Ionophorous Properties of Neutral Diamide Ligands toward Calcium†

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ABSTRACT: The ability of a series of aromatic and alicyclic analogues of 1,2-ethylenedioxydiacetic acids bearing *N,N,N′,N′*-tetra-*n*-propyl amide or *N*-methyl-*N*-carbethoxypentyl amide linkages to enhance the rate of $^{45}Ca^{2+}$ efflux from vesicles was studied. The ligands were less potent in enhancing membrane permeability to Ca^{2+} than A23187 and X537A. Lipid-soluble anions markedly increased the rate and extent of Ca^{2+} transport mediated by these neutral ligands. The abilities of these synthetic diamide ligands and naturally occurring ionophores to transport Ca^{2+} across bilayer membranes were sensitive to the lipid composition of the vesicle. The mechanism of Ca^{2+} transport mediated by this series of synthetic ligands is discussed.

The synthesis and metal ion binding properties of a series of neutral 1,2-ethylenedioxydiacetamide ligands have been discussed in earlier communications (Borowitz et al., 1977; Wun et al., 1977). The abilities of these ligands to solubilize cations in bulk organic phase in a selective fashion (Borowitz et al., 1977) and to form complexes with alkaline-earth metal ions in a binding order largely controlled by the field-strength effect of the cation (except Mg^{2+}) (Wun et al., 1977) led us to determine whether these ligands have ionophorous activity. We describe here the properties of these ligands as carriers of Ca^{2+} across phospholipid bilayer membranes and some aspects of the mechanisms by which they increase Ca^{2+} transport.

The abilities of these neutral ligands to promote Ca^{2+} efflux from vesicles are compared with the activities of two naturally occurring carboxylic ionophorous agents, X537A and A23187. These lipophilic antibiotics form complexes with cations, functioning as mobile carriers of cations in many biological membranes; their biological activity apparently is derived from their ability to effect the equilibration of certain cations across membranes by transporting them in response to concentration gradients (Henderson et al., 1969; Pressman, 1970; Scarpa and Inesi, 1972; Reed and Lardy, 1972; Pressman, 1973). These ionophores have recently become widely used as tools for investigating the influence of Ca^{2+} flux across natural membranes on the regulation of various biological phenomena. The major transported species in X537A- and A23187-mediated Ca^{2+} permeation appears to be uncharged (Reed and Lardy, 1972; Wong et al., 1973; Pfeiffer et al., 1974; Case et al., 1974; Pfeiffer and Lardy, 1976), whereas the synthetic diamide ligands must form positively charged complexes with metal ions. Despite the difference in the charge of the complexes formed between cations and the synthetic and natural ligands, comparison of the ionophorous activity of the synthetic ligands with that of naturally occurring Ca^{2+} ionophores may allow us to predict what molecular features should be built into future synthetic analogues to approach the effects of the ionophorous antibiotics.

Experimental Section

Materials

Egg phosphatidylcholine (PC)[1] was isolated and purified from hen egg yolk and its purity was ascertained as described previously (Bittman and Blau, 1972). Bovine brain phosphatidylserine (PS) was obtained from General Biochemicals, octadecylamine (OA) from K & K Laboratories, and cholesterol from Sigma Chemical Co. Cholesterol was recrystallized twice from methanol. Chlorotetracycline was purchased from Nutritional Biochemical Corp., 8-anilino-1-naphthalenesulfonate (ANS^-) was from Sigma Chemical Co., and sodium tetraphenylborate (Ph_4B^-) and dipicrylamine (DPA^-) were from Aldrich Chemical Co. Calcium-45 was purchased from New England Nuclear Corp. A23187 was donated by Eli Lilly and Co. X537A was donated by Hoffmann-La Roche. Dicyclohexyl-18-crown-6 (DC) was a gift of Dr. H. Frensdorff of Du Pont Central Research. The ligands were synthesized as described previously (Ammann et al., 1975). The structures of these ionophores are shown in Figure 1. They are the *N,N,N′,N′*-tetra-*n*-propyl amides of 1,2-phenylenedioxydiacetic acid (P-PR), 2,3-naphthalenedioxydiacetic acid (N-PR), and *cis*- and *trans*-1,2-cyclohexanedioxydiacetic acids (*c*-C-PR and *t*-C-PR). The structure of the *N*-methyl-*N*-carbethoxypentyl amide of *cis*-1,2-cyclohexanedioxydiacetic acid (*c*-C-5) is also shown.

Methods

Preparation of Phospholipid Vesicles. Vesicles were prepared from rabbit sarcoplasmic reticulum (SR) microsomal phospholipids, egg PC, 80 mol % PC–20 mol % PS, 95 mol % PC–5 mol % OA, and 50 mol % PC–50 mol % cholesterol. SR was prepared by a modification of the procedure of Martonosi et al. (1968). SR microsomal phospholipids were obtained by extracting SR microsomes with chloroform–methanol (2/1, v/v) as described by Folch et al. (1957). The desired amount of lipid was transferred to a vial and organic solvent was removed by a stream of nitrogen to form a thin lipid film. The residue was dispersed in a medium containing 10 mM imidazole buffer, pH 7.0, 0.15 mM $CaCl_2$ (with $^{45}Ca^{2+}$), and 135 mM NaCl by agitation on a Vortex mixer at room temperature with 2 glass beads for 3 min. The dispersion was sonicated for 450 s under nitrogen with circulating cold water using a Branson S110 sonifer at power level 3. The sonicated dispersion was stored at 4 °C for about 2 h prior to molecular sieve chromatography.

Characterization of Egg PC Vesicles. The volume trapped

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[1] Abbreviations used are: SR, sarcoplasmic reticulum; PC, phosphatidylcholine; PS, phosphatidylserine; OA, octadecylamine; DC, dicyclohexyl-18-crown-6; ANS^-, 8-anilino-1-naphthalenesulfonate; DPA^-, dipicrylamine; Pic^-, picrate; Ph_4B^-, tetraphenylborate; DMF, dimethylformamide; EDTA, (ethylenedinitrilo)tetraacetic acid.

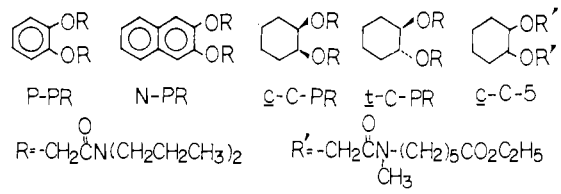

FIGURE 1: Structures of P-PR, N-PR, *c*-C-PR, *t*-C-PR, and *c*-C-5.

in the vesicles in the presence of 0.5 M $K_3Fe(CN)_6$, 10 mM imidazole (pH 7.0), 135 mM NaCl, and 0.15 mM $CaCl_2$ was measured by the method of Newman and Huang (1975), except that chromatography of vesicles was done on a Sephadex G-50 column. The trapped volume was found to be 0.160 ± 0.004 L/mol of phospholipid. The addition of 1.41×10^{-4} M *c*-C-PR or N-PR to the vesicles (4.7 mM egg PC) followed by a 4-h dialysis against a medium containing 10 mM imidazole buffer, pH 7.0, and 135 mM NaCl did not lead to appreciable leakage of the trapped $K_3Fe(CN)_6$. This indicates that the vesicle is not ruptured by the addition of the ionophores at this concentration.

Removal of Untrapped $^{45}Ca^{2+}$. Radioactive ions that were not trapped in the vesicle were removed by gel filtration. The suspension was passed through a column (1.5 × 30 cm) of Sephadex G-50 and eluted with 10 mM imidazole buffer, pH 7.0, containing 135 mM NaCl. The diluted suspension of vesicles thus obtained, free of untrapped $^{45}Ca^{2+}$, was used in leakage experiments.

Assay of $^{45}Ca^{2+}$ *Release.* Stock solutions of the diamide ionophores and DC were prepared in DMF, and A23187 and X537A were dissolved in ethanol. A 10-μL aliquot of ionophore solution (or DMF or ethanol) was added to 1-mL portions of ^{45}Ca-loaded vesicles (approximately 2.5–4.5 μmol of lipid phosphorus). Vesicles were placed in 1-cm diameter Visking tubing. The bags were knotted with air bubbles trapped, and placed in test tubes containing 4 mL of 10 mM imidazole buffer, pH 7.0, and 135 mM NaCl. The sealed test tubes were rotated at 2 rpm. Aliquots (0.1 mL) of the dialysates were taken for radioactive counting at various time intervals. Fluxes are expressed as the percentage of the initial trapped radioactivity lost as a function of time. Experiments with the same preparation of vesicles showed that the Ca^{2+} concentration of the dialysate at a given time did not vary by more than 5%. The initial rate of Ca^{2+} efflux did not vary by more than 5% when different preparations of egg PC vesicles were made in medium containing 0.15 or 1.5 mM $CaCl_2$.

Complexation of Vesicle-Bound Ca^{2+} *by Ionophores Using Chlorotetracycline as Fluorescence Probe.* The method is essentially the same as described by Jilka and Martonosi (1975). Details of the experiment are described in the caption to Figure 8.

Determination of Phospholipid Concentration. Phospholipid determinations were done by digesting 0.1–0.3-mL aliquots of vesicle suspensions with 0.1 mL of concentrated H_2SO_4 at 250–260 °C for 15 min. After a few drops of 30% H_2O_2 were added the sample was redigested for 5 min. The solution was then diluted to give a H_2SO_4 concentration of approximately 1 N. The concentration of inorganic phosphate was determined by the method of Taussky and Shorr (1953).

Measurement of Ionophore Distribution between 1-Butanol and Water. To 5 mL of distilled water saturated with 1-butanol was added 1.5 mL of 1-butanol containing $1–2 \times 10^{-4}$ M N-PR, P-PR, or *c*-C-PR. The mixture was shaken on a Vortex mixer at room temperature for at least 1 min and then centrifuged to separate the organic and aqueous phases. The 1-butanol layer was withdrawn, 10 μL of 1-butanol was added to this layer to reduce the turbidity, and the concentrations of ionophores were calculated from the absorbance using the following extinction coefficients in 1-butanol: N-PR, 3185 M^{-1} cm^{-1} at 323 nm; P-PR, 1593 M^{-1} cm^{-1} at 280 nm; *c*-C-PR, 2065 M^{-1} cm^{-1} at 230 nm. Partition coefficients were obtained by dividing the concentration of ionophore in the 1-butanol layer by the concentration of ionophore in the aqueous layer. The concentration of ionophore in the aqueous layer was obtained by subtracting the ionophore concentration in the organic layer from the total concentration added.

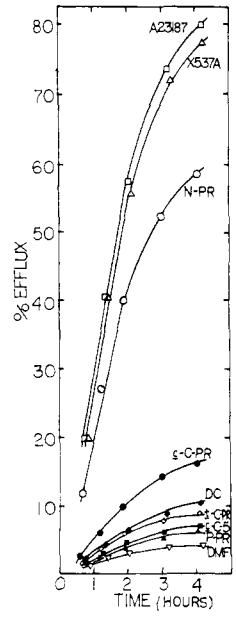

FIGURE 2: Effect of ionophores on the rate of Ca^{2+} efflux from vesicles prepared from SR lipids. The conditions are described under Experimental Section. The SR phospholipid concentration was 2.5 μmol of P/mL. The amount of Ca^{2+} captured in the vesicles was 2.2 nmol of Ca^{2+}/μmol of phospholipid. The temperature was 4 °C. The ionophore concentrations were 1.41×10^{-4} M for N-PR, *c*-PR, *c*-C-5, *t*-C-PR, P-PR, and DC; 8.47×10^{-6} M for X537A; and 4.78×10^{-7} M for A23187. The experiment was performed at 4 °C because the leakage of Ca^{2+} in SR vesicles in the absence of ionophores at room temperature amounts to 53% of the trapped Ca^{2+}.

Measurement of Ionophore Distribution between Egg PC Vesicles and Water. Ionophores were added to 1 mL of vesicles (3.56 mM egg PC in 1 mM imidazole, pH 7.0) contained in dialysis bags and the suspensions were dialyzed against 5 mL of 1 mM imidazole, pH 7.0, for 4–5 h with rotation at 2 rpm. After dialysis, 0.8 mL of vesicles was withdrawn and 1.2 mL of diethyl ether was added to disrupt the vesicles and extract the ionophore. The ether phase was withdrawn after separation by centrifugation, 10 μL of ether was added to the ether layer to reduce the turbidity, and the absorbance was measured. The extinction coefficients of the ionophores in ether were N-PR, 3475 M^{-1} cm^{-1} at 323 nm; P-PR, 2020 M^{-1} cm^{-1} at 280 nm; *c*-C-PR, 1516 M^{-1} cm^{-1} at 230 nm. Under the extraction conditions used, nearly all the *c*-C-PR and P-PR was extracted into the ether layer, while N-PR was only partially extracted. The N-PR has a partition coefficient of ether to water-vesicle layer of about 1.80 ± 0.05. This value was used to calculate the amount of N-PR remaining with the vesicles.

Results

Ionophore-Enhanced Ca^{2+} *Permeability of Bilayer Vesicles.* Figure 2 illustrates the stimulation of Ca^{2+} release

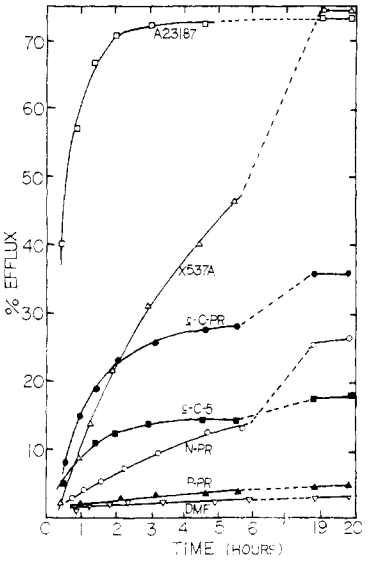

FIGURE 3: Rate of Ca^{2+} efflux from egg PC vesicles in the presence of ionophores. The concentration of phospholipid was 3.6 μmol of P/mL. The amount of Ca^{2+} captured in the vesicle was 0.61 nmol of Ca^{2+}/μmol of phospholipid. The temperature was 26 °C. The ionophore concentrations were the same as in Figure 2.

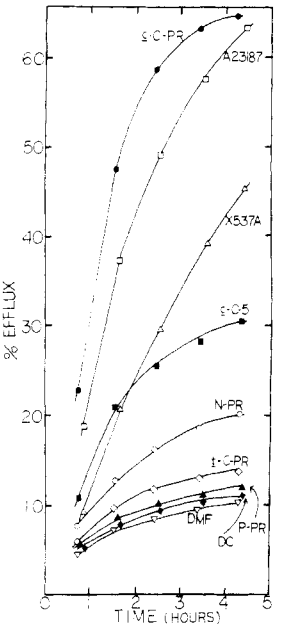

FIGURE 4: Rate of Ca^{2+} efflux from 20 mol % PS-80 mol % egg PC vesicles in the presence of ionophores. The total concentration of phospholipid was 4.5 μmol of P/mL. The amount of Ca^{2+} captured in the vesicles was 1.9 nmol of Ca^{2+}/μmol of phospholipid. The temperature was 26 °C. The ionophore concentrations were the same as in Figure 2.

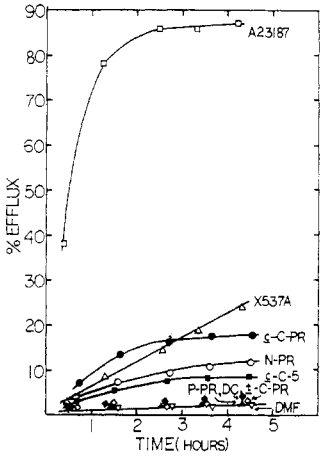

FIGURE 5: Rate of Ca^{2+} efflux from 5 mol % octadecylamine-95 mol % egg PC vesicles in the presence of ionophores. The phospholipid concentration was 3.0 μmol of P/mL. The amount of Ca^{2+} captured in the vesicle was 0.16 nmol of Ca^{2+}/μmol of phospholipid. The temperature was 20 °C. The ionophore concentrations were the same as in Figure 2.

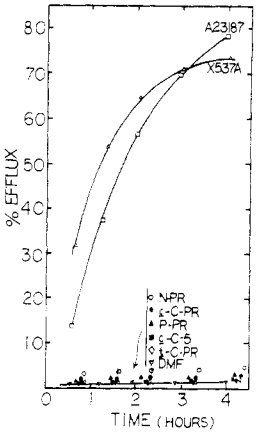

FIGURE 6: Rate of Ca^{2+} efflux from 50 mol % cholesterol-50 mol % egg PC vesicles in the presence of ionophores. The phospholipid concentration was 2.4 μmol of P/mL. The amount of Ca^{2+} captured in the vesicle was 0.35 nmol of Ca^{2+}/μmol of phospholipid. The temperature was 26 °C. The ionophore concentrations were the same as in Figure 2.

produced by addition of various ionophores to vesicles prepared from SR lipids. Of the total lipids of SR microsomes, 89% are phospholipids, in which PC is predominant (71%), followed by phosphatidylethanolamine and sphingomyelin (6%) (Waku et al., 1971). The relative abilities of the ionophores to induce Ca^{2+} release from vesicles prepared from SR lipids (Figure 2) follow the same order as their abilities to decrease chlorotetracycline fluorescence in intact SR microsomes loaded with Ca^{2+}, with the exception of DC (T-C. Wun and R. Bittman, unpublished results). Notably, N-PR is markedly more effective than the other synthetic ionophores in causing Ca^{2+} efflux from SR lipid vesicles.

The abilities of the naturally occurring and synthetic ionophores to transport Ca^{2+} across vesicles prepared from other phospholipids were examined. The kinetics of ionophore-induced transport of Ca^{2+} from vesicles prepared from egg PC are strikingly different from those observed in vesicles derived from SR lipids (Figure 3). Relative to their ability to enhance the rate of passive Ca^{2+} diffusion through SR phospholipid bilayer membranes, X537A and N-PR have markedly depressed potencies in egg PC vesicles. The peculiar leveling off after dialysis of a few hours is not completely understood at present. With vesicles prepared from 20 mol % PS-80 mol % egg PC, *c*-C-PR was found to be highly effective in promoting Ca^{2+} release relative to the other synthetic ionophores (Figure 4). In these negatively charged bilayers, the net efflux of Ca^{2+} induced by A23187 and X537A is somewhat depressed compared with that in egg PC bilayers (Figure 3). In contrast, this lipid environment enhances the abilities of the neutral diamide ionophores (which form positively charged complexes) to catalyze transmembrane efflux of trapped Ca^{2+}. To further examine the influence of bilayer charge on the ability of the ionophores to induce Ca^{2+} transport, vesicles were prepared from 5 mol % OA-95 mol % egg PC. The results obtained for

TABLE I: Influence of Lipid Composition on Ionophore-Induced Efflux of Ca^{2+} from Vesicles.[a]

Lipid Comp of Vesicle	Temp (°C)	Phospholipid Concn (mM)	mmol of Ca^{2+} bound/mol of phospholipid	Rate of Ca^{2+} Efflux (pmol of Ca^{2+} (μmol of ionophore)$^{-1}$ hr^{-1})							
				A23187	X537A	N-PR	*c*-C-PR	*t*-C-PR	*c*-C-5	P-PR	DC
SR	4	2.5	2.2	2920	157	9.9	1.25	0.55	0.20	0.20	0.55
Egg PC	26	3.6	0.61	2670	23.4	0.47	2.18		1.25	~0	
20 mol % PS-80 mol % egg PC	26	3.6	2.5	3201	69.1	2.55	15.6	1.28	5.43	0.64	0.32
5 mol % OA-95 mol % egg PC	20	3.0	0.16	711	2.44	0.12	0.26	~0	0.09	~0	~0
50 mol % Cholesterol-50 mol % egg PC	26	2.4	0.35	474	43.6	0.12	~0	~0	~0	~0	~0

[a] The rate of $^{45}Ca^{2+}$ efflux during the first hour of dialysis following addition of ionophore was measured and corrected for the spontaneous leak of Ca^{2+} during the first hour in the presence of DMF.

Ca^{2+} release from these positively charged bilayers are shown in Figure 5. The rates and extents of Ca^{2+} release induced by all of the ionophores except A23187 are depressed in these vesicles relative to the values obtained in uncharged bilayer membranes. Figure 6 shows the ionophore-induced Ca^{2+} leakage from vesicles prepared from 50 mol % egg PC–50 mol % cholesterol. The $^{45}Ca^{2+}$ efflux rate in the presence of X537A is enhanced compared to the rates observed in the other bilayers derived from egg lecithin. All of the other ionophores have depressed activities relative to their abilities to stimulate Ca^{2+} transport across cholesterol-lacking bilayers.

Examination of the effect of lipid composition on the abilities of ionophores to stimulate Ca^{2+} release reveals several striking features (Table I). First, when the rates of Ca^{2+} efflux from SR vesicles induced by various ionophores are normalized to the rate observed in the presence of 4.78×10^{-7} M A23187, the relative potencies of A23187, X537A, N-PR, *c*-C-PR, *c*-C-5, P-PR, and DC are 100:5.38:0.34:0.043:0.007:0.007:0.019. Very similar values of relative potencies are observed for the abilities of the ionophores to decrease chlorotetracycline fluorescence by 50% in intact SR microsomes loaded with Ca^{2+} (T-C. Wun and R. Bittman, unpublished data). Second, the relative potencies of the ionophores vary when the composition of the lipids in the membrane is altered; for example, N-PR has much higher potency in vesicles prepared from SR lipids than in the vesicles prepared from egg PC alone or from mixtures of egg PC and other lipids. The activity of X537A is depressed in bilayers prepared from egg PC, PS–egg PC, and OA–egg PC relative to the activity in vesicles prepared from SR lipids; however, higher X537A activity was observed in vesicles prepared from an equimolar mixture of egg PC and cholesterol than from egg PC alone. Third, the amount of Ca^{2+} captured by the vesicles depends on the lipid composition. Vesicles containing negatively charged phospholipids (those prepared from SR lipids and PS–egg PC) captured more Ca^{2+}. Egg PC, which is a neutral phospholipid, captured less Ca^{2+}. Egg PC–OA bilayers, which are positively charged, captured the least amount of Ca^{2+}. Fourth, the synthetic ionophores do not increase the permeability of bilayers to Ca^{2+} in proportion to the amount of Ca^{2+} trapped in the vesicle. For example, the presence of 20 mol % PS in egg PC vesicles increased the amount of captured Ca^{2+} to about three times the amount trapped in egg PC vesicles; however, with the exception of A23187 and X537A, the ionophores enhanced the rate of transport by a factor greater than 3. The presence of 5 mol % of positively charged OA decreased Ca^{2+} captured by vesicles by about 3.8 times, but with the exception of A23187 and N-PR the rates of transport did not decrease proportionately. Since A23187 and X537A are negatively charged and the synthetic ionophores are neutral molecules, their complexes with Ca^{2+} differ in charge. The presence of membrane charges may regulate transport of charged complexes to a greater extent than those complexes that are not charged. Fifth, incorporation of cholesterol into the vesicle depressed the potencies of A23187, N-PR, *c*-C-PR, P-PR, *t*-C-PR, *c*-C-5, and DC relative to their activities in pure egg PC bilayers. Since incorporation of cholesterol into egg PC bilayers increases the extension of the fatty acid chains and reduces the amplitude of motion of their long axes (Schreier-Muccillo et al., 1973), transport of these ionophore–Ca^{2+} complexes may be limited by the increased rigidity of the cholesterol-containing bilayers. However, the rate of movement of Ca^{2+}–X537A complex(es) across the membrane is not limited by cholesterol incorporation.

Effect of Lipophilic Anions on Ionophore-Mediated Ca^{2+} Transport. The stoichiometry of ionophore–Ca^{2+} interaction is 1:1 for P-PR and N-PR at low concentration, 2:1 (ligand to metal ion) for *c*-C-PR, and biphasic (1.5:1 and 1:1, depending on the extent of saturation) for *t*-C-PR (Wun et al., 1977). These ionophores are far less potent carriers of Ca^{2+} than the two carboxylic ionophores, X537A and A23187, suggesting that the charge is not fully enveloped in the permeant species. The possibility that lipophilic anions would enhance the movement of Ca^{2+}–ionophore complexes across the bilayer was investigated. Figure 7 shows that the lipid-soluble anions ANS^-, Ph_4B^-, DPA^-, and Pic^- do not appreciably transport Ca^{2+} themselves. However, in the presence of *c*-C-PR, they greatly enhance the rate and extent of Ca^{2+} transport. The order in which these lipophilic anions enhance *c*-C-PR-mediated Ca^{2+} transport is $Ph_4B^- > DPA^- > Pic^- > ANS^-$. (The difference in the ability of Ph_4B^- and DPA^- to enhance *c*-C-PR-mediated Ca^{2+} efflux is clearly observed when lower concentrations of Ph_4B^- and DPA^- are used.) The transport of Ca^{2+} by X537A and A23187 (which are negatively charged at pH 7.0) is not enhanced by Ph_4B^- at the concentration tested (Figure 7B, dashed curves). Hence, neutralization of the charge in the synthetic ionophore–Ca^{2+} complex or formation of lipophilic ion pairs may greatly enhance the transport of Ca^{2+} across the membrane.

Distribution of N-PR, c-C-PR, and P-PR in 1-Butanol–Water and Egg Lecithin Vesicle–Water Systems. Lipophilic ionophorous antibiotics form complexes with cations, increasing cation solubility in the hydrocarbon-like membrane interior and increasing the cation permeability of membranes (Harris, 1972). To characterize the lipophilicity of the synthetic ionophores, their partitioning in 1-octanol–water (Hansch, 1969) was determined. N-PR, *c*-C-PR, and P-PR are nearly completely partitioned into the octanol phase (data

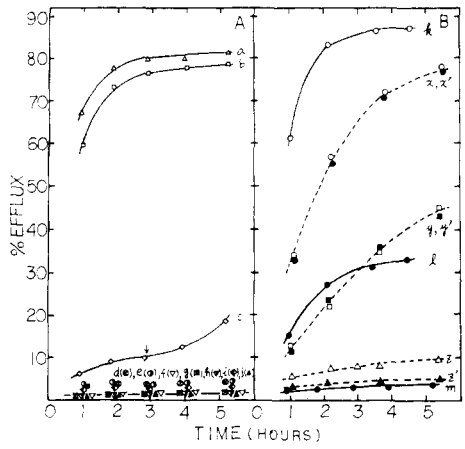

FIGURE 7: Effect of lipophilic anions on ionophore-mediated Ca^{2+} transport across egg PC vesicles. (A) Vesicles were prepared in the medium as described under Experimental Section. The phospholipid concentration and the amount of Ca^{2+} captured were 3.5 μmol of P/mL and 0.35 nmol of Ca^{2+}/μmol of phospholipid, respectively. The concentrations of *c*-C-PR, ANS^-, Ph_4B^-, DPA^-, and Pic^- were 2.82×10^{-5} M. At the point indicated by the arrow, more picric acid was added to the dialysis medium to make the amount of picric acid five times that of *c*-C-PR. The temperature was 25 °C. Addition of (a) *c*-C-PR + Ph_4B^-; (b) *c*-C-PR + DPA^-; (c) *c*-C-PR + Pic^-; (d) *c*-C-PR + ANS^-; (e) *c*-C-PR; (f) DMF; (g) Ph_4B^-; (h) DPA^-; (i) Pic^-; (j) ANS^-. (B) A separate vesicle preparation was used which had a phospholipid concentration of 2 μmol of P/mL, and captured 0.3 nmol of Ca^{2+}/μmol of phospholipid. Addition of (k) *c*-C-PR (1.41×10^{-4} M) and ANS^- (1.41×10^{-4} M); (l) *c*-C-PR (1.41×10^{-4} M); (m) ANS^- (1.41×10^{-4} M). In k and m, the dialysis medium also contained 1.41×10^{-4} M ANS^-. Dashed curves: The experimental conditions were the same as described above except that vesicles were prepared in a medium containing 10 mM imidazole, pH 7.0, 135 mM NaCl, and 1.5 mM $CaCl_2$. The phospholipid concentration was 2.6 μmol of P/mL. The amount of Ca^{2+} captured was 3.32 nmol/μmol of phospholipid. Addition of (x,○) X537A (8.47×10^{-6} M) and Ph_4B^- (1.41×10^{-4} M); (x',●), X537A (8.47×10^{-6} M); (y,□), A23187 (9.56×10^{-8} M) and Ph_4B^- (1.41×10^{-4} M); (y',■), A23187 (9.56×10^{-8} M); (z) Ph_4B^- (1.41×10^{-4} M); (z') ethanol.

not shown). The partition coefficients of these ionophores in a 1-butanol–water system are identical within experimental error (Table II). However, measurement of the partitioning of these ionophores in an egg PC vesicle–water system shows that P-PR has a markedly lower partition coefficient in egg PC vesicles than N-PR and *c*-C-PR (Table III).

Studies of the Complexation of Vesicle-Bound Ca^{2+} with Ionophores. In order to determine whether complexation on the vesicle surface plays an important role in determining the ability of the synthetic ionophores to transport Ca^{2+}, complexation of vesicle-bound Ca^{2+} with *c*-C-PR and N-PR was studied using chlorotetracycline. Vesicles were prepared from egg PC or from a mixture of egg PC and PS in a medium containing Ca^{2+}. On addition of EDTA (1 mM), Ca^{2+} outside the vesicle was chelated (as measured by reduction of chlorotetracycline fluorescence) but Ca^{2+} inside the vesicle was not released. On addition of ionophore, Ca^{2+} inside the vesicle binds to the ionophore and the fluorescence of chlorotetracycline is decreased. Figure 8A shows that the rate of decrease of chlorotetracycline fluorescence induced by addition of *c*-C-PR is slow. Figure 8B shows a log–log plot of the decrease in chlorotetracycline vs. ionophore concentration. N-PR forms complexes with vesicle-bound Ca^{2+} at lower concentrations than *c*-C-PR in both egg PC vesicles and 20 mol % PS–80 mol % egg PC vesicles. The ratio of the slopes of the lines for N-PR to *c*-C-PR is approximately 2 in each vesicle preparation. This indicates that the stoichiometries of ionophore–Ca^{2+} complexes on these vesicle surfaces are different for N-PR and

TABLE II: Partition Coefficients of Ionophores between 1-Butanol and Water.

Ionophore	Partition Coefficient
N-PR	13.4 ± 1.1
P-PR	14.3 ± 1.0
c-C-PR	13.6 ± 1.2

TABLE III: Partitioning of Ionophore between Egg PC Vesicles and Water.

Ionophore	Total Amount of Ionophore Added to the Vesicle (mol)	Amount of Ionophore Remaining in the Dialysis Bag After Dialysis (mol)	% of Ionophore Remaining with the Vesicle
N-PR	2.64×10^{-7}	2.69×10^{-7}	~100
P-PR	2.64×10^{-7}	1.04×10^{-7}	27
c-C-PR	2.64×10^{-7}	2.67×10^{-7}	~100

c-C-PR. It is interesting that UV titration of N-PR and *c*-C-PR with $CaBr_2$ in methanol at low concentration gives a stoichiometry of 1:1 for N-PR–Ca^{2+} and 2:1 for *c*-C-PR–Ca^{2+} (Wun et al., 1977).

Discussion

The kinetics of ionophore-mediated Ca^{2+} transport can be roughly divided into two domains: interfacial reactions and transport of complexes across the membrane interior. The overall transport activity depends not only on the ability of ionophores to form lipophilic complexes with Ca^{2+}, but also on the ability of such complexes to traverse the membrane interior and to dissociate at the other side of the membrane. It is apparent from the results reported here that the potencies of several synthetic and two naturally occurring ionophores to transport Ca^{2+} across different bilayers vary with the lipid composition. The major factors involved in the complicated interplay between the membrane, ionophores, and cation can, in some cases, be elucidated by studying the transport through various types of membranes.

The importance of the phospholipid hydrocarbon chain in determining the rates of ion diffusion across the membrane has been established. Valinomycin-induced leakage of $^{86}Rb^+$ across bilayers was found to depend on the length and degree of unsaturation of the fatty acyl chains (de Gier et al., 1970). The dependence of valinomycin-induced and of nonactin-induced K^+ permeability on temperature was demonstrated (Johnson and Bangham, 1969; Krasne et al., 1971). It has been concluded that membrane fluidity may control the permeation of ionophore–cation complexes. Transport activity may also depend on the concentration of the permeant complexes in the membrane which, in turn, depends on the dielectric constant of the membrane interior, the charge or dipole of the membrane surface, and the polar and nonpolar interactions between ionophore and Ca^{2+} and between lipid and ionophore. Our experiments with this series of neutral ionophores provide some insight into the relationships between stoichiometry of complexation with cation, surface complexation, lipid composition, and Ca^{2+} transport activity. The greater efficiency of the alicyclic ligand *c*-C-PR than the aromatic ligands N-PR and P-PR in enhancing the rate of Ca^{2+} permeation in most vesicles (Figures 3, 4, and 5) except those derived from SR (Figure 2)

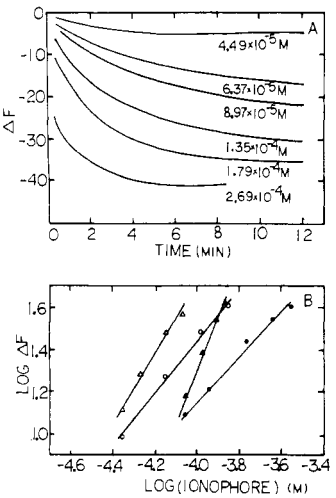

FIGURE 8: Complexation of vesicle-bound Ca^{2+} by ionophores using chlorotetracycline as fluorescence probe. (A) Changes in chlorotetracycline fluorescence upon complexation of vesicle-bound Ca^{2+} by *c*-C-PR. Vesicles (1 mM phospholipid) were prepared by dispersing and sonicating 20 mol % PS and 80 mol % egg PC in a medium containing 10 mM imidazole buffer, pH 7.0, 135 mM NaCl, and 0.2 mM $CaCl_2$. Chlorotetracycline was added to make a final concentration of 1.0×10^{-5} M. Addition of EDTA (final concentration of 1 mM) caused a decrease in fluorescence intensity of chlorotetracycline. After the fluorescence reached a relatively steady level (~2 min), the reaction was started by addition of a concentrated ionophore solution in DMF. The DMF concentration was 1.25% (v/v). The ionophore concentrations are indicated in the figure. The fluorescence intensity was monitored using excitation and emission wavelengths of 390 and 530 nm, respectively. The net fluorescence decrease was obtained from the difference between the fluorescence intensity in the presence of ionophore and DMF and the fluorescence intensity in the presence of DMF alone. The temperature was 27 °C. (B) Log-log plot of the fluorescence decrease vs. ionophore concentration. The complexation of Ca^{2+} bound to PS-egg PC vesicles with N-PR (△) and *c*-C-PR (○) was measured as described in A after the reaction mixture had been incubated for 25 min. The complexation of Ca^{2+} bound to egg PC vesicles with N-PR (▲) and *c*-C-PR (●) was measured as described in A, except that the chlorotetracycline concentration was 2.24×10^{-5} M and the reaction mixture was incubated for 25 min before fluorescence measurements were made. Vesicles from egg PC (1 mM) were prepared in a medium containing 10 mM imidazole buffer, pH 7.0, 135 mM NaCl, and 0.6 mM $CaCl_2$.

may be related to the ability of *c*-C-PR to form a 2:1 ionophore-Ca^{2+} complex, which may shield Ca^{2+} from the bilayer interior better than is possible in a 1:1 complex. The difference in the potencies of *c*-C-PR and N-PR in egg PC vesicles is apparently not related to their abilities to form complexes with Ca^{2+} on the vesicle surface, since Figure 8 shows that their surface complexation properties are not markedly different. The greater efficiency of N-PR than *c*-C-PR in SR vesicles (Figure 2) can be partially attributed to the more extensive degree of complexation of N-PR with Ca^{2+} on the SR membrane (T-C. Wun and R. Bittman, unpublished data). However, the origin of the variation in potencies of these two ionophores in bilayers of varying lipid composition is not fully understood. The lower potency of P-PR observed in all of the vesicles tested is consistent with its lower extent of partitioning into the bilayer (Table III) compared with *c*-C-PR and N-PR. Comparison of *c*-C-5 and *c*-C-PR indicates that the presence of a short apolar side chain terminating in a carbethoxyl group does not result in enhanced Ca^{2+}-transporting activity. In fact, the *N,N,N',N'*-tetra-*n*-propyl amide has a higher potency than the *N*-methyl-*N*-carbethoxypentyl analogue of *cis*-1,2-cyclohexanedioxydiacetic acid in each vesicle system we examined.

Incorporation of cholesterol into the bilayer reduces the valinomycin-facilitated exchange of Rb^+ (de Gier et al., 1970) and decreases the ionophore-induced Ca^{2+} permeability across egg PC bilayers for all of the ionophores examined except X537A (Figure 6). This is consistent with the well-known importance of membrane fluidity in regulating permeability. The permeability of ionophore-Ca^{2+} complexes within the lipid phase may be decreased as a result of the decrease in membrane fluidity caused by cholesterol incorporation. Cholesterol may also modulate membrane permeability to ions by altering the strength and orientation of dipolar groups at the membrane surface, which would make the interior more positive to the bulk aqueous phase than in cholesterol-free phospholipid bilayers (Szabo et al., 1972; Szabo, 1974). It is not yet clear why activity of X537A is enhanced in cholesterol-containing vesicles (Figure 6).

Several possibilities may be considered for the gradual leveling off of the rate of Ca^{2+} efflux noted in the presence of ionophores. (a) The trapped volume in the vesicle is about 6.4×10^{-7} L (1 mL of 4 mM egg PC vesicle) and is negligible compared with the volume of bathing medium (5 mL). It is unlikely that enough Ca^{2+} can leak out from the vesicle to permit return of Ca^{2+} to reduce the net efflux at low percentage of leakage. (b) The transport of Ca^{2+} by the synthetic ionophores may give rise to an electrochemical potential that may compensate for the concentration gradient. Additon of EDTA to the bathing medium or a change to a new dialysis medium did not eliminate the leveling off of Ca^{2+} efflux. This indicates that the electrical potential gradient generated during the ionophore-mediated efflux is not the major factor which gives rise to leveling off. Leveling off was also observed in an isotope flux experiment in which egg PC vesicles containing trapped $^{45}Ca^{2+}$ were dialyzed vs. an equal concentration of unlabeled Ca^{2+}. (c) It is not likely that a minimum intravesicular Ca^{2+} concentration was reached that prevented further release, because a tenfold increase in captured Ca^{2+} does not prevent leveling off (data not shown). (d) The vesicles do not show appreciable aggregation after 4 h of dialysis, as judged from absorbance and light-scattering measurements at 400 nm. Among the remaining possible explanations are a slow aggregation of the ionophores in the vesicle and a time-dependent change in the localization of the ionophores within the vesicle bilayer.

The ligands we used bind selectively to Ca^{2+}, favoring complexation with this ion over the other ions that play a fundamental role in a variety of biological processes (Na^+, K^+, and Mg^{2+}) (Wun et al., 1977). The approaches developed here may be applied to a study of the effects of other more potent synthetic analogues of this type to probe Ca^{2+}-mediated events.

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High-Resolution Nuclear Magnetic Resonance Determination of Transfer RNA Tertiary Base Pairs in Solution. 1. Species Containing a Small Variable Loop†

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ABSTRACT: Eight class I tRNA species have been purified to homogeneity and their proton nuclear magnetic resonance (NMR) spectra in the low-field region (−11 to −15 ppm) have been studied at 360 MHz. The low-field spectra contain only one low-field resonance from each base pair (the ring NH hydrogen bond) and hence directly monitor the number of long-lived secondary and tertiary base pairs in solution. The tRNA species were chosen on the basis of their sequence homology with yeast phenylalanine tRNA in the regions which form tertiary base pairs in the crystal structure of this tRNA. All of the spectra show 26 or 27 low-field resonances approximately 7 of which are derived from tertiary base pairs. These results are contrary to previous claims that the NMR spectra indicate the presence of resonances from secondary base pairs only, as well as more recent claims of only 1-3 tertiary resonances, but are in good agreement with the number of tertiary base pairs expected in solution based on the crystal structure. The tertiary base pair resonances are stable up to at least 46 °C. Removal of magnesium ions causes structural changes in the tRNA but does not result in the loss of any secondary or tertiary base pairs.

Early NMR[1] studies on model base pairs in aprotic solvents revealed that the hydrogen bonded ring NH resonance is greatly deshielded and occurs in the extreme low-field end of the proton spectrum (Katz and Penman, 1966). The pioneering studies of Kearns et al. (1971a,b) showed that, in solutions of tRNA, the solvent-exchangeable base pair ring NH hydrogen bonds were sufficiently long-lived to generate discrete low-field (−11 ppm to −15 ppm) resonances even in H_2O solvents. Since each base pair contains only one ring NH hydrogen bond, low-field NMR spectroscopy appeared an ideal method to study the number of tRNA base pairs in solution under a variety of conditions. During the ensuing 5 years, the extent of base pairing in several class I tRNAs has been studied by this technique; these tRNA species have a four base-paired DHU helix, a five nucleotide variable loop containing m^7G, and contain a total of 19 or 20 secondary base pairs in their cloverleaf structure.

By far the most studied class I D4V5 tRNA species has been yeast $tRNA^{Phe}$, because of its ease of isolation. Over the last

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[1] Abbreviations used are: DHU, dihydrouridine; rT, ribothymidine; m^7G, N^7-methylguanosine; Ψ, pseudouridine; tRNA, transfer ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; BD, benzoylated diethylaminoethyl; UV, ultraviolet; CW, continuous wave; DSS, 2,2-dimethylsilapentane-5-sulfonate.