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CATION TRANSPORT PROPERTIES OF A SYNTHETIC Ca^{2+}-SELECTIVE PEPTIDE IONOPHORE IN PHOSPHOLIPID AND SARCOPLASMIC RETICULUM VESICLES

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Transport by the synthetic cyclic peptide ionophore CYCLEX-2E (Deber, C.M., Young, M.E.M., and Tom-Kun, J. (1980) Biochemistry 19, 6194–6198), which in contrast to Ca^{2+} ionophore A23187 contains no ionizable protons, has been studied with respect to Ca^{2+} and Na^+ transport, and the involvement of exchanged, or counter-transported ions during the transport process. CYCLEX-2E was found to equilibrate Na^+ and Ca^{2+} gradients across phospholipid vesicle membranes. Experiments using the indicator dye Arsenazo III established that calcium ions were indeed reaching the aqueous intravesicular compartments. Absence of metal cations in the external buffer slowed, but did not eliminate, the efflux of Ca^{2+} from phosphatidylcholine vesicles. As an example of its activity in a biological membrane, CYCLEX-2E was shown to be capable of producing Ca^{2+} efflux from sarcoplasmic reticulum vesicles which had been loaded with Ca^{2+} in an ATP-dependent manner. The overall results suggest that in transport by synthetic peptide ionophores typified by CYCLEX-2E, electroneutrality is achieved either through (a) peptide-mediated compensating (but not coupled) fluxes of other cations, or where this is not an option, by (b) transmembrane diffusion of permeant ions such as H^+, OH^-, or Cl^-.

Introduction

Ionophores are increasingly being used as reagents for studies of biological events which may be linked to cation fluxes (for reviews, see Refs. 1 and 2). Most known Ca^{2+} ionophores, of which A23187 is the most noted example, operate via a proton-for-metal exchange mechanism whereby one carboxylic acid proton from each A23187 molecule dissociates upon Ca^{2+} binding [3]. The transported species (i.e., the 2 A23187/1 Ca^{2+} complex in the membrane) has been shown to be electroneutral using conductance measurements on lipid bilayers [4]; in addition, the anticipated pH changes were seen to occur during Ca^{2+} influx into vesicles [5]. Impetus for the present investigation was provided by the development of the calcium transporting peptide CYCLEX-2E [6,7], which, in contrast to A23187, contains no ionizable protons. This peptide was shown to be calcium-selective in competition experiments performed in Pressman Cells ('thick liquid membranes') and to produce efflux of calcium ions from the aqueous interior of phospholipid vesicles. Thus, analogously to the K^+-selective cyclic depsipeptide ionophore valinomycin [1], the CYCLEX-2E/cation complex may have a net positive charge during some stage of the overall transport process.

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In the present work, we have studied the role of Na^+ as an exchanged or countertransported ion during Ca^{2+} transport by the peptide ionophore CYCLEX-2E, and compared some aspects of our findings with electroneutral Ca^{2+} transport by ionophore A23187. We further demonstrate the peptide's capability of producing Ca^{2+} efflux from sarcoplasmic reticulum vesicles as an example of its possible utility in a biological membrane system.

Materials and Methods

Materials. Egg lecithin (phosphatidylcholine, PC) was obtained from Avanti (Birmingham, AL). Radioisotopes (calcium-45, sodium-22, carbon-14, phosphorus-32, and tritiated sucrose) were obtained from New England Nuclear (Cambridge, MA). Arsenazo III was obtained from Sigma (St. Louis, MO). The cyclic octapeptide CYCLEX-2E (structure: cyclo(Glu(OBz)Sar-Gly-(*N*-cyclohexyl)Gly)$_2$; OBz = benzyl ester, Sar = *N*-methylglycine) was synthesized using procedures described previously [8]. Ionophore A23187 was a gift from Dr. R. Hamill, Eli Lilly and Co. (Indianapolis, IN). [γ-^{32}P]ATP was obtained from Amersham Corporation (Arlington Heights, IL).

Preparation of phosphatidylcholine vesicles. Dried phosphatidylcholine was vortexed with 0.5–1 ml of 2 mM Hepes buffer (pH 7.4) in the presence of the appropriate salt and/or radioisotope. The resulting suspension was sonicated in a Bransonic bath at room temperature until the sample cleared. The unilamellar vesicles thus formed were eluted from a Sephadex G-50 column (30 mm × 1.2 mm diameter) (eluting buffer 2 mM Hepes, 1 mM EGTA) to remove external salt and/or isotope. Fractions were counted on a Beckman LS8100 or Isocap/300 scintillation counter in 10 ml aquasol to determine $^{45}Ca^{2+}$, ^{14}C, or ^{3}H. $^{22}Na^+$ was determined by a γ-counter (NCS Instrumentation, Inc.). Lipid vesicles were shown to elute in the void volume on Sephadex G-50 columns by phosphorus assay [9].

Cation efflux from PC vesicles. To measure cation efflux from vesicles, 2.5 mg of unilamellar PC vesicles in 1 ml buffer were placed in a dialysis sac, and dialysed vs. 0.5–1 liter of buffer. Aliquots (25 μl) were withdrawn from inside the sac at desired time intervals for counting of radioisotopes. Peptide (CYCLEX-2E) (125 μg) was added as a 5–10 mg/ml solution in dimethylformamide (approx. 25 μl). Controls using dimethylformamide and/or peptide + [^{14}C]sucrose were performed to establish vesicle integrity and extent, if any, of non-specific membrane damage over the course of the experiment.

Cation influx into PC vesicles. The phosphatidylcholine vesicles (2.5 mg) + ionophore (215 μg) were placed in 1 ml buffer for the desired incubation time. The vesicles were then eluted from a Sephadex G-50 column, from which lipid and free cation fractions were collected and counted for radioactivity.

Arsenazo III experiments. Residual calcium was removed from the dye by two passages over Na · Chelex-100 columns. Lipid was sonicated, as described above, with 10 mM Arsenazo III [10]. Unincorporated dye was removed on a G-50 Sephadex column using an elution buffer containing 2 mM Hepes and 1 mM EGTA, the latter present to insure that no divalent cations are associated with the external vesicular surface at the time of Ca^{2+} addition. Difference spectra for PC vesicles + Ca^{2+} with and without peptide were recorded on a Beckman Acta MVI double beam spectrometer.

Measurement of Ca^{2+} and oxalate fluxes in sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were prepared according to methods previously described [11]. Ca^{2+} uptake and release was assayed by the Millipore filtration method [12]. Oxalate release was measured by the radioactivity remaining on Millipore filters [13].

Results

Internal volume of sonicated PC vesicles

Calcium has been reported to bind to membrane surfaces even of neutral lipids such as PC [14]. To measure the relative amounts of calcium adhering to internal and external vesicle surfaces, as well as to determine the internal aqueous volume of the vesicles, 15 mg PC was sonicated with $^{45}CaCl_2$ (50 mM) and ^{3}H-labelled sucrose. The assumption was made that sucrose does not bind to the membrane so that the % of sucrose incorporated during sonication is representative of the

true volume included by the lipid. Unincorporated salt and sucrose were removed by gel chromatography. Half of the lipid was eluted on one column with 50 mM NaCl, and the other half on a second column with salt-free buffer. For the salt-free sample, the $^{45}Ca^{2+}/[^{3}H]$sucrose ratio in the eluted lipid fraction was 0.124 compared to an initial value of 0.092. This indicates that 25.8% of the total calcium is bound non-specifically to the membrane. Similar results were obtained in the presence of external NaCl. From these data and the specific activity of lipid fractions, the average value of the internal aqueous volume was calculated to be 0.45 μl/mg sonicated PC vesicles. Osmolarity changes occurring after sonication of the vesicles (e.g., salt-free elution media) did not affect the total counts recovered in the determination of intravesicular volume, either of $^{45}Ca^{2+}$ or $[^{3}H]$sucrose, suggesting that the PC vesicles are not ruptured nor become leaky where non-isotonic conditions were employed.

Calcium influx into PC vesicles by CYCLEX-2E

Vesicles containing either 10 mM $CaCl_2$, 15 mM NaCl, or metal-free buffer, were incubated with peptide (125 μg) + 10 mM $^{45}Ca^{2+}$ for 1–3 h, passed through Sephadex columns, and counted for $^{45}Ca^{2+}$ uptake. Under these conditions, calcium, sodium, and metal-free loaded vesicles accumulated 22.5, 23.5, and 25.8 nmol Ca^{2+}, respectively. No additional uptake was observed if the vesicles were incubated for longer times (up to 24 h). From these data, values of internal $[Ca^{2+}]$ after peptide-mediated influx, corrected as above, were calculated to be 13.9, 14.5, and 16 mM for Ca^{2+}, Na^{+}, and metal-free loaded vesicles, respectively.

To demonstrate that this lipid-associated Ca^{2+} is being transported and released into the intravesicular aqueous compartment, we employed the calcium-sensitive Arsenazo III, which forms a 1 : 1 water-soluble Ca^{2+} complex. The absorption maximum of the free dye shifts from 560 nm to 605 nm and 660 nm upon complexation [10]. Our interest in obtaining data in the early stages of influx in systems with relatively low intravesicular aqueous volume prompted the use of the high affinity indicator Arsenazo III rather than murexide [15]. Calibration of A_{605} vs. internal $[Ca^{2+}]$ (not shown) was performed using A23187 with vesicles suspended in media of varying external $CaCl_2$ concentration, and found to be most sensitive to change at low (1.0 μM–1.0 mM) $[Ca^{2+}]$. Difference spectra (550–700 nm) for PC vesicles in the presence and absence of peptide CYCLEX-2E are illustrated in Fig. 1. Using the above calibration, the extent of CYCLEX-2E-mediated influx of Ca^{2+} ions into vesicles as a function of time was determined as shown in Fig. 1 (inset).

Sodium transport by CYCLEX-2E

Calcium-loaded PC vesicles (50 mM $CaCl_2$) or sodium-loaded PC vesicles (50 mM NaCl) suspended in 100 mM ^{22}NaCl and incubated overnight in the presence of CYCLEX-2E are depleted of Ca^{2+} and accumulate Na^{+}. The final concentration of influxed Na^{+} was independent of the cation initially present inside the vesicles, i.e. for 50.9 nmol Ca^{2+} exiting from vesicles (calculated from the initial concentration and internal volume of the vesicles), 86.3 nmol $^{22}Na^{+}$ were taken up, while in the $^{22}Na^{+}$/unlabelled Na^{+} experiment, 93.4 nmol $^{22}Na^{+}$ (calculated from the specific activity of $^{22}Na^{+}$) were influxed for 50.9 nmol unlabelled Na^{+}. Thus, in both experiments, the internal and external Na^{+} concentrations are essentially equilibrated.

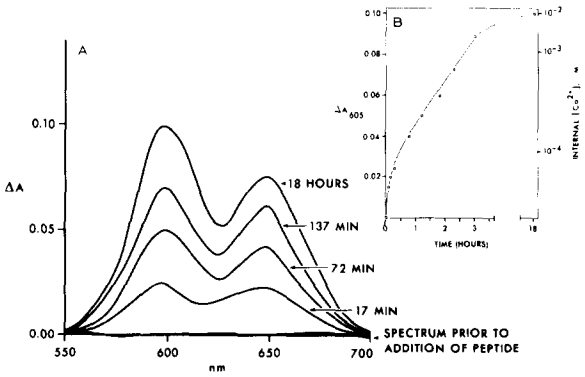

Fig. 1. Ca^{2+} influx monitored by Arsenazo III difference spectra. PC vesicles contain 10 mM Arsenazo III, 50 mM NaCl, 5 mM Hepes (pH 7.4). The external buffer contains 2 mM Hepes (pH 7.4), 1 mM EGTA, and 10 mM $CaCl_2$. (A) Difference spectra were recorded between 2.5 mg PC vesicles to which 125 μg CYCLEX-2E has been added and those without peptide. (B) the changes in absorbance (at 605 nm), and internal $[Ca^{2+}]$ vs. time are shown.

Time course of $^{22}Na^+$ influx and $^{45}Ca^{2+}$ efflux in PC vesicles

The intravesicular concentrations of Ca^{2+} and Na^+ at several times during transport by CYCLEX-2E are shown in Table I. In parallel experiments, $^{45}Ca^{2+}$-loaded vesicles were suspended in $^{22}Na^+$ or unlabelled NaCl and CYCLEX-2E (125 μg) added. At each indicated time, one Sephadex G-50 column was used to determine the $^{45}Ca^{2+}$ remaining in the lipid fraction and another to measure simultaneously the $^{22}Na^+$ influxed into the vesicles. Although the rates of Ca^{2+} efflux and Na^+ influx appear to differ by less than an order of magnitude, the results in Table I do not suggest a direct 2/1 'coupled' exchange of two sodium ions for one calcium ion. The final data point (20 h) parallels the outcome of the experiments described in the previous section.

Effect of external salt on calcium efflux by CYCLEX-2E and A23187

When Ca^{2+} efflux from liposomes was measured in the presence and absence of NaCl in the external dialyzing medium, the rates of efflux to the metal-free buffer were reduced not only for CYCLEX-2E but for the electroneutral ionophore A23187 (Fig. 2). The more rapid Ca^{2+}-efflux rate was restored in the presence of external Na^+, and (at least for CYCLEX-2E) by addition of external Ca^{2+} [7].

Similar experiments with CYCLEX-2E were performed using the chloride salt of choline (a bulky organic cation of structure similar to the head group of PC) as the only salt in the external dialyzing medium. PC vesicles were found to retain [^{14}C]choline when suspended in metal-free or 15 mM NaCl buffer, both in the presence and absence of peptide. Upon addition of 15 mM choline chloride to PC vesicles suspended in metal-free buffer, the more rapid rate of CYCLEX-2E-mediated Ca^{2+} efflux (data not shown, but similar to NaCl curve in Fig. 2A) was restored.

Activity of CYCLEX-2E in sarcoplasmic reticulum vesicles

The ability of CYCLEX-2E to transport Ca^{2+}

TABLE I

CONCENTRATIONS OF CATIONS INSIDE PC VESICLES DURING Na^+/Ca^{2+} TRANSPORT BY CYCLEX-2E

2.5 mg PC containing 50 mM $CaCl_2$ ($+^{45}CaCl_2$), and 2 mM Hepes (pH 7.4) were suspended in 1 ml 100 mM NaCl (unlabelled), 2 mM Hepes (pH 7.4), 1 mM EDTA + 125 μg peptide, and then passed over G-50 columns after the indicated times to measure the amount of $^{45}Ca^{2+}$ remaining in the lipid fraction. Simultaneously, identical samples suspended in 100 mM NaCl ($+30$ μC ^{22}NaCl) were eluted from G-50 columns to measure the $^{22}Na^+$ influx at each time. Concentrations are based on the measured internal volume, 1.13 μl/2.5 mg PC.

Time	$[Na^+]$ (mM)	$[Ca^{2+}]$ (mM)
0 min	0	50
2 min	6.2	44
17 min	7.9	39
32 min	8.0	36
152 min	23.4	33
20 h	87.5	5.8

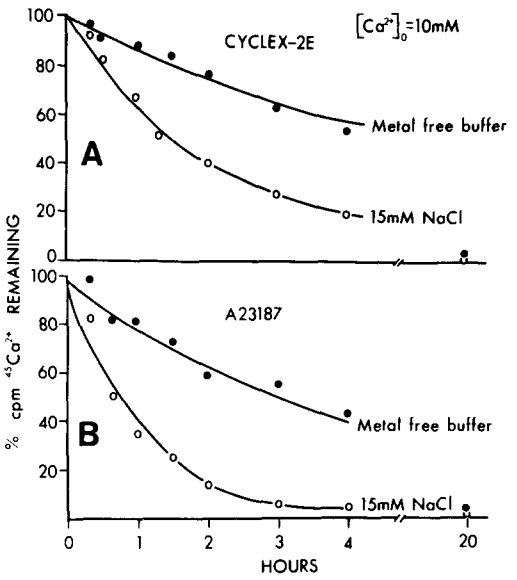

Fig. 2. $^{45}Ca^{2+}$ efflux mediated by CYCLEX-2E and A23187 in NaCl or metal-free buffers. Vesicles (2.5 mg PC) containing 10 mM $CaCl_2$ ($+^{45}Ca^{2+}$), 2 mM Hepes (pH 7.40) were dialyzed vs. 15 mM NaCl, 2 mM Hepes (pH 7.4), 1 mM EGTA (○); or 2 mM Hepes (pH 7.4), 1 mM EGTA only (●). (A) CYCLEX-2E (125 μg) added to each 1 ml sample. (B) A23187 (10 μg) added to each 1 ml sample. Since aliquots are taken from inside dialysis sacs, the rates of Ca^{2+} efflux are limited by the rate of Ca^{2+} diffusion through the sacs [6].

in sarcoplasmic reticulum vesicles is shown in Fig. 3. When CYCLEX-2E was administered after ATP-initiated Ca^{2+} uptake was complete, Ca^{2+} was observed to exit from the vesicles. The behaviour of the electroneutral ionophore A23187 in the same system is shown for comparison.

To aid in confirming the specificity of the observed Ca^{2+} release, sarcoplasmic reticulum vesicles were suspended in 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7) and loaded with radiolabelled oxalate (1 mM potassium oxalate + 20 μCi [^{14}C]oxalic acid). When these sarcoplasmic reticulum vesicles (250 μg protein) were treated with CYCLEX-2E at a dose which promotes Ca^{2+} release (130 μM peptide), there was no increase in the passive permeability of the sarcoplasmic reticulum membrane to oxalate compared with the control (data not shown); in both cases, the vesicles had lost approx. 50% of original oxalate after 3–4 min in the presence or absence of peptide. In view of the approximately comparable half-life of CYCLEX-2E-mediated Ca^{2+} loss from sarcoplasmic reticulum vesicles (Fig. 3), the oxalate controls must be regarded as indicative, rather than as a conclusive demonstration, of the absence of peptide-related non-specific effects.

Results of a (Ca^{2+}-Mg^{2+})-ATPase assay (presented in Table II) demonstrate that the inorganic phosphate generated by sarcoplasmic reticulum

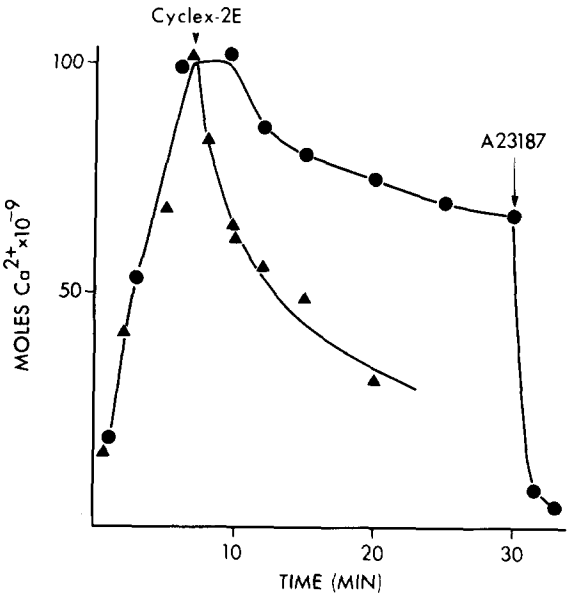

Fig. 3. Ca^{2+} release from sarcoplasmic reticulum vesicles by CYCLEX-2E and A23187. Vesicles (250 μg protein) are suspended in 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris maleate (pH 6.8), 50 mM potassium phosphate, 0.5 mM EGTA, 0.5 mM $CaCl_2$ + 50 μC $^{45}CaCl_2$. To initiate uptake, 250 μl 0.1 M ATP is added to 5 ml assay media. Ionophores are added as indicated by arrows. When ATP-mediated Ca^{2+} uptake peaked (at approx. 7 min), CYCLEX-2E was added (▲) (final concentration, 130 μM) as a solution in dimethylformamide. As a control, dimethylformamide alone was added at 7 min (●); in this latter experiment, A23187 was added (final concentration, 2 μM) at 30 min as a solution in ethanol.

TABLE II

ASSAY OF (Ca^{2+}-Mg^{2+})-ATPase ACTIVITY IN SARCOPLASMIC RETICULUM VESICLES IN THE PRESENCE OF IONOPHORES

Procedures used are similar to those described in Avron [25]. Each assay contains 20 μg sarcoplasmic reticulum protein in 0.5 ml of 100 mM histidine chloride buffer (pH 6.8) and either 0.5 mM EGTA + 0.5 mM $CaCl_2$ or the same but with no $CaCl_2$ and 1 mM EGTA. 25 μl 0.1 M ATP initiates the reaction. The incubation is for 10 min at 37°C. After extraction of ^{32}P with 5 ml isobutyl benzene, 1 ml is counted to determine the amount of ATP hydrolyzed. Each value is average of two determinations. The average error is ±4%.

Buffer	Ionophore added (μg)		nmol P_i/μg sarcoplasmic reticulum protein per min
	CYCLEX-2E	A23187	
$-Ca^{2+}$	–	–	0.6
$+Ca^{2+}$	–	–	4.4
$-Ca^{2+}$	200	–	0.7
$+Ca^{2+}$	200	–	5.2
$-Ca^{2+}$	–	10	0.7
$+Ca^{2+}$	–	10	6.9

vesicle suspensions in Ca^{2+}-containing buffer is not diminished by the addition of ionophore, and hence that there is no inhibition of the enzyme's activity in the presence of CYCLEX-2E or A23187. In fact, some stimulation of activity by both ionophores was noted (right-hand column, Table II).

Discussion

Examples are abundant in living systems of Ca^{2+}-Na^+ exchange across membranes. Although mechanisms differ widely in detail, it has been reported to occur, for instance, in squid axon [16,17]; in heart sarcolemma [18–20]; and in cardiac mitochondria [21]. In the present work, synthetic peptide ionophore CYCLEX-2E has been found to mediate the influx of Na^+ during Ca^{2+} efflux such that the concentration gradients for both ions across phosphatidylcholine vesicle membranes tend to be dissipated. However, since (a) CYCLEX-2E was also found to equilibrate $Na^+/^{22}Na^+$ across the PC membrane in the absence of other cations (see Results), and (b) the results of the time-course study in Table I do not suggest the occurrence of an obligatory 2 Na^+-1 Ca^{2+} exchange, transport by the peptide of Ca^{2+} and Na^+ may not be directly coupled and/or alternative mechanisms may operate for CYCLEX-2E-mediated transport of each individual cation.

With respect to influx of Ca^{2+} into PC vesicles, experiments using Ca^{2+}-indicator dye Arsenazo III (Fig. 1) established that calcium ions are indeed reaching the vesicle interior aqueous compartments; difference spectra appeared as quickly as measurements could be taken (approx. 3 min) and the net absorption difference eventually approached that displayed by an A23187 standard *.

Previous studies had noted that the rate of net Ca^{2+} efflux from PC vesicles did not appear to be limited specifically by the replacement of chloride on both sides of the membrane by the relatively impermeant sulfate anion [7], and the present studies confirm that an oppositely-directed peptide-mediated cation (e.g., Na^+) flow accompanies Ca^{2+} transport. Nevertheless, CYCLEX-2E-mediated Ca^{2+} transport is not halted by the absence of salt outside the vesicles during Ca^{2+} efflux (note the 20 h point in Fig. 2A), or by the absence of salt inside the vesicles during Ca^{2+} influx (see Results). Thus, in these systems, fluxes of H^+, OH^-, or Cl^- (added as $^{45}CaCl_2$) must contribute to the eventual dissipation of the Ca^{2+} gradient, the PC vesicle bilayer being permeable to all of these ions [22,23]. These results suggest that in salt-free buffers, non-specific transmembrane diffusion of one or more of these permeant ions becomes the rate-limiting process for Ca^{2+} efflux.

Experiments with ionophore A23187, for which an electroneutral transport mechanism is expected and therefore the involvement of compensatory ion fluxes is not postulated, demonstrated that absence of external cations similarly retards the rate of A23187-mediated Ca^{2+} efflux (Fig. 2B). Also, addition to extravesicular salt-free media of choline cations, which were shown not to be transported by CYCLEX-2E, was observed to restore the normal rate of CYCLEX-2E-mediated Ca^{2+} efflux. Thus, unless morphological or other changes in PC vesicles in non-isotonic media are impeding cation movement, a rate-determining step common to the overall transport processes of both types of calcium ionophores may well involve cation- (or anion-)-facilitated dissociation of the Ca^{2+}-ionophore complex at the membrane surface.

Conclusion

Our results suggest that CYCLEX-2E-mediated transmembrane Ca^{2+} transport in phosphatidylcholine vesicles is accompanied by a counter-directed (but not coupled) flow of (e.g., Na^+) cations. In cases where this is not an option, Ca^{2+} efflux is slowed as its rate becomes limited by

* When Arsenazo III-containing vesicles were treated with CYCLEX-2E (125 μg) in the presence of internal 50 mM $^{22}Na^+$, and the vesicles eluted from Sephadex G-50 columns after Ca^{2+} influx was complete (20 h), 75% of initial $^{22}Na^+$ was retained. However, corresponding vesicles treated with A23187 (10 μg) were found to have retained only 19% of initial $^{22}Na^+$. Controls using [^{14}C]sucrose established that the integrity of PC vesicular membranes had not been compromised by 10 μg of A23187/2.5 mg lipid. These results indicate the possibility of measurable Na^+ transport by ionophore A23187.

transmembrane diffusion of permeant ions such as H^+, OH^-, or Cl^-. These findings, in conjunction with the observation that peptide-mediated Ca^{2+} efflux proceeds in the presence of membrane-impermeant SO_4^{2-} anions [7], suggest that the class of synthetic ionophoric peptides [8] typified by CYCLEX-2E could therefore function by a primary electrogenic mechanism where the active transporting species is the positively charged peptide/cation complex, but in which electroneutrality is ultimately achieved through compensating fluxes of other cations or anions, rather than via 'obligatory' cation/cation exchange.

Sarcoplasmic reticulum vesicles provided a convenient example of a biological membrane in which to examine Ca^{2+}-ionophore CYCLEX-2E, particularly because these vesicles can be loaded with calcium in an ATP-dependent manner. Since oxalate controls suggested that CYCLEX-2E causes no immediate non-specific damage to sarcoplasmic reticulum vesicle membranes (see Results), and the findings in Table II demonstrated that the peptide does not inhibit (Ca^{2+}-Mg^{2+})-ATPase, the peptide's effect on Ca^{2+} levels in sarcoplasmic reticulum vesicles is probably attributable to the ability of CYCLEX-2E to transport calcium ions. Sarcoplasmic reticulum membranes have a high passive Na^+ and Cl^- permeability [24], and the rate of Ca^{2+} release by CYCLEX-2E in the sarcoplasmic reticulum vesicles is at least as slow as either of these (Na^+, Cl^-) ion fluxes. Thus, any electrochemical gradient developed by an electrogenic type of CYCLEX-2E-mediated Ca^{2+} transport could readily be dissipated by the passive equilibration of other ions in the system.

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