Ca^{2+} content on Ca^{2+} concentration in the external medium are of hyperbolic type. In the presence of 2 μM ionophore A23187, the curve is slightly shifted upward. The dependences can be linearized in double-reciprocal plots ($1/[Ca^{2+}]_{tot}$ vs. $1/[Ca^{2+}]_e$), giving the number of binding sites per mg protein and the dissociation constant, K_d (Fig. 4B). Note that ionophore A23187 did not significantly change the number of Ca^{2+} binding sites or the K_d.

As mentioned above, vesicular Ca^{2+} content can be taken to consist of the amount of Ca^{2+} in

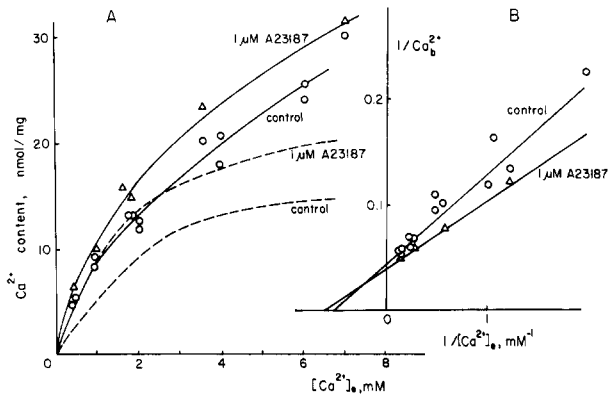

Fig. 4. A. Dependence of total Ca^{2+} content in sarcolemmal vesicles on Ca^{2+} concentration in the incubation medium. B. Dependence of Ca^{2+} binding to sarcolemmal vesicles on Ca^{2+} concentration in double-reciprocal plots. Dotted lines show calculated curves for bound Ca^{2+} content obtained by subtraction of the amount of Ca^{2+} contained in the internal water volume taken equal to 1.5 μl per mg from total Ca^{2+} content. The concentration of sarcolemmal protein was 5.18 mg/ml.

the intravesicular water volume and of the Ca^{2+} bound with the membrane. For such a distribution of Ca^{2+} inside the vesicles, the total content of calcium in the vesicles can be described by the equation (see also Ref. 25):

$$Ca^{2+}_{tot} = Ca^{2+}_{f} + Ca^{2+}_{b} = \vartheta[Ca^{2+}]_e + \frac{N[Ca^{2+}]_e}{K_d + [Ca^{2+}]_e}$$

where Ca^{2+}_{tot} is total vesicular Ca^{2+} content, and Ca^{2+}_{f} and Ca^{2+}_{b} the content of free and membrane-bound Ca^{2+} inside the vesicles, respectively; $[Ca^{2+}]_e$ is the Ca^{2+} concentration in the external medium equal to intravesicular Ca^{2+} concentration, $[Ca^{2+}]_i$, at equilibrium (mmol/l); ϑ is the internal vesicular water volume (l/mg); N is the number of Ca^{2+} binding sites (nmol/mg) and K_d the Ca^{2+} dissociation constant (mmol/l). This equation is applicable if following assumptions hold: first, the inner vesicular volume (ϑ) is much smaller than external medium volume, this being always so in practice; second; there is only one type of Ca^{2+} binding site characterized by one K_d, and Ca^{2+} binding to different binding sites is independent. Analysis of experimental data on the basis of this equation (see Fig. 4A, B) gave an internal volume equal to about 1.5 μl/mg, a number of binding sites, N, of 20 ± 4.6 nmol/mg and a K_d of 1.85 ± 0.21 mM for our preparations. When the internal vesicular volume was not taken into account, values of N and K_d were somewhat overestimated in comparison with those presented above: $N = 31.7 \pm 7.6$ nmol/mg and $K_d = 3.03 \pm 0.46$ mM.

(3) Passive Ca^{2+} efflux from sarcolemmal vesicles

To investigate passive Ca^{2+} efflux from inside-out-oriented sarcolemmal vesicles preloaded with Ca^{2+} by long-term incubation in isotonic buffer containing various Ca^{2+} concentrations (0.5–10 mM) as described above (see Fig. 2), a Ca^{2+} gradient was generated across the sarcolemmal vesicle membranes by dilution of the vesicle suspension 20-times with Ca^{2+}-free medium containing EGTA at concentrations maintaining concentration of free Ca^{2+} outside the vesicles under 0.1 μM ($K_d^{CaEGTA} = 0.15$ μM at pH 7.3 [19]). Fig. 5A demonstrates the kinetics of Ca^{2+} efflux from sarcolemmal vesicles at an intravesicular Ca^{2+} concentration of 1.6 mM and 37°C. As follows from this figure, vesicular Ca^{2+} content drops to approximately a half for the first 30 s. Representation of these data in semilogarithmic plots (log $[Ca^{2+}]$ vs. time) shows that the kinetics of Ca^{2+} efflux are not described by one exponential but that it is a multiphase process (Fig. 5A). The initial rate of Ca^{2+} efflux determined for the first 5 s was equal to 0.3 nmol/mg per s, and the pseudo-first-order rate constant was 2 min^{-1}. The minimal time interval available for measurements of initial Ca^{2+} efflux from the vesicles with the technique used was 2–3 s when Ca^{2+} efflux was triggered immediately on the filter. For this initial time interval, the efflux rate was determined to be 0.8 nmol/mg per s. Addition of ionophore A23187 (5 μM) and gramicidin (10 μg/ml) in the Ca^{2+} loading medium and in the dilution buffer accelerated efflux of this cation more than 4-times, as can be seen from Fig. 5B. A similar accelerating effect was produced by dilution of vesicular suspension in hypotonic medium without sucrose (Fig. 5B). These data allows us to conclude that the observed decrease in the total vesicular Ca^{2+} content was a result of transmembrane Ca^{2+} movement from inside the vesicles to the external medium, since any treat-

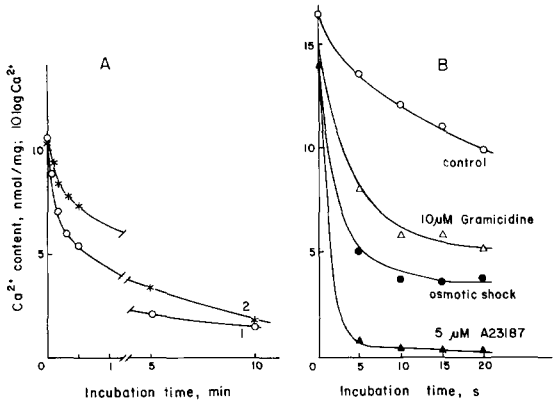

Fig. 5. A. Kinetics of passive Ca^{2+} efflux from the sarcolemmal vesicles. (1) dependence of vesicular Ca^{2+} content on time; (2) dependence of log $[Ca^{2+}]$ on time. B. Effects of ionophores and osmotic shock on the rate of passive Ca^{2+} efflux from the vesicles. Vesicles (4.4 mg/ml) were equilibrated with 1.8 mM Ca^{2+}. To produce osmotic shock, sucrose was omitted from the dilution medium.

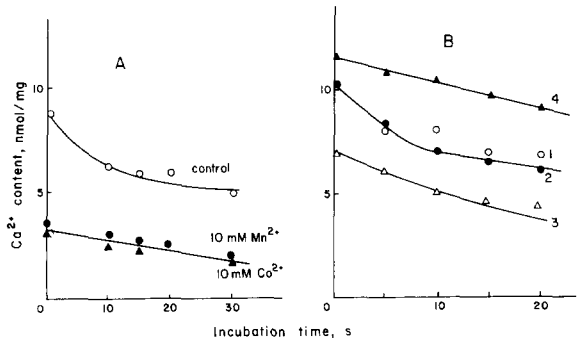

Fig. 6. Inhibition of passive Ca^{2+} efflux from sarcolemmal vesicles by Mn^{2+}, Co^{2+} and verapamil. A. Effect of Mn^{2+} and Co^{2+}. B. Verapamil effect: (1) control; (2) 0.1 mM verapamil added to extravesicular space; (3) vesicles isolated with 0.1 mM verapamil which was also present in the external medium in the same concentration; (4) no EGTA in the dilution medium at 85 μM external Ca^{2+} concentration. $MnCl_2$ and $CoCl_2$ were added to the Ca^{2+} loading medium at the concentrations indicated simultaneously with 1.7 mM $CaCl_2$. After equilibration with these cations for several hours at 0°C and following 10 min prewarming at 37°C, Ca^{2+} efflux was initiated by 20-fold dilution in the medium containing 0.2 mM EGTA and 0.5 mM EDTA. Therefore, the external dilution medium contained free Mn^{2+} and Co^{2+} in negligible concentrations and about 0.1 μM free Ca^{2+}. The control curve 1 shown in A was determined in the presence of 0.5 mM Mg·EDTA.

ment increasing sarcolemmal membrane permeability (either by ionophores or by mechanical disruption by osmotic shock) resulted in augmentation of the rate of Ca^{2+} efflux from the vesicles. The most simple mechanism for this Ca^{2+} movement is its leakage through membrane water channels emerging, for instance, from disturbances of the membrane structure. For such a mechanism of passive diffusion, the rate of Ca^{2+} efflux should depend linearly on its intravesicular concentration at constant external Ca^{2+} and in the simplest case should be independent of other cations. The data presented in Fig. 6A show that simultaneous preincubation of sarcolemmal particles with Ca^{2+} and 10 mM Mn^{2+} or 10 mM Co^{2+} decreased vesicular Ca^{2+} content by 60–70%, thus leading to a several-fold decrease in the rate of Ca^{2+} efflux. The same decrease in intravesicular Ca^{2+} content was observed when these divalent cations were added after equilibration of the vesicles with Ca^{2+}, which may be taken to show competition between Ca^{2+} and divalent cations for binding sites inside the vesicles. Since the dilution medium contained 0.5 mM EDTA, i.e., at a concentration equimolar with respect to Mn^{2+} and Co^{2+} concentrations in the dilution medium after 20-fold dilution of the initial suspension, the inhibitory action of divalent cations was most probably related to their binding to the internal surface of the vesicles. The Ca^{2+} antagonist, verapamil (0.1 mM) added outside the vesicles into the incubation medium simultaneously with Ca^{2+} and into the dilution medium, did not suppress Ca^{2+} binding and efflux (Fig. 6B). At the same time, sarcolemmal preparations isolated in the presence of verapamil (0.1 mM in the homogenization medium) and therefore containing it, were characterized by a decreased rate of Ca^{2+} efflux, with a less significant decrease in the initial Ca^{2+} content (about 30%) Thus, Ca^{2+} antagonists such as Mn^{2+}, Co^{2+} and verapamil competing with Ca^{2+} for binding sites on the sarcolemmal membrane [27–30] were able to block Ca^{2+} efflux from the vesicles when they were inside the vesicles.

It is necessary to note that the rate of Ca^{2+} efflux from the vesicles was decreased by factor of 3 when the external Ca^{2+} concentration was enhanced up to 85 μM (by omitting EGTA from the dilution medium), although the magnitude of the

Ca^{2+} concentration gradient across the vesicle membrane was practically unchanged, since $[Ca^{2+}]_i$ was always much greater than $[Ca^{2+}]_e$.

Fig. 7A demonstrates the dependence of the Ca^{2+} efflux rate on free Ca^{2+} concentration inside the vesicles. This dependence is described by a hyperbolic curve giving a straight line in double-reciprocal plots (Fig. 7B). As can be seen, K_m for this process is close to the K_d found for equilibrium Ca^{2+} binding to sarcolemma ($K_d = 1.85 \pm 0.21$ mM). Ionophore A23187 augmented only the maximal rate of Ca^{2+} efflux (by 3–4 times (not shown), see Table II), not affecting the K_m value. These results suggest that Ca^{2+} efflux from the vesicles is somehow controlled by its binding to the inner surface of the vesicle membrane. Actually, there is a linear relationship between the Ca^{2+} efflux rate and the total Ca^{2+} content in preparations (Fig. 7C).

This conclusion is consistent with inhibition of Ca^{2+} efflux from the vesicles with Mn^{2+}, Co^{2+} and verapamil. All these results show that Ca^{2+} efflux from the vesicles represents a process more complex than simple diffusion across a leaky membrane and is somewhat controlled or mediated by Ca^{2+} binding with sarcolemma.

(4) Effects of ATP and its analogues on the Ca^{2+} efflux rate

Preincubation of the sarcolemmal vesicles with

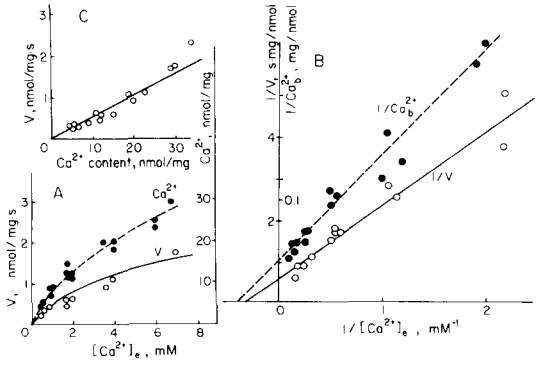

Fig. 7. A. Dependence of Ca^{2+} efflux velocity from sarcolemmal vesicles on intravesicular Ca^{2+} concentration. B. Data shown in A are given in double-reciprocal plots. C. Correlation between Ca^{2+} efflux rate and vesicular Ca^{2+} content. For comparison, the dependences of Ca^{2+} binding with sarcolemmal vesicles on Ca^{2+} concentration taken from Fig. 4 are shown by dotted lines.

0.2 mM MgATP at 30°C for 10 min before their loading with Ca^{2+} resulted in some increase in vesicular Ca^{2+} content (by $13.5 \pm 4.3\%$) and in its efflux rate 1.7-times (1.73 ± 0.49, $n = 15$, see Table II). Turnover number of Ca^{2+} binding sites defined as $V/[Ca^{2+}]_b$ also increased 1.5-times after treatment of sarcolemmal preparations with ATP. The latter observation means that the accelerating effect of ATP cannot be explained by simple enhancement of the number of Ca^{2+}-binding sites as compared to control preparations, but relates either to modification of existing binding sites or to appearance of new sites with higher turnover number. In fact, the increase in the rate ($\Delta V = V_{ATP} - V$) correlated with the rise in Ca^{2+} content ($\Delta[Ca^{2+}]_b = [Ca^{2+}]_{b,ATP} - [Ca^{2+}]_b$), giving a turnover number ($\Delta V/\Delta[Ca^{2+}]_b$) for new binding sites (0.49 ± 0.21 s^{-1}) that is 5-times higher than that for control preparations (0.093 ± 0.054 s^{-1}). Also, ATP did not cause any essential change in the K_d for Ca^{2+} and the K_m for Ca^{2+} efflux from the vesicles. Verapamil introduced into the vesicles by its addition into the homogenization medium (0.1 mM) completely suppressed ATP-stimulated Ca^{2+} efflux (see Table II). ATP-stimulated Ca^{2+} efflux was dependent on an electric potential difference across the membrane created by a transmembrane K^+ gradient in the presence of valinomycin, as shown in Fig. 8. This figure demonstrates the ATP-induced relative enhancement of Ca^{2+} efflux rate from the vesicles ($\Delta V/V$) as a function of membrane potential. In the absence of membrane potential ATP produced the maximal stimulating effect. This effect was not observed at extreme potential values (± 80 mV). Non-monotonic dependence of the ATP-stimulated Ca^{2+} efflux rate on membrane potential may indicate involvement of membrane potential-sensitive ion channels in this process. The dependence found has a semiquantitative character: first, the presented values of the potentials were calculated using the Nernst equation ($\varphi = (RT/F) \cdot \ln [K^+]_i/[K^+]_e$) and second, these values were changed with time as the K^+ gradient dissipated. Note that the rate of Ca^{2+} efflux in control preparations (without preincubation with ATP) was practically independent of membrane potential over the range tested (see Fig. 8). It would be attractive to explain the observed effect of ATP by

TABLE II

THE EFFECT OF ATP, ADP + P_i, Ado*PP*[NH]*P* AND A23187 ON THE CALCIUM BINDING AND ITS EFFLUX FROM CARDIAC SARCOLEMMAL VESICLES

In this table means ± S.E. are shown. In brackets, the number of determinations is given equal to number of sarcolemmal preparations for which these determinations were performed. Each determination is average of 3–5 measurements for every preparation. Temperature, 37°C; pH 7.3.

Experimental conditions	V/V_0	$[Ca^{2+}]_b/[Ca^{2+}]_{b,0}$	$\Delta V/[\Delta Ca^{2+}]_b$ (s^{-1})	$TN = V/[Ca^{2+}]_b$ (s^{-1})	TN/TN_0
1 Control	1.0	1.0	–	0.093 ± 0.054 ($n = 16$)	1.0
2 0.2 mM ATP	1.73 ± 0.49 ($n = 15$)	1.135 ± 0.043 ($n = 12$)	0.49 ± 0.21 ($n = 14$)	0.138 ± 0.063 ($n = 15$)	1.53 ± 0.34 ($n = 16$)
3 0.2 mM ATP + verapamil	0.99 ± 0.32 ($n = 6$)	1.01 ± 0.05 ($n = 6$)	–	0.098 ± 0.017 ($n = 4$)	0.99 ± 0.29 ($n = 6$)
4 0.2 mM ADP + 0.2 mM P_i	1.79 ± 0.28 ($n = 4$)	1.043 ± 0.019 ($n = 4$)	1.03 ± 0.48 ($n = 4$)	–	1.59 ± 0.45 ($n = 5$)
5 0.2 mM Ado*PP*[NH]*P*	15 ± 5.9 ($n = 8$)	2.3 ± 0.52 ($n = 8$)	0.42 ± 0.08 ($n = 9$)	0.259 ± 0.044 ($n = 8$)	6.4 ± 1.5 ($n = 8$) [a]
6 1–2 μM A23187	3.43 ± 0.62 ($n = 7$)	1.17 ± 0.08 ($n = 11$)	–	–	–

[a] Ratios of turnover numbers (TN/TN_0) were calculated using control values of turnover numbers (TN_0) detected only for preparations for which this effector was tested.

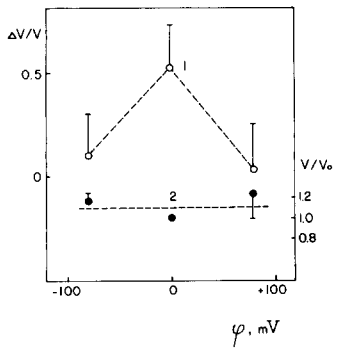

Fig. 8. Dependence of the relative increase in the velocity of Ca^{2+} efflux from the vesicles, stimulated with ATP ($\Delta V/V$) and relative efflux rate without ATP stimulation on the membrane potential. (1) dependence of $\Delta V/V$ on φ, left axis; (2) V/V_0 as function of φ, right axis. Membrane potential was generated by K^+ gradient in the presence of 2 μg/ml valinomycin and was calculated using Nernst equation. $\Delta V = V_{ATP} - V$, where V_{ATP} and V are the rates of Ca^{2+} efflux from the vesicles preincubated with and without ATP, respectively; V_0 is the rate of Ca^{2+} efflux at zero potential in the absence of ATP. Each point is mean ± S.E. obtained for five sarcolemmal preparations. Values of Ca^{2+} efflux rate for each sarcolemmal preparation are averages of 3–5 measurements.

phosphorylation of some sarcolemmal proteins in the protein kinase reaction catalyzed by membrane-bound sarcolemmal protein kinase existing in our preparations [12]. Actually, in our preparations phosphorylation of low molecular weight sarcolemmal protein with M_r approx. 11 500 occurred and the phosphoprotein formed was stable enough [12]. It must be noted that the accelerating effect of ATP on Ca^{2+} efflux was not related to its extrusion from right-side-out vesicles by sarcolemmal Ca^{2+} ATPase since, first, ATP does not penetrate into the intact vesicles and, second, all ATP (0.2 mM) added to a plasma membrane suspension (2–5 mg/ml) was hydrolyzed for 10 min with incubation at 30°C. In other words, Ca^{2+} efflux from the vesicles was determined in the presence of ADP and phosphate. Therefore, similar experiments were carried out in which ATP was substituted by an equimolar mixture of ADP and P_i. It appeared that ADP plus phosphate as well as ADP alone increased the rate of Ca^{2+} efflux to the same extent as did ATP (see Table II). Since sarcolemmal preparations contained some adenylate kinase activity, it is possible to imagine that some smal amount of ATP was formed from ADP sufficient for sarcolemmal protein phosphorylation. However, addition of an excess of hexokinase and glucose to the incubation medium did not eliminate the ADP effect. To resolve this point we used nonhydrolyzable ATP analogue adenylyl β,γ-imidodiphosphate (Ado*PP*[NH]*P*). Taken in the same concentration as ATP (0.2 mM), this compound caused a 2–3-fold increase in the vesicular Ca^{2+} content together with a 15-fold enhancement of Ca^{2+} efflux rate and a 6-fold rise in the average turnover number of Ca^{2+}-binding sites (see Table II). It means that Ado*PP*[NH]*P* induced the formation of new Ca^{2+}-binding sites which are characterized by a higher turnover number ($\Delta V/\Delta[Ca^{2+}]_b$) than the binding sites in control preparations. The turnover number for Ado*PP*[NH]*P*-induced binding sites is close to that for ATP-induced binding sites (see Table II). Ado*PP*[NH]*P* effect was not produced by cAMP formed from Ado*PP*[NH]*P* in the adenylate cyclase reaction, since cAMP itself taken in concentrations of 10 μM and 200 μM or in combination with ATP did not stimulate significantly the Ca^{2+} efflux from the vesicles. Thus, there is a certain similarity between ATP, ADP and Ado*PP*[NH]*P* effects on Ca^{2+} efflux from sarcolemmal vesicles. This similarity was displayed in the simultaneous increase in the vesicular Ca^{2+} content and the rate of Ca^{2+} efflux after treatment of sarcolemmal vesicles with these substances. Enhancement of the rate of Ca^{2+} efflux correlated with the increase in Ca^{2+} content and turnover number of these newly formed binding sites. This number was approximately the same for all these compounds and several times higher than that for control preparations (see Table II). Acceleration of Ca^{2+} efflux from the vesicles in the presence of the non-hydrolyzable ATP analogue (Ado*PP*[NH]*P*) shows that the mechanism of action of this substance is not related to the phosphorylation of sarcolemmal proteins.

Discussion

The procedure for isolation of the sarcolemmal vesicles used in this work provided a preparation of mostly inside-out particles from rat and guinea-pig hearts. It is necessary to note here that different research teams obtain sarcolemmal prepara-

tions with opposite membrane orientation: inside-out [31–33] and right-side-out [23–25,34]. First, this can result from application of different methods for isolation of sarcolemma from muscles of different animals, and second, from application of different criteria for estimation of vesicular membrane sidedness. One such method of sidedness examination is based on the detection of the number of ouabain-binding sites of intact vesicles in the absence and in the presence of detergent making the membrane permeable to components of the incubation medium [23,24]. In works in which this method was used, ouabain binding was determined in mixture of ATP/Mg^{2+}/Na^+ [23,24]. However, since ouabain and ATP binding centres are located on the opposite sides of the vesicular membrane, it is possible to determine only the content of open vesicles without detergent and not the content of right-side-out particles as the authors believed [24]. Using of Mg^{2+} and P_i for measurement of ouabain binding to sarcolemmal vesicles for sidedness estimation provides more adequate results.

The results of this study show that the sarcolemmal vesicles' membrane in the isolated preparation is specifically permeable for Ca^{2+} and allows its passive movement along its concentration gradient by a mechanism involving the binding of calcium ions to specific binding sites on the membrane. According to our estimations, the number of Ca^{2+}-binding sites on the inner vesicular membrane surface was about 20 nmol/mg (pH 7.3, 37°C); Ca^{2+} binding to these sites was characterized by a dissociation constant of about 2 mM. The data obtained in this work may be compared with similar results from Langer's laboratory [25,26]. They investigated Ca^{2+} binding with sarcolemmal vesicles isolated from rabbit hearts [25,26]. However, their sarcollemal vesicles were mostly right-side-out oriented [26]. Despite of the essential differences in sarcolemmal preparations used and the experimental conditions, the value of the dissociation constant for Ca^{2+} found in this work ($K_d \approx 2$ mM) is close to that determined in the Langer's laboratory ($K_d = 2.7$ mM [26]).

Passive Ca^{2+} efflux from sarcolemmal vesicles does not seem to be a matter of simple Ca^{2+} diffusion across the membrane, but is apparently controlled and/or mediated by Ca^{2+} binding to the internal vesicular surface. This point of view is supported by the following observations. First, divalent cations such as Mn^{2+} and Co^{2+}, known as competitors for Ca^{2+} in the process of binding with sarcolemma [27], decreased Ca^{2+} binding by about 60% and simultaneously decreased the rate of Ca^{2+} efflux from the vesicles. Second, the rate of Ca^{2+} efflux was hyperbolically dependent on the intravesicular concentration of free Ca^{2+}; the K_m value for this process was very close to the K_d value obtained for equilibrium Ca^{2+} binding with the vesicles. In addition, reagents which elevated vesicular Ca^{2+} content (ATP, Ado*PP*[NH]*P*) accelerated its efflux from the vesicles; there was good correlation between ΔV and $\Delta[Ca^{2+}]_b$ (see Fig. 7C and Table II).

The rate of Ca^{2+} efflux from the vesicles may be controlled by Ca^{2+} binding in two ways: (1) Ca^{2+} binding to the membrane may change its properties and thereby increase membrane permeability to Ca^{2+} located in the vesicular water space; (2) Ca^{2+} binding is an obligatory step of its transfer across the membrane according to the following scheme:

$$Ca_i^{2+} + L^{n-} \overset{K_d}{\rightleftarrows} Ca \cdot L^{(n-2)-} \rightarrow Ca_e^{2+} + L^{n-}$$

where Ca_i^{2+} and Ca_e^{2+} are intra- and extravesicular Ca^{2+}, respectively, and L^{n-} is ligand binding Ca^{2+} on the membrane surface faced to extracellular medium. According to this scheme, the rate of Ca^{2+} efflux from the vesicles is proportional to concentration of the complex $Ca \cdot L^{(n-2)-}$. In this case, any changes in the concentration of $Ca \cdot L^{(n-2)-}$, for instance, either by addition of competing ions, Mn^{2+}, Co^{2+} and verapamil or by the increasing (or decreasing) Ca^{2+} concentration inside the vesicles, should lead to corresponding changes in the rate of Ca^{2+} efflux.

Ca^{2+} efflux from sarcolemmal vesicles was also stimulated by pretreatment of the vesicles with ATP, ADP and Ado*PP*[NH]*P*. If the mechanisms of action of all these substances are similar, then such a mechanism is not directly related to sarcolemmal protein phosphorylation. A possible alternative mechanism of the activating effect of adenine nucleotides may consist of their binding

with some sarcolemmal components, thus leading to the elevation of Ca^{2+} permeability. The fact that ATP-stimulated Ca^{2+} efflux from sarcolemmal vesicles was inhibited with verapamil and nonmonotonically dependent on membrane potential may indicate possible involvement of some potential-dependent mechanism in this process, analogous to ion channels. In this respect it is worth mentioning the experiments of Atsuko et al. [35] in which ATP, GTP and Ado*PP*[NH]*P* were found to increase slow inward Ca^{2+} current in frog myocardium [35].

Additionally, it has very recently been shown that a slow inward calcium current in snail neurons is stimulated by ATP, ADP and Ado*PP*[NH]*P* when, however, they are introduced extracellularly [36]. These results have been discussed in terms of 'purinergic' receptors [36].

A stimulatory effect of ATP on Ca^{2+} binding to sarcolemmal membrane has been observed earlier [30,37,38]. However, it is not clear from these works which binding was measured: Ca^{2+} binding to the membrane surface or ATP-dependent Ca^{2+} accumulation inside the vesicles catalyzed by Ca^{2+}-ATPase. It was also found that verapamil partially inhibited passive and ATP-dependent Ca^{2+} binding with sarcolemmal preparations [30]. These effects of ATP and verapamil on Ca^{2+} binding seem to be similar to those observed in our work.

The mechanism of passive Ca^{2+} movement across the sarcolemmal membranes described in this work is in a good accordance with the current concept of the role of Ca^{2+} binding with sarcolemma in the control of cardiac muscle contraction [25–27,39,40]. Thus, Philipson et al. [25,26] showed that the rate of contraction force development in rabbit heart (dT/dt) is directly related to the degree of occupancy of sarcolemmal binding sites with Ca^{2+}. At the present time it is not clear, however, in which way the passive Ca^{2+} movement observed in our study may be related to some physiological mechanism of regulation of cardiac contraction.

References

1 Reuter, H. (1973) Progr. Biophys. Mol. Biol. 26, 3–43
2 Langer, G.A. (1973) Annu. Rev. Physiol. 35, 55–86
3 Langer, G.A., Frank, J.S. and Brady, A.J. (1976) Int. Rev. Physiol. 9, 191–237
4 Niedergerke, R., Ogden, D.C. and Page S. (1976) in Calcium in Biological Systems, Vol. 30, Symposia of the Society for Experimental Biology., Englefield Green, 1975, pp. 381–395, Cambridge University Press, Cambridge
5 Sperelakis, N. and Schneider, J.A. (1976) Am. J. Cardiol., 37, 1079–1085
6 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 590–594
7 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235
8 Hui, C.-W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415–427
9 Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27–39
10 Caroni, P. and Carafoli, E. (1980) Nature 283, 765–767
11 Preobrazhensky, A.N., Kupriyanov, V.V., Saks, V.A., Grosse, R. and Spitzer, E. (1982) Biokhimia 47, 126–136
12 Preobrazhensky, A.N. and Saks, V.A. (1981) Biokhimia 46, 1681–1693
13 Kidwai, A.M., Radcliffe, M.A., Duchon, G. and Daniel, E.E. (1971) Biochem. Biophys. Res. Commun. 45, 901–910
14 Kidwai, A.M., Radcliffe, M.A. and Daniel, E.E. (1971) Biochim. Biophys. Acta 233, 538–549
15 Levitsky, D.O., Aliev, M.K., Kuzmin, A.V., Levchenko, T.S., Smirnov, V.N. and Chazov, E.I. (1976) Biochim. Biophys. Acta 443, 468–484
16 Steiner, A.L., Pagliara, A.S., Chase, L.R. and Kipnis, D.M. (1972) J. Biol. Chem. 247, 1114–1120
17 Pitts, B.J.R., Wallick, E.T., Van Winkle, W.B., Allen, J.C. and Schwatz, A. (1977) Arch. Biochem. Biophys. 184, 431–440
18 Wallick, E.T., Pitts, B.J.R., Lane, L.K. and Schwartz, A. (1980) Arch. Biochem. Biophys. 202, 442–449
19 DiPolo, R., Requena, F., Brinley, J.R., Mullins, L.F., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433–467
20 Hartree, E.F. (1972) Anal. Biochem. 48, 422–427
21 Schwartz, A., Nagano, K., Nakao, M., Lindenmayer, G.E. and Allen, J.C. (1971) in Methods in Pharmacology (Schwartz, A., Ed.), Vol. 1, pp. 361–388, Meredith, New York
22 Stekhoven, F.S. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76
23 Jones, L.R., Maddock, S.W. and Besch, H.R., Jr. (1980) J. Biol. Chem., 255, 9971–9980
24 Bers, D.M., Philipson, K.D. and Nishimoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358–371
25 Philipson, K.D. and Langer, G.A. (1979) J. Mol. Cell. Cardiol., 11, 857–875
26 Philipson, K.D., Bers. D.M., Nishimoto, A.Y. and Langer, G.A. (1980) Am. J. Physiol., 12, 231–239
27 Bers, D.M. and Langer, G.A. (1979) Am. J. Physiol. 237, H332–H341
28 Williamson, J.R., Woodrow, M.L. and Scarpa, A. (1975) in Recent Advances in Studies on Cardiac Structure and Metabolism. Basic Functions of Cations in Myocardial Activity (Fleckenstein, A. and Dhalla, N.S., eds.), Vol. 5, p. 61, University Park Press, Baltimore

29 Nayler, W.G., Dunnet, J. and Sullivan, A. (1976) in Recent Advances in Studies on Cardiac Structure and Metabolism The Sarcolemma (Roy, P.-E. and Dhalla, N.S., eds.), Vol. 9, pp. 53–70, University Park Press, Baltimore
30 Nayler, W.G., Mas-Oliva, J. and Williams, A.J. (1980) Circ. Res. Suppl. I, 46, 161–166
31 Lüllman, H. and Peters T. (1976) in Recent Advances in Studies on Cardiac Structure and Metabolism. The Sarcolemma. (Roy, P.-E. and Dhalla, N.S., eds.), Vol. 9, pp. 311–328, University Park Press, Baltimore
32 Grosse, R., Spitzer, E., Kupriyanov, V.V., Saks, V.A. and Repke, K.R.H. (1980) Biochim. Biophys. Acta 603, 142–156
33 Mas-Oliva, J., Williams, A.J. and Nayler, W.G. (1980) Anal. Biochem. 103, 222–226
34 Philipson, K.D. and Nishimoto, A.Y. (1981) J. Biol. Chem. 256, 3698–3702
35 Atsuko, Y., Masayosi, G. and Yasuo, T. (1978) Jap. J. Physiol. 28, 47–61
36 Yatani, A., Tsuda, Y., Akaike, N. and Brown, A.M. (1982) Nature 296, 169–171
37 Morcos, N.C. and Jacobson, A.L. (1979) Can J. Physiol. Pharmacol. 57, 529–534
38 Mas-Oliva, J., Williams, A.J. and Nayler, W.G. (1979) Biochem. Biophys. Res. Commun. 87, 441–447
39 Langer, G.A. (1980) J. Mol. Cell. Cardiol. 12, 231–239
40 Tillisch, J.H., Fung, L.K., Hom, P.M. and Langer G.A. (1979) J. Mol. Cell. Cardiol. 11, 137–148