Synthetic Cation Transport Peptides: Calcium Transport across Phospholipid Membranes†

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ABSTRACT: Molecular aspects of peptide-mediated calcium transport are examined through the study of the cation transport properties of a series of synthetic cyclic octapeptides. These peptides, of general structure cyclo[Glu(OR_1)-Sar-Gly-(*N*-R_2)Gly]$_2$ (R_1 = H or benzyl ester; R_2 = cyclohexyl, *n*-hexyl, or *n*-decyl) (and an Asp analogue), contain central binding cavities of geometry and dimensions similar to calcium-binding sites in proteins. Transport in Pressman cells ("thick liquid membranes") demonstrated the ionophorous activity of the synthetic peptides; among physiologically abundant cations, the order of selectivity was $Ca^{2+} > Na^+$, $K^+ \gg Mg^{2+}$. Cation competition studies further showed that cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ (CYCLEX-2E) is essentially a calcium-specific transport peptide whenever calcium is present. When the CYCLEX-2E peptide was added to a suspension of $^{45}Ca^{2+}$-loaded sonicated phosphatidylcholine (PC) vesicles in a dialysis sac, the vesicles were completely emptied of internal calcium. Controls using [^{14}C]sucrose established that CYCLEX-2E caused no nonspecific membrane damage. Calcium efflux experiments using several salts of calcium (including $^{36}Cl^-$, [^{14}C]acetate, [^{14}C]succinate, and $^{35}SO_4^{2-}$) suggested that these anions do not specifically accompany the Ca^{2+}–peptide active transporting species across the phospholipid membrane. However, when $^{45}Ca^{2+}$-loaded PC vesicles were suspended in metal-free buffer and treated with CYCLEX-2E peptide, calcium efflux did not occur until calcium or sodium chloride was added to the external medium.

Cation transport across biological membranes—whether mediated by pores, channels, conformational transitions of membrane proteins, or mobile carrier molecules—should at some stage involve binding of the transported cation to liganding sites typical of protein functional groups. We have undertaken an investigation of these phenomena on the molecular level through development of peptides which render membranes selectively permeable to calcium, an ion of universal importance as a factor in cell communication [for a review, see Rasmussen & Goodman (1977)].

Calcium-selective transport peptides can, in principle, (a) provide new information as to the molecular prerequisites for calcium binding and selectivity by proteins and also (b) give rise to a new series of calcium ionophores which would be useful probes as reagents in a variety of biochemical systems [for a review of ionophore biochemistry, see Pressman (1976)]. Yet, of over one hundred naturally occurring or synthetic ion transport materials [surveyed by Ovchinnikov et al. (1974)], few have been reported to display calcium transport activity. These latter substances are largely nonpeptidic materials, such as the widely used ionophore A23187, an antibiotic isolated from *Chartreusensis streptomyces* [for a review, see Pfeiffer et al. (1978)], ionomycin (Liu & Hermann, 1978; Beeler et al, 1979), and synthetic Ca^{2+}-liganding agents (Caroni et al., 1977; Umen & Scarpa, 1978a,b; Wierenga et al., 1979). Calcium transport by peptidic materials has been described for the depsipeptide antibiotic beauvericin (Hamilton et al., 1975) and for a 3000-dalton protein derived from the inner mitochondrial membrane of calf heart (Jeng et al., 1978).

In considering structures of choice as potentially calcium-binding peptides, we examined calcium-binding proteins for which X-ray crystallographic data are available (including conconavalin A, thermolysin, rabbit skeletal muscle troponin, and carp parvalbumin) [summarized by Kretsinger (1976)]. Calcium-binding sites were found to be comprised of liganding atoms derived from sequentially distant residues held in proximity via the native protein conformation. Invariably, these liganding sites consisted of six oxygen atoms arising from a combination of glutamic or aspartic acid carboxylate groups and backbone peptide carbonyl groups coordinated to calcium in approximately octahedral geometry. These basic structural criteria are met, in part, by cyclic octapeptides of sequence cyclo[Glu-Sar-Gly-(*N*-R)Gly]$_2$ (and corresponding Asp analogues) which we have now synthesized (Deber & Adawadkar, 1979). Cyclization of the peptide chain creates a binding cavity lined with four potentially coplanar peptide carbonyl groups and also brings diametrically opposed Glu (or Asp) carboxyl side-chain groups into proximity. Membrane solubility of these peptides (and especially of their metal complexes) was achieved through incorporation of covalently linked lipophilic "tails" using *N*-alkylglycine residues. Further, the procedures for their synthesis allow us to generate two distinct categories of materials: one series containing Glu or Asp side chains in the carboxylate acid form, where proton(s) for metal exchange can lead to neutral peptide–cation complexes with divalent cations, and a second series of corresponding (benzyl) ester derivatives, which may form charged peptide–cation complexes. Calcium binding and transport properties of peptides of the former type, particularly cyclo[Glu-Sar-Gly-(*N*-decyl)Gly]$_2$ (DECYL-2), have been reported (Deber et al., 1978, 1980). In the present work we describe the calcium-selective transport activity of the ester materials and report that one peptide, cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ (CYCLEX-2E) (Figure 1) transports calcium across phospholipid membranes. Since the Glu residues in this membrane-active peptide contain (nonionizable) ester side chains, the mechanism by which CYCLEX-2E transports calcium is examined with respect to the role of available ions in balancing the overall charge.

Materials and Methods

Materials. Egg phosphatidylcholine (PC) was purchased from Serdary Research Laboratories. Radioisotopes $^{45}Ca^{2+}$, $^{22}Na^+$, $^{35}SO_4^{2-}$, and $^{36}Cl^-$ and [^{14}C]sucrose, [^{14}C]succinate, and [^{14}C]acetate were obtained from New England Nuclear,

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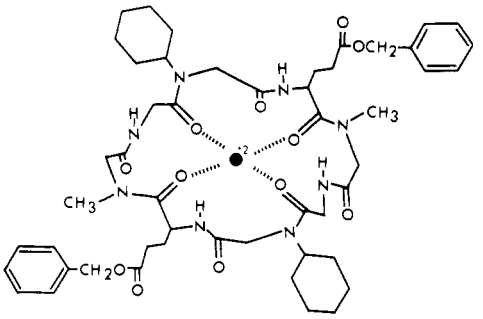

FIGURE 1: Schematic representation of divalent cation complex of cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ (CYCLEX-2E). Four coplanar peptide carbonyl oxygen atoms can coordinate the cation; additional liganding can occur from above or below the plane of the molecule. Anion(s) are not shown. The analogue cyclo[Asp-(OBz)-Sar-Gly-(*N*-decyl)Gly]$_2$ (ASP-DECYL-2E) contains one less methylene group vs. Glu in side chains and two *n*-decyl [$-(CH_2)_9CH_3$] substituents instead of cyclohexyl groups. Sar = *N*-methylglycine.

Cambridge, MA. All chemicals and solvents used were reagent grade. Ionophore A23187 was a gift from Dr. R. Hamill, Eli Lilly & Co., Indianapolis, IN.

Synthesis of Peptides. The cyclic octapeptides described in this paper were synthesized in our laboratory by Dr. P. D. Adawadkar according to the methods we have described (Deber & Adawadkar, 1979). *N*-Alkylglycine residues were prepared through the reaction of iodoacetic acid with the corresponding alkylamine. Peptide coupling reactions generally employed mixed anhydride procedures with Z (benzyloxycarbonyl) or *t*-Boc (*tert*-butyloxycarbonyl) N protection and OBz (benzyl ester) C-terminal and Glu (or Asp) side chain protection. Peptide chains were cyclized either as the octapeptide *p*-nitrophenyl ester or cyclodimerized from the tetrapeptide *p*-nitrophenyl ester. Products were characterized by (a) survival after mixed-bed ion-exchange resin treatment, (b) homogeneity on thin-layer chromatography (TLC), (c) appropriate infrared spectra, (d) appropriate NMR spectra both before and after addition of cations, and (e) molecular ion peak obtained from mass spectrometry. The synthesis of cyclo[Asp(OBz)-Sar-Gly-(*N*-decyl)Gly]$_2$ followed the same overall procedures as those described for its Glu analogue.

Transport in Pressman Cells. In a typical experiment, aqueous phase 1 contained 100 mM picric acid, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 20 mM cation chloride, in a volume of 2 mL. This was placed in one arm of a U-tube apparatus (Ashton & Steinrauf, 1970) containing 10 mL of organic phase (usually chloroform) with dissolved peptide (concentration, 20 μM). Aqueous phase 2, consisting of 2 mL of 10 mM Hepes buffer, was placed in the second arm of the tube. Both aqueous phases were at pH 7 (adjusted with lithium hydroxide). The organic phase was stirred. After desired time periods, 100-μL aliquots were taken from phase 2 for absorption spectroscopy reading and/or 25-μL aliquots were taken for scintillation counting. For reading of picrate concentration, samples were brought to 1 mL with 10 mM Hepes, and the optical density was read at 355 nm; a standard curve was used to convert the readings to micromoles of picric acid. For scintillation counting, the aliquots were read in 10 mL of Aquasol (with the counts corrected for quenching as necessary in samples with high picric acid concentrations). Organic phases could similarly be monitored for (metal) picrate by optical density (read at 342 nm) or for $^{45}Ca^{2+}$ by scintillation counting.

Transport in Unilamellar Phospholipid Vesicles. In a typical experiment, lipid (usually 2 mg of PC) was dried to a thin film on the walls of a test tube. An aqueous solution (0.5 mL) of 50 mM calcium chloride (or other calcium salt) buffered by 2 mM Hepes at pH 7.4 was added to the tube, along with an aliquot of radiolabeled Ca^{2+} or the anion under investigation. The contents of the tube were vortexed (2 min) to suspend the lipid completely (as multilamellar liposomes). Sonication of the sample in a Bransonic bath at room temperature for ~1 h, or until the sample cleared, resulted in the formation of unilamellar PC vesicles. To remove external radioisotope not incorporated into vesicle interiors, we eluted the samples through a 24 × 1 cm column of Sephadex G-50, from which the vesicles elute in the void volume. The column elution buffer contained 75 mM NaCl, 2 mM Hepes, and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4. After elution from the column, vesicle-containing fractions (0.8–1-mL volume each) were placed in open-ended dialysis sacs. Peptide was added [typically 75 μg in 15 μL of dimethylformamide (DMF) solvent]. Aliquots for scintillation counting were removed periodically from inside the sac. Dialysis was carried out against 1 L of elution buffer, usually for a period of 3–5 h. Appropriate controls established the stability of the vesicles over the course of the experiment. Cations, anions, and suspending media were varied as reported in Figures 3–5.

Results

Experiments with Thick Liquid Membranes (Pressman Cells). Transport in Pressman cells [U-tubes containing two aqueous phases separated by an organic layer (Ashton & Steinrauf, 1970)] has been shown to be an excellent indicator of cation selectivity and organic solubility of cation complexes of potential ionophorous substances; such materials may behave analogously in phospholipid bilayers and biological membranes. An initial aqueous phase typically contains 20 mM cation chloride and 100 mM picric acid (which acts as a lipophilic anion to solubilize the peptide–cation complex) buffered to pH 7 (see Materials and Methods). The organic phase—usually chloroform—contains 20 μM cyclic peptide. Cells are monitored for accumulation of cation in aqueous phase 2, either by radioisotope assay (e.g., $^{45}Ca^{2+}$ or $^{22}Na^{+}$) or through measurement of picrate concentration using visible absorption spectroscopy.

Cation transport to aqueous phase 2 becomes most efficient after the organic phase is "saturated" with peptide–cation complex, which usually requires 50–100 h due to the limited cross-sectional area of the interface between layers (U-tube inner diameter $\simeq$ 1 cm). Typical values for $^{45}Ca^{2+}$ content of chloroform phases at saturation were 0.07–0.13 μM/0.2 μM peptide. Relative extents of cations transported after a post-equilibrium time interval are reported in Table I for Pressman cell experiments involving cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ and cyclo[Asp(OBz)-Sar-Gly-(*N*-decyl)Gly]$_2$ in combination with four physiologically abundant cations: sodium, potassium, magnesium, and calcium. Reported values are adjusted for background picric acid transport and putative lithium transport via control experiments without added cation chloride. The results demonstrate not only the intrinsic cation capability of these peptides but also indicate their selectivity. Between the two divalent cations, the cyclohexyl compound selects for Ca^{2+} by ~1 order of magnitude over Mg^{2+}; magnesium transport could not be detected at all for the decyl compound.

Both peptides transported Na^+ and K^+ in addition to Ca^{2+}, a result which prompted us to investigate transport on a competitive basis. Thus, Pressman cell experiments were conducted in which aqueous phase 1 initially contained either Na^+ alone or Na^+ in the presence of equimolar Mg^{2+}, K^+, and

Table I: Cation Transport in Pressman Cells by Synthetic Cyclic Octapeptides[a]

peptide	cation				
	Na^+	K^+	Mg^{2+}	Ca^{2+}	$^{45}Ca^{2+}$
CYCLEX-2E[b]	0.54	0.44	0.08	0.83	1.0
ASP-DECYL-2E[c]	0.28	0.09	0	0.95	1.0

[a] Values were obtained by setting micromoles of $^{45}Ca^{2+}$ accumulated after 260 h in aqueous phase 2 equal to unity and expressing others as ratios vs. calcium. Other than $^{45}Ca^{2+}$, transport was determined from concentrations of picric acid in aqueous phase 2 (see Materials and Methods). In these instances, values given are corrected for background picrate resulting from putative lithium transport by peptides assuming no suppression of Li^+ transport due to competitive cations. Typically, in Ca^{2+} transport experiments, background amounted to 15–20% of total picrate accumulated in aqueous phase 2. Picrate transport in the absence of peptide was negligible. Cation concentration in aqueous phase 1 = 20 mM. Peptide concentration in chloroform phase = 20 μM. Absolute amounts of cation transport to aqueous phase 2 under similar conditions are given in Figure 2 and its legend. [b] CYCLEX-2E = cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$. [c] ASP-DECYL-2E = cyclo[Asp(OBz)-Sar-Gly-(*N*-decyl)Gly]$_2$.

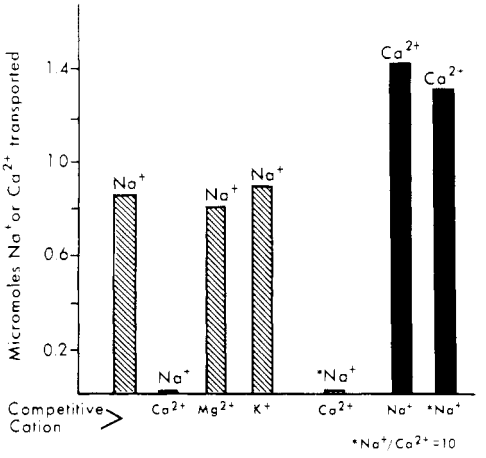

FIGURE 2: Competitive cation transport in Pressman cells mediated by CYCLEX-2E peptide. In the U-tube apparatus (see Materials and Methods), aqueous phase 1 contained 20 mM $^{22}Na^+$ (or "cold" Na^+) and, where indicated, equimolar concentrations of the competitive cation. Also present were 100 mM picric acid and 10 mM Hepes buffer. The organic (chloroform) phase contained 20 μM CYCLEX-2E. For calcium competition experiments, identical runs contained either $^{22}Na^+$ or $^{45}Ca^{2+}$ as desired. Samples marked (*) in the diagram contained sodium at a 10-fold excess concentration (200 mM) over calcium. Aliquots taken after 168 h from aqueous phase 2 were counted and converted to micromoles of cation transported. After 336 h, similar samples yielded the following micromoles transported: Na^+ alone, 2.8; Na^+ ($+Ca^{2+}$), 0.08; Na^+ ($+Mg^{2+}$), 2.7; Na^+ ($+K^+$), 2.7; 200 mM Na^+ ($+Ca^{2+}$), 0.05; Ca^{2+} ($+Na^+$), 3.4; Ca^{2+} (+200 mM Na^+), 3.1.

Ca^{2+}. In addition, one set of experiments with 10-fold excess Na^+ over Ca^{2+} was carried out. Assay for cations appearing in aqueous phase 2 was made directly by using $^{22}Na^+$ or $^{45}Ca^{2+}$. The results are shown in Figure 2. It was observed that the presence of equimolar Mg^{2+} or K^+ did not affect Na^+ transport. However, *Na^+ transport was eliminated when Ca^{2+} was present.* This was similarly the outcome in the presence of a 10-fold excess of sodium. Concomitantly, the copresence of equimolar or 10-fold excess Na^+ did not significantly diminish net calcium transport. Thus, on the basis of cation competition experiments, CYCLEX-2E can be described as an essentially *calcium-specific transport peptide whenever calcium is present.* In related experiments using the peptide ASP-DECYL-2E, but measuring only the accumulation of $^{45}Ca^{2+}$ in aqueous phase 2, it was similarly observed that net

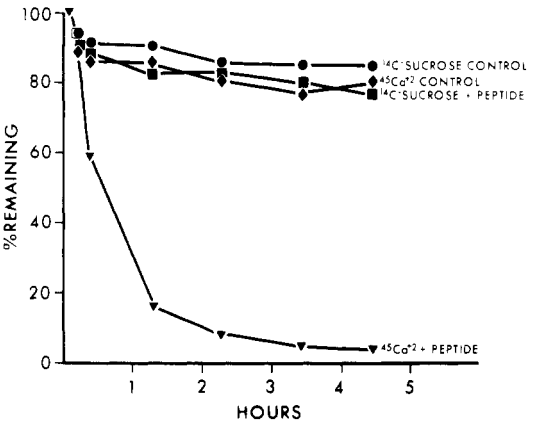

FIGURE 3: The efflux of calcium ions from sonicated unilamellar phosphatidylcholine vesicles, mediated by CYCLEX-2E. Each sample consisted of ~2 mg of lipid suspended in 0.8 mL of buffer (see Materials and Methods) to which CYCLEX-2E was added (75 μg in 15 μL of DMF). All four samples shown were prepared in an identical manner, except that radioactive forms of sucrose and calcium were alternated and only DMF (but no peptide) was added to both controls.

Ca^{2+} transport was not affected in the presence of equimolar Mg^{2+}, Na^+, or K^+ ions.

Transmembrane Calcium Transport by Cyclo[Glu(OBz)-Sar-Gly-(N-cyclohexyl)Gly]$_2$ in Unilamellar Phospholipid Vesicles. Sonicated (unilamellar) vesicles of egg phosphatidylcholine (PC) were prepared in the presence of $^{45}CaCl_2$ and passed down a column of Sephadex G-50 to remove external calcium as described under Materials and Methods. Sucrose (10 mM) was added as a control for nonspecific leakage of calcium (e.g., due to peptide-induced detergent activity). Simultaneously, identical samples were prepared with "cold" Ca^{2+} in the presence of [^{14}C]sucrose. Experiments using CYCLEX-2E produced the results shown in Figure 3. It was observed that the addition of peptide mediated the complete exit of internal $^{45}Ca^{2+}$ from the vesicles. Because these experiments were performed by sampling from inside dialysis sacs—and since the rate of $^{45}Ca^{2+}$ egress from the sac in the absence of phospholipid was found to be comparable to the rate in Figure 3 (data not shown)—the experiments provide no direct measurement of the actual rate of $^{45}Ca^{2+}$ efflux from the PC vesicles [see also Deber (1980)]. [^{14}C]Sucrose egress controls demonstrated not only that the vesicles were intact over several hours, but also that the addition of peptide caused no nonspecific sucrose efflux. Experiments performed monitoring Ca^{2+} efflux in the presence of equimolar internal Mg^{2+} established that the rate of calcium efflux was unaffected by the presence of magnesium. As a further control, the naturally occurring K^+–ionophore valinomycin (Ovchinnikov et al., 1974) which has not been reported to interact specifically with calcium was found to have no effect on calcium transport under conditions where CYCLEX-2E emptied the vesicles of $^{45}Ca^{2+}$, thus reinforcing the observation that calcium efflux is a specific consequence of the presence of CYCLEX-2E peptide.

Influence of Anion on Calcium Efflux from Phospholipid Vesicles. To obtain information about the mechanistic details of calcium transport, we examined the effect of several anions in these systems. For instance, charge balance during calcium efflux could be achieved by corresponding Cl^- efflux (although Cl^- ion need not physically accompany the peptide–calcium complex across the membrane). By use of $^{36}Cl^-$ (and "cold" calcium) in experiments similar to those depicted in Figure 3, it was observed that the vesicles nonspecifically and rapidly

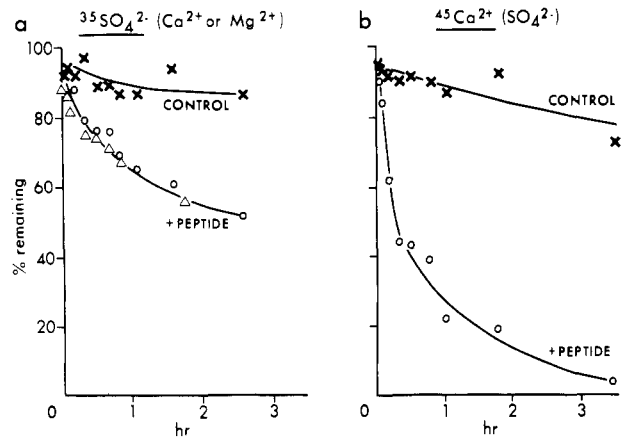

FIGURE 4: The efflux of ions from sonicated unilamellar phosphatidylcholine vesicles. (a) Loss of $^{35}SO_4^{2-}$ anions from vesicles (loaded with calcium or magnesium sulfate) in the absence or presence of peptide (CYCLEX-2E). (b) Efflux of $^{45}Ca^{2+}$ mediated by CYCLEX-2E in the presence of sulfate anions.

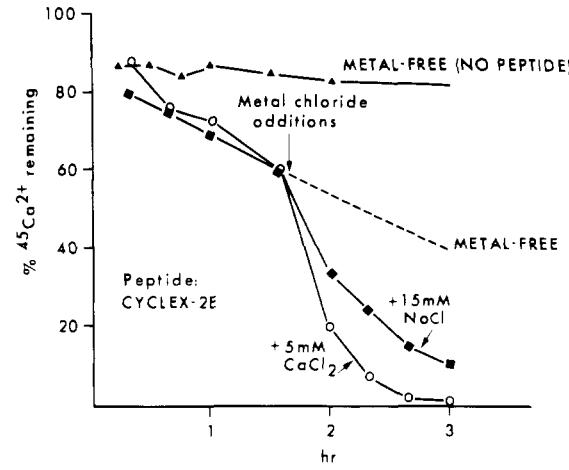

FIGURE 5: The efflux of calcium ions from sonicated unilamellar phosphatidylcholine vesicles suspended in "metal-free" buffer (2 mM Hepes and 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), in double-distilled water). The pH was adjusted to 7.4 with ammonium hydroxide. The curves represent the extents of $^{45}Ca^{2+}$ efflux in the absence of peptide, in the presence of peptide (125 μg of CYCLEX-2E/2.5 mg of PC), and after additions of 5 mM $CaCl_2$ and 15 mM NaCl. Other details of the experiment were as described under Materials and Methods.

equilibrated $^{36}Cl^-$ ion in the presence or absence of peptide. Similar results were obtained by using [^{14}C]acetate ion.

In further attempts to identify an anion whose rate of efflux was comparable to or slower than calcium efflux in the presence of peptide, we examined divalent anions. Despite its net charge of −2, we found that in experiments where [^{14}C]-succinate replaced Cl^- as the anion throughout the experiment, the vesicles were permeable to succinate either by itself or the the presence of peptide. When $^{45}Ca^{2+}$ efflux was measured in the presence of "cold" succinate, the vesicles were impermeable to Ca^{2+} in the absence of peptide but were depleted of calcium in its presence.

However, extension of these experiments to another divalent anion, sulfate, demonstrated (Figure 4a) that the vesicles were not permeable to $^{35}SO_4^{2-}$ over the 3-h course of the measurements. Although addition of peptide appeared to cause some leakage of $^{35}SO_4^{2-}$, other evidence suggests that this leakage may be related to the presence (along with peptide) of the dimethylformamide (DMF) solvent used in administering the compound to the vesicles. As shown in Figure 4b, parallel experiments with sulfate, but where $^{45}Ca^{2+}$ efflux was monitored, demonstrated that Ca^{2+} efflux did not take place nonspecifically, but upon addition of peptide, occurred at approximately the same rate as observed for the other anions.

Specific Role of External Cations in Peptide-Mediated Calcium Transport. A counter-directed (peptide-mediated) cation flow could also act as the charge-balancing factor in the overall CYCLEX-2E calcium transport mechanism. To investigate this possibility, we prepared unilamellar PC vesicles loaded with $^{45}Ca^{2+}$ and suspended them in "metal-free" buffer as shown in Figure 5. Controls established the stability of the vesicles in this suspending medium. The gradual slope of the metal-free curve is probably an indication that the medium is not rigorously metal-free and/or that ammonium or large organic cations may have a measurable effect on the rate of $^{45}Ca^{2+}$ efflux. *Addition of peptide did not induce significant efflux of calcium until either $CaCl_2$ or NaCl was added to the external medium.* Efflux of $^{45}Ca^{2+}$ from the vesicles then proceeded as previously observed (e.g., Figure 3). Similar experiments involving the addition of 5 mM external NaCl (vs. 15 mM shown in Figure 5) produced a $^{45}Ca^{2+}$-efflux curve of slope comparable to that measured under metal-free conditions, suggesting that the cation selectivity of the peptide (i.e., $Ca^{2+} > Na^+$) may also play a role in this phase of the transport process.

Discussion

Experiments with the synthetic cyclic octapeptides cyclo-[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ (CYCLEX-2E) and cyclo[Asp(OBz)-Sar-Gly-(*N*-decyl)Gly]$_2$ (ASP-DECYL-2E) demonstrate the ionophorous activity of this class of peptides. Among physiologically abundant cations, both peptides select for calcium and, to a lesser extent, transport sodium and potassium. Magnesium transport is poorest and was not detected in ASP-DECYL-2E experiments. Competition experiments in Pressman cells using CYCLEX-2E proved that this peptide will transport calcium essentially *specifically* in the presence of equimolar or 10-fold excess sodium ions. Results presented in Figures 3-5 establish cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$ as a membrane-active calcium ionophore; i.e., the peptide mediates the total exit of $^{45}Ca^{2+}$ from the aqueous interior of phosphatidylcholine vesicles. Among several octapeptides examined in this laboratory, which contain virtually identical cation-binding cavities and display similar cation transport profiles in Pressman cells (including analogues with two *n*-decyl chains and two *n*-hexyl chains), only CYCLEX-2E displayed the desirable combination of transmembrane calcium transport without nonspecific (detergent-like) membrane damage. In contrast, we noted especially the tendency of peptides with "decyl tails" to act as detergents, a property reminiscent of the mechanism of membrane activity attributed to the family of polymyxin antibiotics (Storm et al., 1977). Thus, calcium transport activity of a substance in phospholipid membranes must depend upon additional factors such as physical size and shape of the transporting species and probably the solubility of the peptide–cation complex in the bilayer.

In elucidating the overall mechanism by which CYCLEX-2E mediates transmembrane calcium transport, we note first that it must differ in detail from the mode of calcium transport by ionophore A23187 (Chaney et al., 1974). By a protons for metal exchange mechanism, the carboxylate acid A23187 forms electroneutral (uncharged) complexes with calcium to produce a two A23187 to one Ca^{2+} species (Deber & Pfeiffer, 1976) which will, ideally, equalize the Ca^{2+} concentrations on both sides of a membrane. Indeed, A23187 does act as a calcium transport agent in the unilamellar PC vesicles systems

employed here (data not shown). Since CYCLEX-2E (a dibenzyl ester) lacks exchangeable protons, its mode of cation transport may be more formally analogous to the prototypic K^+ ionophore valinomycin (Ovchinnikov et al., 1974). The latter substance contains 12 (alternating) amino and hydroxy acids vs. 8 (alternating) amino and imino acids in CYCLEX-2E but similarly lacks an exchangeable proton. Thus, both substances initially generate positively charged species upon complexation with cations, which require a compensating process of charge balance to complete a transport cycle. In Pressman cells transport by CYCLEX-2E requires picrate (trinitrophenol) as a lipophilic anion to solubilize cation complexes in the organic phases, as well as to assure the overall charge balance. However, valinomycin membrane transport may differ from CYCLEX-2E membrane transport in view of the report that valinomycin did not produce net K^+ egress from egg lecithin (PC) micelles until an "uncoupler" [dinitrophenol (DNP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)] had been added to the membrane to allow proton equilibration to occur (Chappell & Haarhoff, 1966).

Although valinomycin does not transport Ca^{2+} (vide infra), experiments using CYCLEX-2E in Pressman cells show that this octapeptide can transport K^+. In a preliminary experiment to examine CYCLEX-2E K^+ transport in a biological membrane, we suspended rat liver mitochondria [prepared according to Johnson & Lardy (1967)] in potassium acetate solutions under standard conditions where valinomycin causes swelling of mitochondria upon addition of an uncoupler of oxidative phosphorylation (Henderson, 1971). Additions of CYCLEX-2E up to 30 nM had no nonspecific effect on the mitochondria in the absence of uncoupler; when the uncoupler CCCP was added, swelling phenomena were essentially complete at 20 nM peptide, paralleling valinomycin activity. Nonspecific permeability effects precluded adaption of these mitochondria experiments to calcium transport studies.

From the CYCLEX-2E experiments with PC vesicles, one could infer initially that transport requires a functional anion which can facilitate transport either *directly* by accompanying the (positively charged) peptide–calcium complex across the membrane (as a neutral or at least singly charged species) or perhaps *indirectly* via a charge-balancing anion flux. However, to a first approximation, data presented herein suggest that $^{45}Ca^{2+}$ transport by CYCLEX-2E is essentially independent of directly accompanying anions, since (a) similar $^{45}Ca^{2+}$ egress rates were observed whether the anion present was chloride, acetate, succinate, or sulfate but particularly (b) sulfate efflux was minimal during peptide-mediated $^{45}Ca^{2+}$ efflux (Figure 4). These observations do not rule out hydroxide as a functional anion for the CYCLEX-2E–Ca^{2+} complex.

The finding (Figure 5) that when PC vesicles are suspended in metal-free buffer, $^{45}Ca^{2+}$ transport by CYCLEX-2E occurs only after Ca^{2+} or Na^+ ions have been added to the external medium suggests the possibility that an *inwardly directed cation flow* (whether or not peptide mediated) accounts for the experimental outcome. However, it remains to be verified whether this is the case, or whether the results reflect indirect ionic strength and/or binding effects of cations on the membrane outer surface.

We are presently investigating the detailed properties of CYCLEX-2E and a number of its analogues in both artificial and biological membranes. The calcium selectivity and membrane transport exhibited by CYCLEX-2E, and its potential ability to act as a transmembrane cation-exchange peptide, should make this peptide useful as a probe in a variety of systems where biochemical events are linked to calcium fluxes.

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