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Ionophore-mediated loading of Ca^{2+} into large unilamellar vesicles in response to transmembrane pH gradients

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Summary

The Ca^{2+} translocating properties of the carboxylic ionophores A23187, ionomycin and lasolocid A (X537A) have been investigated by employing large unilamellar vesicles that exhibit a pH gradient (acidic interior). An analysis of Ca^{2+} uptake at equilibrium reveals that Ca^{2+} accumulation is an electroneutral process, whereby one Ca^{2+} ion is transported in for every two H^+ ions transported out. A kinetic analysis shows that both A23187 and ionomycin transport Ca^{2+} in the form of a 1:1 cation:ionophore complex, whereas a 1:2 complex is observed for lasolocid A. The specificity of the ionophores for transporting Ca^{2+} is reflected by the influence of exterior Na^+ ions that inhibit Ca^{2+} uptake for lasolocid A but do not influence ionomycin-mediated uptake.

Keywords: A23187, ionomycin, transmembrane pH gradient, liposome.

Introduction

Recent studies from this laboratory have shown that the presence of transmembrane pH gradients across the membranes of large unilamellar vesicles (LUVs) can result in the induction of asymmetric transbilayer distributions of certain lipids [1-3], as well as the net accumulation of a variety of lipophilic amino-containing drugs [4, 5] and certain peptides [6]. These processes, which reflect the highly membrane permeable nature of the neutral form of certain weak acids and bases, can lead to extremely high concentrations of entrapped materials. Lipophilic amines accumulated in response to a ΔpH of three units (inside acidic), for example, can exhibit interior concentrations that are 1000-fold higher than the external concentration, as the equilibrium transbilayer concentration gradient of the amine reflects the proton gradient.

Related processes can be used to drive the uptake of divalent cations such as Ca^{2+} ions, leading to high entrapped cation concentrations $\geq$200 mM [7]. In the present study, the kinetic and equilibrium behaviour of this uptake process is examined for A23187 (Figure 1A) and the generality of this protocol is explored for two other carboxylic Ca^{2+} ionophores, namely ionomycin (Figure 1B) and lasolocid A (Figure 1C), that exhibit varying Ca^{2+} specificity.

Results

A23187-mediated uptake of Ca^{2+}

The first set of experiments was aimed at showing that the A23817-mediated equilibrium uptake of Ca^{2+} into LUVs with an acidic interior could be described employing the formalism detailed in Experimental procedures. This calculation assumes that for each Ca^{2+} imported two H^+ ions are exported and thus a plot of the concentration of imported Ca^{2+} versus the effective concentration of H^+ ions exported should result in a straight line with a slope of 2. This was tested by measuring the Ca^{2+} uptake and interior pH at equilibrium for a variety of interior citrate buffer concentrations (Figure 2A). The effective concentration of H^+ ions released can be calculated from equation 4 employing the known initial internal proton concentration and the measured equilibrium pH following Ca^{2+} uptake. As shown in Figure 2B, the resulting plot of $[Ca^{2+}]_i$ versus the effective concentration of H^+ ions exported gives a straight line with a slope of 2. A related plot of interest concerns the relation between the exterior and interior gradients of Ca^{2+} and protons. As indicated under Experimental procedures, if the accumulated Ca^{2+} remains in a free (non-precipitated) form, then at equilibrium the relation $[Ca^{2+}]_i/[Ca^{2+}]_0 = [H^+]_i^2/[H^+]_0^2$ should hold. As shown in Figure 2C, a plot of $\log([Ca^{2+}]_i^{eq}/[Ca^{2+}]_0^{eq})$ versus $\log([H^+]_i^{eq}/[H^+]_0^{eq})$ yields a straight line with a slope of 2.

To characterize the mechanism of A23187-mediated uptake further, the influence of the A23817 concentration on

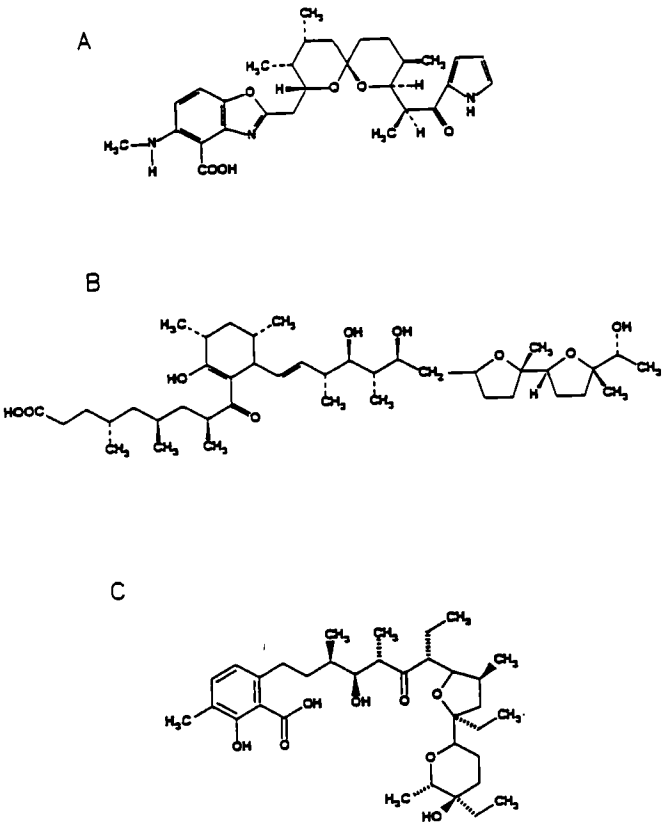

Figure 1. Structures of the carboxylic ionophores A23187 (A), ionomycin (B) and lasolocid A (C).

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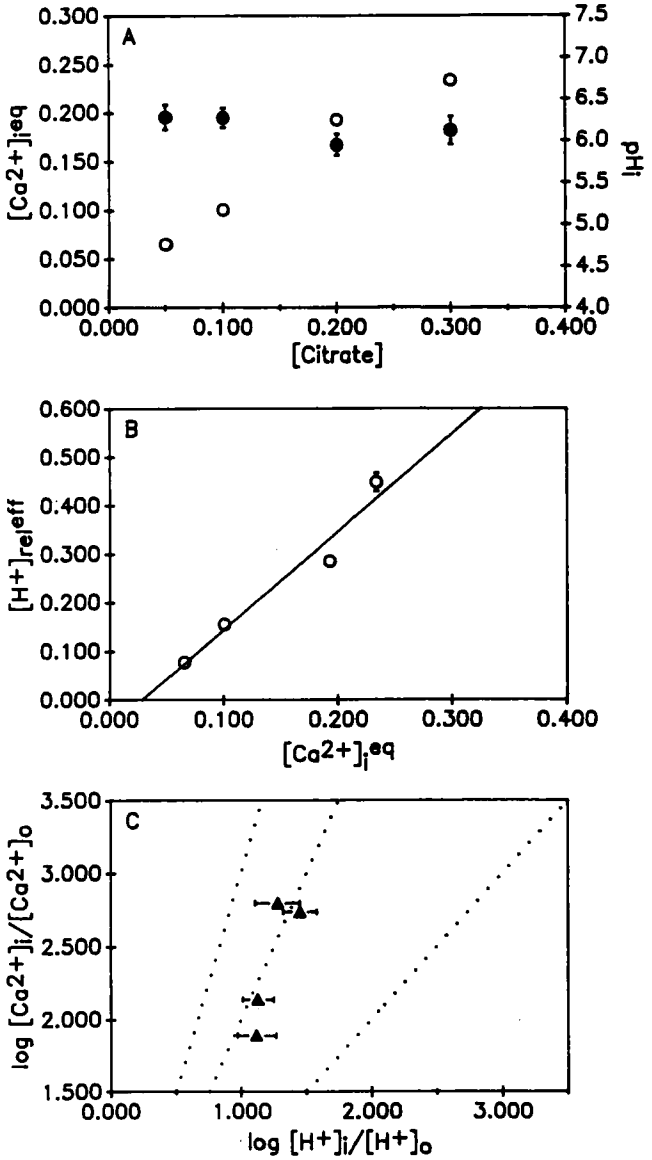

Figure 2. (A) Effect of internal citrate concentration on Ca^{2+} uptake (○) near equilibrium and internal pH (●) of EPC LUVs (100 nm) experiencing a transmembrane pH gradient 20 mM HEPES (pH 7·5) in the external medium and 300 nM citrate (pH 4·0) in the internal medium), in the presence of A23187 (2 μg/ml) and 1·0 mM external Ca^{2+}. Measurements were made after 1 h and the phospholipid concentration was 2·0 mM. (B) Comparison of the internal calcium concentration at equilibrium $[Ca^{2+}]^{eq}$ and the relative concentration of protons ($[H^+]_{rel}$) exported as calculated according to Experimental procedures (slope = 2·02). (C) Comparison of $[Ca^{2+}]_i^{eq}[Ca^{2+}]_0^{eq}$ and $[H^+]_i^{eq}[H^+]_0^{eq}$ as described in Experimental procedures. The dotted lines represent theoretical values for a slope of 1 (right), 2 (middle) or 3 (left). The experimental data indicate that there is a square dependence between the H^+ and Ca^{2+} ratios inside and outside the vesicles at equilibrium and that two H^+ are transported out for every Ca^{2+} transported in. Data in (A)–(C) are given as the mean ± SD of three experiments performed in duplicate. All concentrations given are molar. Where no error bars are shown, the error was smaller than the symbol size.

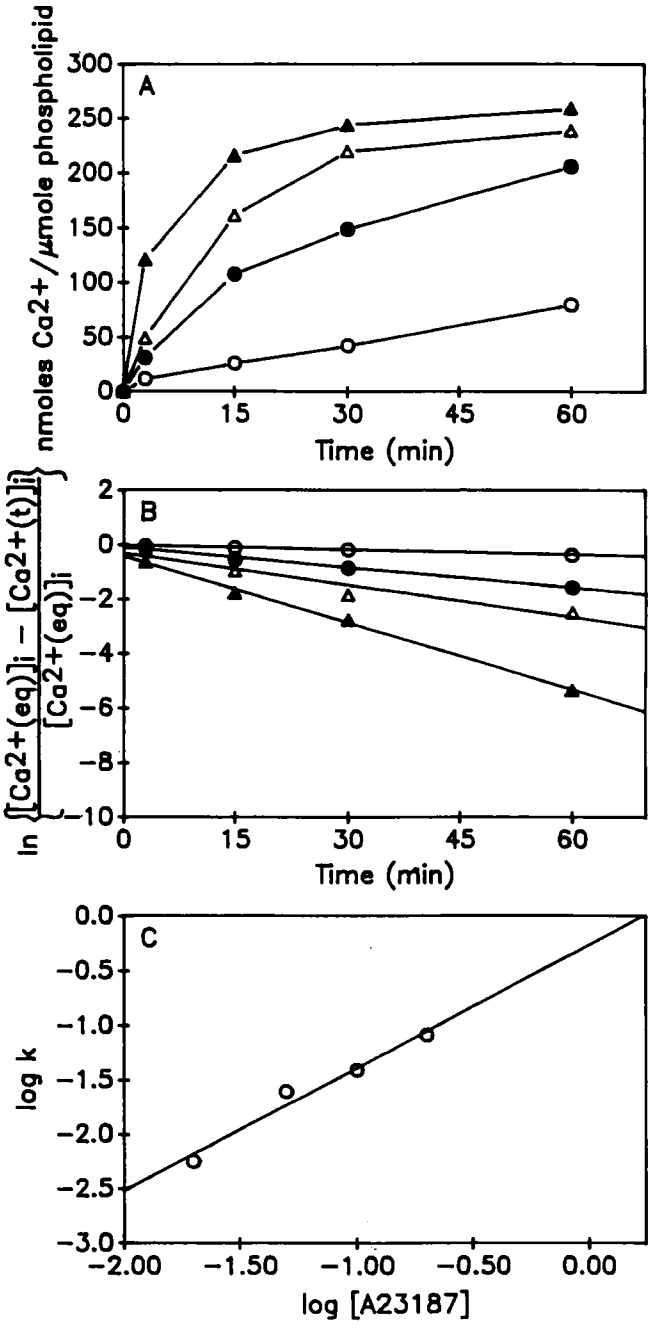

Figure 3. Effect of A23187 concentration on Ca^{2+} uptake into DOPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM HEPES (pH 7·4) in the external medium and 300 mM citrate (pH 4·0) in the internal medium) in the presence of 0·5 mM Ca^{2+} (A). A23187 concentrations used were 0·02 (○), 0·05 (●), 0·1 (△) and 0·2 μg/ml (▲). Initial rates were taken from (A) by assuming a first-order rate with respect to Ca^{2+} (see Experimental procedures) (B) and the log rate constant versus log A23187 concentration was plotted (C). The slope of the line (= 1·1) was taken as the stoichiometry of A23187 to Ca^{2+}. The experiment was repeated three times.

the rate of Ca^{2+} accumulation was investigated (Figure 3A). As indicated under Experimental procedures, the slope of a plot of log (k) (where k is the rate constant) versus [A23187] provides a measure of the stoichiometry of the A23817–Ca^{2+} transport complex. For this and subsequent studies, standard uptake conditions where the initial $[Ca^{2+}]_0 = 0·5$ mM and phospholipid concentration = 2 mM,

were employed. As shown in Figure 3A, increasing the A23187 concentration from 0·02 to 0·2 μg/ml results in a marked increase in the uptake rate of $6{\cdot}63 \times 10^{-3}$/min to $8{\cdot}16 \times 10^{-2}$/min. A plot of the rate constants as determined from the data of Figure 3B against the A23187 concentration yields a slope of nearly 1 (Figure 3C).

Ionomycin-mediated uptake of Ca^{2+}

It is of interest to compare the ionophoretic properties of A23187 with other carboxylic Ca^{2+} ionophores, which may be expected to also allow loading in response to ΔpH. Previous reports have indicated that ionomycin exhibits a higher specificity for Ca^{2+} than does A23187 [8] . In this regard, in previous work [7] it was found that to achieve stable A23187-mediated uptake of Ca^{2+} into LUVs with an acidic interior required the use of the sucrose buffer employed above, rather than an external saline buffer. This was attributed to A23187-mediated Na^+ import (in exchange for H^+ ions) that would deplete the interior buffering capacity. The enhanced Ca^{2+} specificity of ionomycin may be expected to mitigate against such non-specific effects, and this is illustrated in Figure 4. LUVs composed of dioleoyl phosphatidylcholine (DOPC) exhibiting a pH gradient ($pH_i = 4{\cdot}0$, $pH_o = 7{\cdot}4$) and containing 140 mM NaCl in the exterior medium allow the ionomycin-mediated uptake of Ca^{2+} to reach >200 nmol/μmol lipid. This entrapment is stable with no measurable leakage over 3 h at 25°C. This behaviour is mirrored by that of the transmembrane pH gradient, which exhibits a stable value at equilibrium of approximately 1·4 pH units.

Corresponding ionomycin-mediated Ca^{2+} uptake experiments were conducted on LUVs experiencing no transmembrane pH gradient using an interior buffer of either 300 mM citrate at pH 7·4 or 150 mM NaCl, 20 mM HEPES at pH 7·4. This resulted in uptake levels of only 20 and 5 nmol Ca^{2+}/μmol phospholipid respectively, after 3 h incubation (Figure 4).

In order to determine the stoichiometry of the Ca^{2+}-ionomycin transport complex, the effect of the ionomycin concentration on the rate of Ca^{2+} uptake was determined. Initial rates were determined for various ionomycin concentrations over a 30-min time course (Figure 5A). The slope of 0·8 obtained from a plot of log ionomycin concentration versus the log of the rate associated with uptake (Figure 5B) indicates that ionomycin transports Ca^{2+} in a 1:1 complex.

X537A-mediated uptake of Ca^{2+}

The smallest of carboxylic antibiotic ionophores is lasolocid A (X537A). In the absence of Na^+ ions in the external medium, uptake levels >250 nmol Ca^{2+}/μmol phospholipid are achieved within 30 min (Figure 6), which is equivalent to effectively 100% entrapment. This is >10-fold the rate observed for lasolocid A-mediated Ca^{2+} transport into LUVs in the presence of 150 mM NaCl where the established transmembrane pH gradient was completely dissipated within 2 min. In contrast with the results obtained for ionomycin, significant Ca^{2+} loading was observed for LUVs

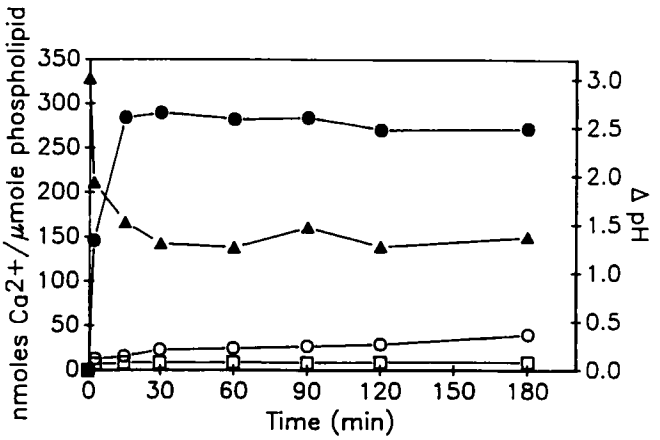

Figure 4. Accumulation of Ca^{2+} into EPC LUVs (100 nm) experiencing a transmembrane pH gradient and 0·5 mM external Ca^{2+} in the presence of 0·1 μg ionomycin/ml (●). EPC LUVs (2·0 mM phospholipid) were prepared in 300 mM citrate buffer (pH 4·0) and the untrapped (exterior) buffer exchanged for 150 mM NaCl and 20 mM HEPES (pH 7·4). Ca^{2+} uptake was quantitated as described in Experimental procedures. The magnitude of the transmembrane pH gradient (▲) was determined by measuring the transmembrane distribution of [^{14}C]methylamine according to Experimental procedures. Experiments conducted on LUVs experiencing no transmembrane pH gradient employed an interior buffer of either 300 mM citrate (pH 7·4) (○) or 150 mM NaCl, 20 mM HEPES (pH 7·4) (□).

experiencing no transmembrane pH gradient, but containing citrate in the vesicle interior (300 mM citrate, pH 7·4 inside). The extent of lasolocid A-mediated Ca^{2+} uptake was 60 nmol Ca^{2+}/μmol phospholipid at 2 h.

The effect of X537A concentration on the rate of Ca^{2+} uptake is illustrated in Figure 7. Initial rates were determined for ionomycin over a 30-min time course (Figure 7A). The slopes obtained from a plot of log ionophore concentration versus log rate (Figure 7B) indicated that X537A transports Ca^{2+} in a 1:2 (Ca^{2+}: ionophore) complex.

Discussion

In a previous study we have shown that the carboxylic ionophore A23187 could mediate rapid and efficient entrapment of Ca^{2+} into egg phosphatidylcholine (EPC) LUVs experiencing a pH gradient [7]. It was of interest to further characterize the transport properties of A23187 under these conditions as well as the carboxylic ionophores ionomycin and lasolocid A(X537A) with a view to understanding mechanisms of ionophore-mediated uptake and stoichiometry with respect to entrapped buffer.

The results of the present investigation show that, in contrast with A23187, ionomycin-mediated Ca^{2+} uptake is not significantly affected by NaCl in the external medium. This can be attributed to the fact that ionomycin is a more specific Ca^{2+} ionophore than is A23187 [9] and does not transport monovalent cations appreciably. In contrast, lasolocid A-mediated Ca^{2+} uptake is inhibited by NaCl (150 mM) giving rise to significantly lower uptake levels. This is attributed to the fact that lasolocid A is not a specific ionophore and is known to transfer a wide range of monovalent, divalent and

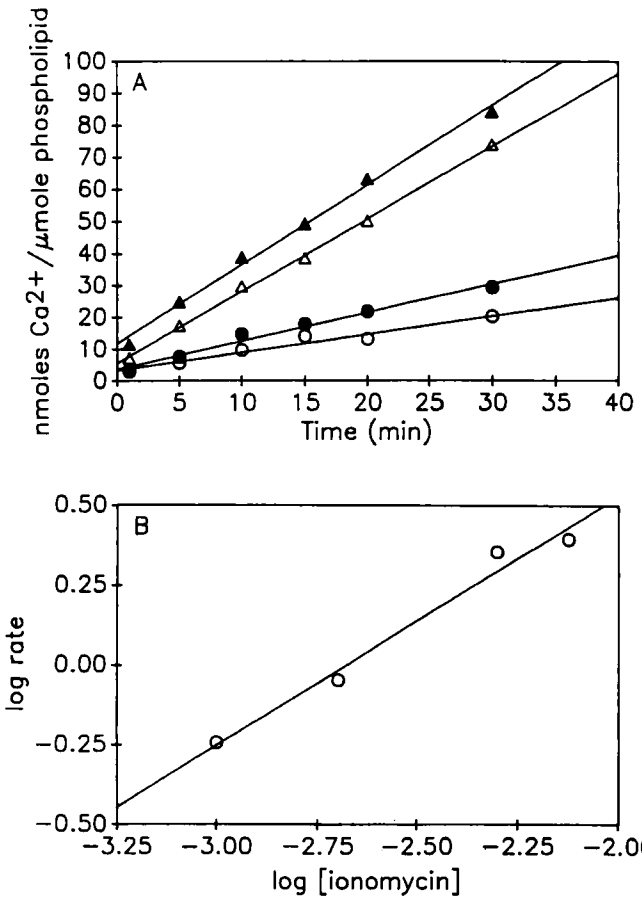

Figure 5. Effect of ionomycin concentration on Ca^{2+} uptake into DOPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM HEPES (pH 7·4) in the external medium and 300 mM citrate (pH 4·0) in the internal medium) in the presence of 0·5 mM Ca^{2+} (A). Ionomycin concentrations used were 0·001 (○), 0·002 (●), 0·005 (△), and 0·0075 μg/ml (▲). Initial rates were taken from (A) and the log rate versus log ionomycin concentration was plotted (B). The slope of the line in (B) (=0·8) was taken as the stoichiometry of ionomycin to Ca^{2+} as described in Experimental procedures. The experiment was repeated three times.

trivalent cations including Na^+ [10]. Transfer of Na^+ rapidly dissipates the pH gradient and thus inhibits pH-driven Ca^{2+} translocation. In addition, results presented have shown that lasolocid A binding to Ca^{2+} is weak enough that the chelating property of internal citrate is sufficient to drive Ca^{2+} uptake. This was not observed with A23187 or ionomycin.

In an effort to understand pH-driven, carrier-mediated Ca^{2+} transport better, an uptake model based on the buffering capacity of the internal contents and the external and internal pH was developed (see Experimental procedures). The observed agreement with this model indicates that Ca^{2+}/H^+ exchange is an electroneutral process in which one Ca^{2+} ion is transported in for every two H^+ ions transported out. In contrast, lasolocid A-mediated Ca^{2+} transport in the presence of 150 mM NaCl is not consistent with this model, presumably because Na^+ is also being transported in exchange for protons thus essentially 'uncoupling' the Ca^{2+}/H^+ exchange. In contrast,

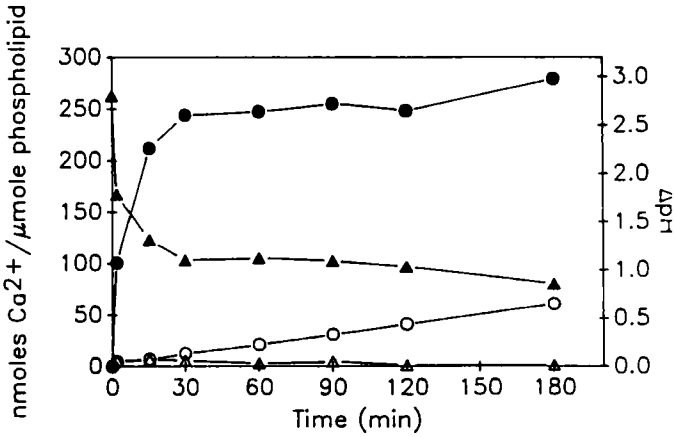

Figure 6. Accumulation of Ca^{2+} into EPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM HEPES (pH 7·4) or 150 mM NaCl, 20 mM HEPES (pH 7·4) in the external medium and 300 mM citrate (pH 4·0) in the internal medium) in the presence of 0·5 mM Ca^{2+} and 5 mg lasolocid A/ml. Ca^{2+} uptake with 300 mM sucrose, 20 mM HEPES (pH 7·4) in the external medium (●), and the residual pH gradient (▲); 150 mM NaCl, 20 mM HEPES (pH 7·4) in the external medium (○), and the corresponding residual pH gradient (△).

Ca^{2+} transport by ionomycin was well coupled in the presence of 150 mM NaCl consistent with a low ability to transport Na^+. Surprisingly, the model indicates that nearly all of the entrapped Ca^{2+} is in the free form. Although citrate is known to be a good chelator of Ca^{2+}, it appears that under the conditions studied very little is bound. The solubility of calcium citrate ($Ca_3(Cit)_24H_2O$) is only about 4 mM in H_2O. According to Davies and Hoyle [11] the calcium dissociation constants for citrate are $1·27x10^{-5}$, $8·9x10^{-4}$ and 0·07 M^{-1} for $CaCit^-$, CaHCit and CaH_2Cit^+ respectively. We have found that when 200 mM $CaCl_2$ is added to 300 mM citric acid at pH 4 that a precipitate is formed very slowly over a period of days or more quickly if the solution is heated to 60°C. The unique nature of the liposome interior environment may be an additional factor in the apparent lack of association of Ca^{2+} with citrate. However, when Ba^{2+} was transported into distearoyl phosphatidylcholine:cholesterol (55:45) LUVs via A23187 at 60°C an electron-dense precipitate was formed indiating that Ba^{2+} was complexing with citrate [12]. We were not able to detect such a precipitate in the Ca^{2+}-loaded vesicles described in this study.

It would seem apparent that citrate is binding Ca^{2+} somewhat since significant loading of Ca^{2+} occurred with lasolocid A in the absence of a pH gradient but with 300 mM citrate inside. This was attributed to the fact that lasolocid A has a relatively low affinity for Ca^{2+} as compared with A23187 or ionomycin allowing citrate to compete for the Ca^{2+} bound to the ionophore. It was not likely caused by an exchange of internal Na^+ for Ca^{2+} ions since the internal citrate was titrated with the free base, arginine, and not NaOH. A direct correlation was also observed between the strength of the internal chelator for Ca^{2+} and the rate and extent of ionomycin- and lasolocid A-mediated Ca^{2+} uptake in the order: EGTA>EDTA>citrate>HEPES.

It is generally thought that A23187 transports Ca^{2+} in 1:2 complex [9], i.e. that two molecules of A23187 are

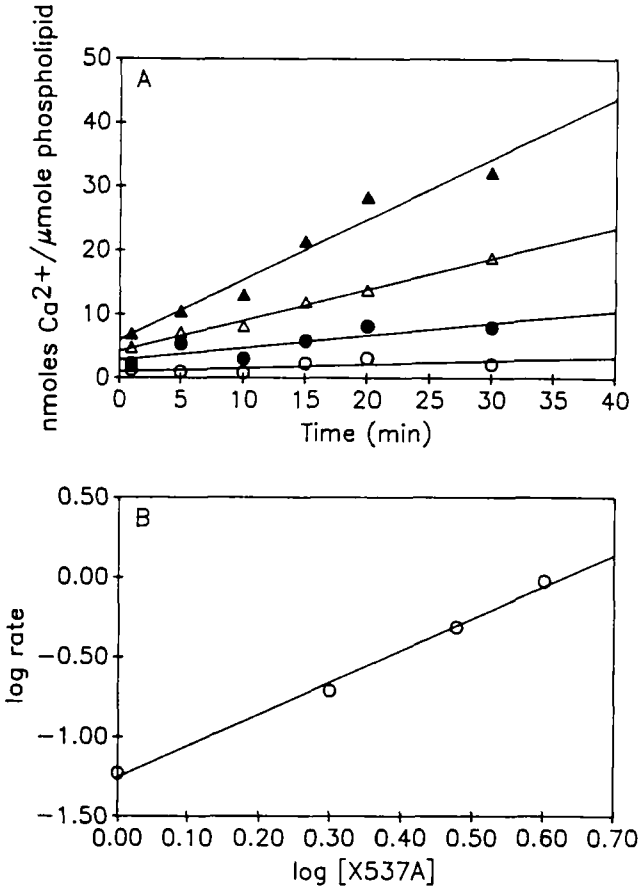

Figure 7. Effect of lasolocid A concentration on Ca^{2+} uptake into DOPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM HEPES (pH 7·4) in the external medium and 300 mM citrate (pH 4·0) in the internal medium) in the presence of 0·5 mM Ca^{2+} (A). Lasolocid A concentrations used were 1 (○), 2 (●), 3 (△) and 4 μg/ml (▲). Initial rates were taken from (A) and the log rate versus log lasolocid A concentration was plotted (B). The slope of the line in (B) (=2·0) was taken as the stoichiometry of lasolocid A to Ca^{2+} as described in Experimental procedures. The experiment was repeated three times.

required to transport Ca^{2+} ion. The transport stoichiometry we obtained for A23187 was approximately one A23187 molecule to one Ca^{2+} for two different batches of A23187. Previous workers have reported the existence of a 1:1 stoichiometry between A23187 and Ca^{2+} [13–16]. Since A23187 carries one negative charge at pH 7, the complex has a +1 charge and is not a likely candidate for transport across a lipid bilayer. It is not likely that the low A23187 concentrations used (38–382 mM) are the reason for the apparent stoichiometry observed since Blau *et al.* [9] showed a 1:2 (Ca^{2+}: ionophore) complex at ionophore concentrations >3 mM. It should be noted, however, that the methyl ester of A23187 showed a 1:1 transport stoichiometry for EPS LUVs [9]. Our findings for a 1:1 transport stoichiometry as measured by the kinetic analysis described is not without precedent. Wulf and Pohl [17] showed that an electroneutral transport of Ca^{2+} across Muller-Rudin membranes was obtained, but that the rate varied linearly with A23187 concentration implying a 1:1 stoichiometry, while optical absorption measurements indicated a 1:2 (Ca^{2+}; onophore) binding stoichiometry. Furthermore, Hyono *et al.* [18] found that, at low concentration, A23187 appears to transport Ca^{2+} in a 2:1 (Ca^{2+}: ionophore) complex.

The transport stoichiometry of ionomycin and lasolocid A was found to be one and two molecules of ionophore respectively to one molecule of Ca^{2+} which is in agreement with previous measurements [8, 9].

In summary, we have shown that Ca^{2+} uptake at equilibrium in a well-coupled system can be predicted from a simple model for a given internal buffer and pH. The results of this investigation suggest a transport stoichiometry of Ca^{2+}: ionophore of 1:1, 1:1 and 1:2 for A23187, ionomycin and X537A respectively. The utility of this approach lies in the many potential applications of cation loading. High concentrations of radionucleotides may be loaded into vesicles for use in radiation therapy or for determination of the biodistribution of vesicles in *in vivo* studies. Furthermore, in the case of cations such as Ba^{2+} which form precipitates after loading, vesicles can be rendered electron-dense and thus visible in the electron microscope [12].

Experimental procedures

Materials

Egg yolk phosphatidylcholine (EPC) and dioleoyl phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Pelham, AL, USA), and cholesterol was purchased from Sigma Chemical Company (St Louis, MO, USA). All lipids were used without further purification after verification of purity by thin-layer chromatography (TLC). The ionophores A23187, ionomycin and lasolocid A (X537A) were obtained from Sigma. [^{14}C]methylamine and $^{45}CaCl_2$ were purchased from New England Nuclear (Missasauga, Ontario, Canada), and all other reagents were analytical grade or equivalent.

Liposome production

Large unilamellar vesicles (LUVs) were produced employing the method of Hope *et al.* [19] that involves extrusion of frozen and thawed lipid dispersions through polycarbonate filters (Nuclepore, Pleasanton, CA, USA) with a 0·1 μm pore size, employing an extrusion device (Lipex Biomembranes, Vancouver, BC, Canada). Vesicles prepared in this manner exhibit trapped volumes of 1·5 μl/ μmol phospholipid and a mean diameter of 90 nm. Phospholipid concentrations were determined by analysis of lipid phosphorous as described previously [20]. Vesicle size was routinely determined by quasi-elastic light scattering using a Nicomp Model 200 Laser Particle Sizer [19].

Transmembrane pH gradients were generated by initially preparing LUVs in the presence of a low pH buffer with high buffering capacity (300 mM citrate, pH 4·0, unless indicated otherwise). Untrapped buffer was then removed by passing the LUVs through a Sephadex G-50 column pre-equilibrated with the external pH 7·4 buffer (300 mM sucrose, 20 mM HEPES, or 150 mM NaCl, 20 mM HEPES, unless indicated otherwise).

Uptake of Ca^{2+}

The experimental protocol involved adding a defined amount of ionophore (in a chloroform stock solution) to a test tube. The solvent was removed under a stream of nitrogen followed by incubation at low pressure. The appropriate extravesicular buffer system containing $CaCl_2$ (0·5 mM unless indicated otherwise) and a trace of ^{45}Ca (1·0 μCi/ml) was added to the test tube, and the dispersion was mixed thoroughly. To this was added the vesicle suspension (2·0 mM phospholipid) at a time that marked the start of the experimental time course.

Vesicle-entrapped Ca^{2+} was separated from extravesicular Ca^{2+} by gel filtration. Aliquots (100 μl) were withdrawn at appropriate times and extravesicular Ca^{2+} removed by pasage over 1 ml Sephadex

G-50 mini spin columns, pre-equilibrated and washed in the appropriate buffer as described previously [7]. The trapped probe was determined by liquid scintillation counting and the phospholipid concentration by phosphate assay [20].

Calculation of transmembrane pH gradients

The magnitude of the transmembrane pH gradients was determined by measuring the transmembrane distribution of the weak base [^{14}C]methylamine. Methylamine was added to the LUVs (typically 2·0 mM phospholipid) to achieve a concentration of 1 μM containing 1 μCi/ml of [^{14}C]methylamine. At appropriate time intervals aliquots (100 μl) were withdrawn, and untrapped probe removed employing 1 ml Sephadex G-50 mini spin columns as described previously [19]. The trapped probe was determined by liquid scintillation counting, and the phospholipid concentration by phosphate assay [20]. Transmembrane pH gradients were calculated according to the relationship $pH = \log [MeAM]_i/[MeAM]_o$, where the subscripts i and o represent the inside and outside of the liposome respectively [21].

Kinetic analysis of Ca^{2+} uptake

The transport of Ca^{2+} by ionophores was analysed assuming that n molecules of ionophore (I) complex with one Ca^{2+} ion. The rate of influx of Ca^{2+} ions per unit membrane area (A_m) can then be written as

$$\frac{dN(Ca^{2+})}{dt} = -KA_m([I_nCa^{2+}]_o - [I_nCa^{2+}]_i), \quad (1)$$

where K is the rate constant for transport of the complex and the subscripts o and i indicate the outer and inner monolayer respectively. We assume that $[I_nCa^{2+}]_o \gg [I_nCa^{2+}]_i$, which can be maintained by chelating internalized Ca^{2+} (by trapping Ca^{2+} chelators inside) or by maintaining an acidic interior (see Results). Writing the dissociation constant for Ca^{2+} and ionophore as $K_{Ca} = [I]^n[Ca^{2+}]/[I_nCa^{2+}]$ we then obtain

$$\frac{d[Ca^{2+}]_o}{dt} = \frac{-KA_m[I]^n[Ca^{2+}]_o}{K_{Ca}V_o}, \quad (2)$$

where V_o is the outside volume. Employing the relation $d[Ca^{2+}]_o/dt = -(V_i/V_o)d[Ca^{2+}]_i/dt$ where V_i is the interior (trapped) volume, it is then straightforward to show that

$$[Ca^{2+}(t)]_i = [Ca^{2+}(eq)]_i(1 - e^{-kt}), \quad (3)$$

where $k = (A_m/V_o)(K[I]/K_{Ca})[I]^n$ and $[Ca^{2+}(eq)]_i$ is the interior Ca^{2+} concentration at equilibrium. Thus a plot of $\ln(([Ca^{2+}(eq)]_i - [Ca^{2+}(t)]_i/[Ca^{2+}(eq)]_i$ versus t should yield a straight line with a slope $-k$. The rate constants k were determined by applying a nonlinear, least-squares analysis to the data using a commercially available plotting program (Sigmaplot, Jandel Scientific, 1986). The best fits to the data were obtained by employing k and $[Ca^{2+}(eq)]_i$ as variables. Since $k = (A_m/V_o)(K[I]/K_{Ca})[I]^n$, a plot of log k versus log [I]should yield a straight line with a slope of n, allowing the ionophore-cation stoichiometry to be determined.

Equilibrium analysis of Ca^{2+} uptake

Assuming that each Ca^{2+} ion accumulated results in the A23187-mediated release of two H^+ ions, the uptake of Ca^{2+} into LUVs with an acidic interior will result in an increase in the internal pH. For the citrate buffer employed in the present study, which exhibits three pK's ($pK_1 = 3·06$, $pK_2 = 4·74$, $pK_3 = 5·4$), the effective concentration of H^+ ions released, $[H^+]^{eff}_{rel}$, for a change in internal H^+ ion concentration from the initial concentration, $[H^+]^a$, to the final concentration, $[H^+]^b$, can be written by combining equations 11-15 from Harrigan *et al.* [22] as:

$$[H^+]^{eff}_{rel} = [B]^{tot}\left(K_1\frac{1}{[H^+]^b f([H^+]^b)} - \frac{1}{[H^+]^a f([H^+]^a)}\right) + K_1K_2\left(\frac{1}{([H^+]^b)^2 f([H^+]^b)} - \frac{1}{([H^+]^a)^2 f([H^+]^a)}\right) + K_1K_2K_3\left(\frac{1}{([H^+]^b)^3 f([H^+]^b)} - \frac{1}{([H^+]^a)^3 f([H^+]^a)}\right) \quad (4)$$

where $f([H^+]) = 1 + K_1/[H^+] + K_1K_2/[H^+]^2 + K_1K_2K_3/[H^+]^3$ and $[B]^{tot}$ is the total (internal) citrate concentration. The internal pH at equilibrium can be determined employing methylamine as indicated above. Thus, the internal Ca^{2+} concentration at equilibrium is given by

$$[Ca^{2+}]_i^{eq} = \frac{[H^+]^{eff}_{rel}}{2} \quad (5)$$

Under the assumption that only the neutral form of the ionophore-proton or ionophore-Ca^{2+} complex is membrane permeable, at equilibrium it would be expected that

$$\frac{[Ca^{2+}]_i^{eq}}{[Ca^{2+}]_o^{eq}} = \frac{([H^+]_i^{eq})^2}{([H^+]_o^{eq})^2}, \quad (6)$$

Solving equations 5 and 6 simultaneously yields $[Ca^{2+}]_i^{eq}$ and $[H^+]_i^{eq}$ for any given internal buffer concentration $[B]^{tot}$.

Acknowledgement

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