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A novel method for the efficient entrapment of calcium in large unilamellar phospholipid vesicles

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A technique for the efficient entrapment of high concentrations of Ca^{2+} in large unilamellar phospholipid vesicles (LUVs), using the carboxylic acid antibiotic ionophore A23187 (calcimycin) is demonstrated. It is shown that rapid A23187-mediated entrapment of Ca^{2+} , corresponding to essentially 100% sequestration of the extravesicular cation may be achieved for egg yolk phosphatidylcholine LUVs (100 nm) in the presence of a transmembrane proton gradient (acidic interior). Interior-exterior concentration cation gradients of over 400-fold may be readily achieved, with interior Ca^{2+} concentrations in excess of 250 mM. It is shown that the extent and efficiency of the A23187-mediated uptake process is affected by the intravesicular buffering capacity and the extravesicular Ca^{2+} concentration in a manner that is consistent with a Ca^{2+} - H^+ exchange process. In the absence of a pH gradient, or the presence of a reversed gradient (basic interior), only background levels of cation uptake are detected. The driving force for A23187-mediated uptake of Ca^{2+} is shown to depend on the intravesicular proton pool rather than on a chelation process. This protocol provides a novel method for the efficient entrapment of high concentrations of Ca^{2+} and other cations in phospholipid vesicles.

Introduction

We have previously demonstrated that transmembrane electrochemical gradients ($\Delta\psi$ and ΔpH) can result in the rapid and efficient accumulation of a variety of lipophilic cations into the intravesicular space of large unilamellar phospholipid vesicles (LUVs) [1–3]. This can result in extremely high interior concentrations of certain lipophilic cationic drugs (for example, doxorubicin), in combination with trapping efficiencies approaching 100%. In addition it has been shown that asymmetric transbilayer distributions of simple acids that exhibit weak acid or base characteristics, as well as certain acidic phospholipids, can be induced in LUVs experiencing a transmembrane pH gradient [4,5].

In this regard the lipophilic ionophore antibiotic

A23187 (calcimycin) is capable of divalent cation transport across biological and model membranes, with an overall electroneutral transport arising from a M^{2+} for 2H^+ exchange [6–9]. In view of the previous findings, it was considered that Ca^{2+} transport mediated by the carboxylic ionophore should respond to a transmembrane pH gradient. In particular for LUVs with an acidic interior, the presence of A23187 should allow the exchange of interior H^+ ions for exterior Ca^{2+} ions, resulting in net Ca^{2+} accumulation.

In this report we show that A23187-mediated Ca^{2+} accumulation into LUVs can be driven by transmembrane pH gradients, resulting in trapping efficiencies of up to 100% and interior Ca^{2+} concentrations which are over two orders of magnitude greater than initial extravesicular Ca^{2+} concentrations. The results are consistent with a Ca^{2+} - 2H^+ exchange process.

Materials and Methods

Egg yolk phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Pelham, AL), and used without further purification after verification of purity by thin-layer chromatography. The ionophore A23187

Abbreviations: LUV, large unilamellar vesicle; EPC, egg phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EDTA, ethylenediaminetetraacetic acid.

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was obtained from Calbiochem (Calgary) and [^{14}C]methylamine and $^{45}\text{CaCl}_2$ were purchased from New England Nuclear. All other reagents were analytical grade or equivalent.

Large unilamellar vesicles were produced by extrusion [10] of frozen and thawed lipid dispersions through $0.1\ \mu\text{m}$ polycarbonate filters (Nuclepore), employing an extrusion device (Lipex Biomembranes, Vancouver). Vesicles prepared in this manner had trapped volumes of $1.5\ \mu\text{l}/\mu\text{mol}$ phospholipid, employing [^{14}C]inulin as an aqueous marker, and an average diameter of 90 nm as measured by quasi elastic light scattering and freeze-fracture electron microscopy [10]. Phospholipid concentrations were determined by analysis of lipid phosphorous as previously described [11]. Vesicle size was routinely determined by quasi elastic light scattering using a Nicomp Model 200 Laser Particle Sizer [12].

Transmembrane pH gradients were generated by initially preparing EPC LUVs in the presence of a low pH buffer (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 neutral pH buffer (300 mM sucrose, 20 mM Hepes (pH 7.4) or 150 mM NaCl, 20 mM Hepes (pH 7.4) unless indicated otherwise).

The experimental protocol involved adding a defined amount of A23187 (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 amount of ^{45}Ca ($1.0\ \mu\text{Ci}/\text{ml}$) 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 which marked the start of the experimental time course. All experiments were conducted at 25°C unless indicated otherwise.

Vesicle entrapped Ca^{2+} was separated from extravesicular Ca^{2+} by gel filtration. Aliquots ($100\ \mu\text{l}$) were withdrawn at appropriate times and extravesicular Ca^{2+} removed by passage over 1 ml Sephadex G-50 mini spin columns, pre-equilibrated and washed in the appropriate buffer as previously described [10]. Within 20 s, the spin columns were eluted by centrifugation at $2500 \times g$ for three minutes. The LUVs and associated Ca^{2+} were subsequently analyzed for ^{45}Ca by liquid scintillation counting employing a Packard 2000 CA liquid scintillation counter, and for lipid phosphorus [11]. All data shown are representative of multiple experiments ($n > 3$). All experiments were performed above the gel to liquid crystalline transition temperature of the lipid.

The magnitude of the transmembrane pH gradient was determined by measurement of the equilibrium 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 $1\ \mu\text{M}$ containing $1\ \mu\text{Ci}/\text{ml}$ of [^{14}C]methylamine. At appropriate time intervals aliquots ($100\ \mu\text{l}$) were withdrawn, and untrapped probe removed employing 1 ml Sephadex G-50 mini spin columns as previously described [10]. The trapped probe was determined by liquid scintillation counting, and the phospholipid concentration by phosphate assay [11]. Transmembrane pH gradients were calculated according to the relationship $\Delta\text{pH} = \log[\text{MeAM}]_i/\text{MeAM}]_o$ [13].

Results

Initial transport experiments were designed with the aim of establishing whether a transmembrane pH gradient could drive A23187-mediated uptake of Ca^{2+} . As shown in Fig. 1, the LUVs experiencing a transmembrane pH gradient (pH 7.4 outside, pH 4.0 inside), exhibit a remarkable ability to accumulate Ca^{2+} in the presence of A23187 ($50\ \mu\text{g}/\text{ml}$). Within two minutes Ca^{2+} is loaded to interior levels of $220\ \text{nmol}\ \text{Ca}^{2+}/\mu\text{mol}$ phospholipid, or 148 mM (given a trapped volume of $1.5\ \mu\text{l}/\mu\text{mol}$ phospholipid [10]), compared to an initial extravesicular Ca^{2+} concentration of 0.5 mM. This corresponds to over 99% entrapment of extravesicular Ca^{2+} . Corresponding experiments conducted on vesicle systems experiencing no pH gradients, pH 4.0 inside and outside (see Fig. 1), or pH 7.4 inside and outside (see below), showed only background levels of Ca^{2+} uptake, typically less than $5\ \text{nmol}\ \text{Ca}^{2+}/\mu\text{mol}$ phospholipid. Experiments conducted on vesicular systems experiencing a reversed pH gradient (basic interior) again resulted in only background Ca^{2+} uptake ($< 2\ \text{nmol}\ \text{Ca}^{2+}/\mu\text{mol}$ phospholipid, results not shown). The data of Fig. 1 also shows that after the initial rapid accumulation of Ca^{2+} , a time-dependent leakage of the entrapped cation occurred. As much as 60% of the loaded Ca^{2+} was released from the LUVs within two hours. In this regard, the specificity of A23187 for Na^+ is much less than that of Ca^{2+} [14]. However, the relatively high extravesicular Na^+ concentration (150 mM) with respect to Ca^{2+} (0.5 mM) initially present in the extravesicular space could conceivably result in significant A23187-mediated Na^+ uptake. This would serve to contribute to dissipation of the internal proton pool via $\text{Na}^+\text{-H}^+$ exchange. Two potential methods of reducing this dissipation involve decreasing the concentration of the A23187, or removing the extravesicular Na^+ .

Fig. 1 shows the effect of decreasing the ionophore concentration to $0.1\ \mu\text{g}/\text{ml}$ on the rate of Ca^{2+} loading into the LUVs. The results indicate that the rate of uptake was substantially slower at lower ionophore concentrations. However, greater than 99% entrapment was still achievable, but over a longer period of time. No leakage at the lower ionophore concentration was

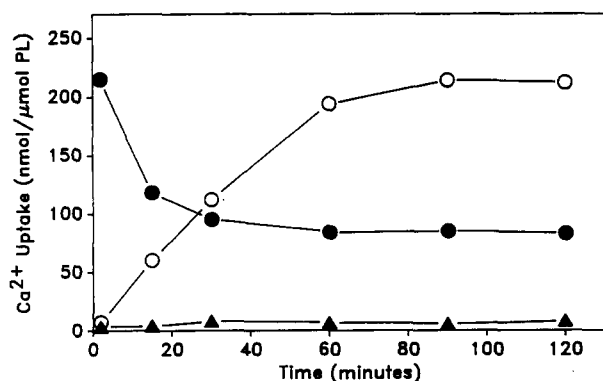


Fig. 1. Time-course of the accumulation of Ca^{2+} into EPC LUVs (100 nm) experiencing a transmembrane pH gradient and 0.5 mM external Ca^{2+} in the presence of 50 $\mu\text{g}/\text{ml}$ (●) and 0.1 $\mu\text{g}/\text{ml}$ (○) A23187. EPC LUVs (2.0 mM phospholipid) were prepared in 300 mM citrate buffer (pH 4.0) and the untrapped (exterior) buffer exchanged for 150 mM Na_2SO_4 and 20 mM Hepes (pH 7.4). Ca^{2+} uptake was quantitated as described in Materials and Methods. The solid triangle indicates ionophore mediated cation uptake in the absence of a transmembrane pH gradient (pH 4.0 in and out), as described in Materials and Methods.

observed over a period of several hours, in contrast to the situation at high A23187 levels.

Data supporting a direct role of extravesicular Na^+ in dissipation of the transmembrane pH gradient is shown in Fig. 2. A Na^+ -free vesicle system was used, in which the external Na_2SO_4 was replaced with sucrose (300 mM) and the internal and external pH was adjusted with arginine (free base). As shown in Fig. 2 the Na^+ -free LUVs exhibited no significant Ca^{2+} leakage after the initial uptake even at high A23187 levels. These results are consistent with an ability of the ionophore to transport Na^+ as well as Ca^{2+} , which contributes to the dissipation of the transmembrane pH gradi-

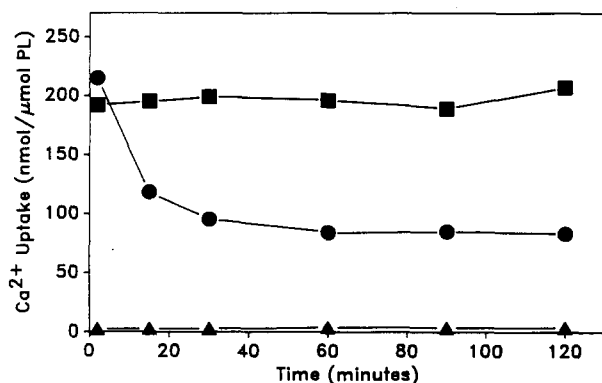


Fig. 2. Time-dependent A23187-mediated uptake of Ca^{2+} into EPC LUVs (100 nm) experiencing a transmembrane pH gradient (exterior pH 7.4, interior pH 4.0), in the presence of 50 $\mu\text{g}/\text{ml}$ A23187 and 0.5 mM external Ca^{2+} , for LUVs (2.0 mM phospholipid) containing Na^+ in the external medium (150 mM Na_2SO_4 , 20 mM Hepes) (●) and LUVs that contain no Na^+ in the external medium (300 mM sucrose, 20 mM Hepes) (○) (see Materials and Methods for details). The solid triangle indicates Ca^{2+} uptake in the absence of a transmembrane pH gradient (pH 4.0 in and out) for LUVs in the absence of Na^+ .

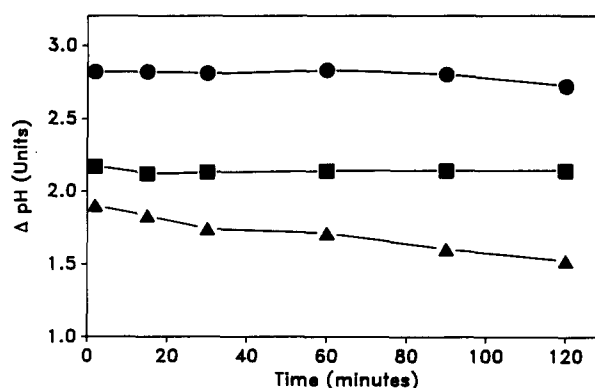


Fig. 3. Magnitude of the transmembrane pH gradient as measured by [^{14}C]methylamine (see Materials and Methods), for EPC LUVs (100 nm) at a final phospholipid concentration of 2.0 mM, in the absence of ionophore (●); EPC LUVs in the presence of A23187 (1.0 $\mu\text{g}/\text{ml}$) and 0.5 mM external Ca^{2+} for LUVs containing Na^+ in the external medium (150 mM Na_2SO_4 , 20 mM Hepes) (▲), and LUVs containing no Na^+ in the external medium (300 mM sucrose, 20 mM Hepes) (■).

ent. The Na^+ -free LUVs in the absence of a transmembrane pH gradient, or in the presence of a reverse pH gradient (basic interior) indicated no significant Ca^{2+} uptake (see Fig. 2).

In order to confirm that exterior Na^+ resulted in the dissipation of the transmembrane pH gradient, the pH probe [^{14}C]methylamine was used to measure the residual pH gradient across the Na^+ -free and Na^+ -containing LUVs after Ca^{2+} uptake (see Materials and Methods). At high ionophore concentrations (50 $\mu\text{g}/\text{ml}$) after two hours no residual transmembrane pH gradient was detectable across the LUVs with exterior Na^+ , whereas a pH gradient of approximately 2.0 units was measured across the Na^+ free vesicle system. The relationship between the magnitude of the residual transmembrane pH gradient, and A23187-mediated Ca^{2+} uptake was also investigated at low ionophore concentrations (1.0 $\mu\text{g}/\text{ml}$). As indicated in Fig. 3 for EPC LUVs in the absence of ionophore (Na^+ -containing LUVs), a stable transmembrane pH gradient of 2.8 units was observed. In the absence of ionophore similar values were also obtained using the Na^+ -free LUVs (results not shown). In the presence of ionophore (1.0 $\mu\text{g}/\text{ml}$) the magnitude of the pH gradient for the Na^+ free LUVs was 2.2 units, and remained effectively constant over two hours. The initially decrease in pH is due to the rapid ionophore mediated $\text{Ca}^{2+}\text{-H}^+$ exchange across the bilayer, where 100% entrapment occurs within two minutes. In the case of the Na^+ -rich LUVs, however, the magnitude of the pH gradient was determined to be 1.9 after two minutes, and further decreased as a function of time. The latter observation is likely due to $\text{Na}^+\text{-H}^+$ ionophore mediated exchange.

The effects of the initial extravesicular Ca^{2+} concentration on A23187-mediated cation uptake and the corresponding trapping efficiencies are depicted in Fig.

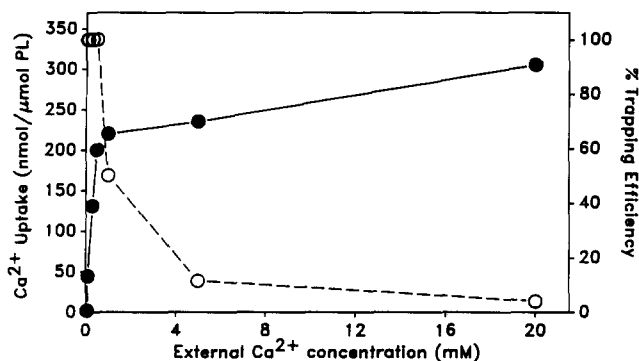


Fig. 4. The effect of initial extravesicular Ca^{2+} concentration on A23187 (10 $\mu\text{g}/\text{ml}$) mediated Ca^{2+} uptake (●) and percentage trapping efficiency (○), into EPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM Hepes (pH 7.4) in the external medium and 300 mM citrate (pH 4.0) in the internal medium). Measurements were made at two hours, and the phospholipid concentration was 2.0 mM.

4. All data reported were collected after two hours. At low initial extravesicular Ca^{2+} concentrations (0.05–0.5 mM), greater than 99% trapping efficiency was observed at the phospholipid concentration used (2.0 mM). This indicated essentially a complete sequestration of the external Ca^{2+} pool. This corresponded to entrapped Ca^{2+} levels of between 2.3 and 200 nmol per μmol phospholipid. Under these experimental conditions a residual transmembrane pH gradient was detected at the completion of the time course (two hours) (results not shown). At higher extravesicular Ca^{2+} concentrations (0.5–20 mM), however, the trapping efficiency decreased significantly, and cation uptake levels plateau at about 350 nmol $\text{Ca}^{2+}/\mu\text{mol}$ phospholipid. No residual transmembrane pH gradient was measured under these experimental conditions, which limits further cation uptake. Deleers and Malaisse observed similar enhancement in ^{45}Ca exchange diffusion across multilamellar liposomal membranes experiencing no pH gradient at increasing concentrations of external calcium [15].

The pH-dependent uptake of Ca^{2+} mediated by A23187 was further studied to determine the influence of internal buffering capacity. The effects of changing the intravesicular buffering capacity from 10 mM to 500 mM citrate at a constant extravesicular Ca^{2+} concentration (0.5 mM), on Ca^{2+} uptake and the trapping efficiency are shown in Fig. 5. All data reported were collected after two hours. The sucrose concentration in the extravesicular buffer was also adjusted to be isotonic with respect to the vesicle interior. The data of Fig. 5 indicated that a low citrate concentrations (< 300 mM), the buffering capacity limited A23187-mediated cation uptake, and trapping efficiencies were well below 100%. Furthermore, no residual transmembrane pH gradient was measured at these low citrate concentrations at the conclusion of the experimental time course.

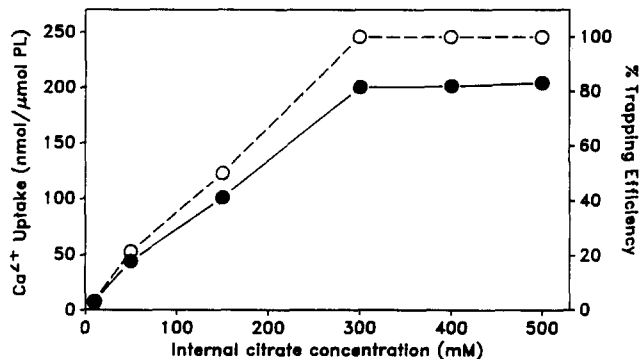


Fig. 5. The effect of internal citrate concentration on Ca^{2+} uptake (●) and percentage trapping efficiency (○) into EPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose, 20 mM Hepes (pH 7.4) in the external medium and 300 mM citrate (pH 4.0) in the internal medium), in the presence of A23187 (10 $\mu\text{g}/\text{ml}$) and 0.5 mM external Ca^{2+} . Measurements were made at two hours, and the phospholipid concentration was 2.0 mM.

At higher internal citrate concentrations, however, the available extravesicular Ca^{2+} limited uptake, and greater than 99% trapping efficiencies were achieved. A residual transmembrane pH gradient of approximately two units was still present at the higher citrate concentrations (> 300 mM), after maximal uptake levels had been attained (results not shown). Furthermore, under conditions where external calcium was in excess (> 2.0 mM), maximum A23187-mediated Ca^{2+} uptake was proportional to the intravesicular buffering capacity (results not shown).

The effect of temperature on A23187-mediated Ca^{2+} uptake in the presence of a transmembrane pH gradient is shown in Fig. 6. The ionophore concentration was decreased to 0.05 $\mu\text{g}/\text{ml}$, in order to follow the kinetics. The results show a strong temperature dependence. Despite the relatively low ionophore concentration used (0.05 $\mu\text{g}/\text{ml}$) maximal cation entrapment was achieved

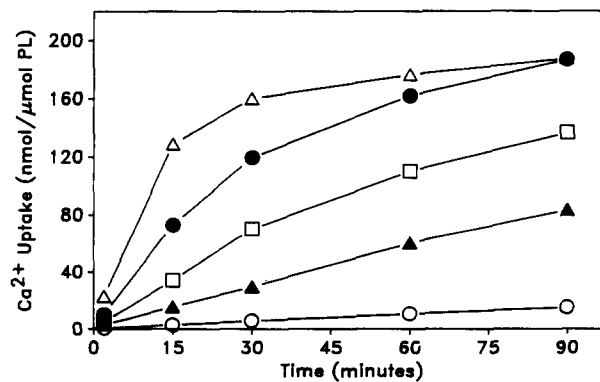


Fig. 6. The effect of temperature on Ca^{2+} accumulation into EPC LUVs (100 nm) experiencing a transmembrane pH gradient (300 mM sucrose (pH 7.4) in the external medium and 300 mM citrate (pH 4.0) in the internal medium), and in the presence of A23187 (0.05 $\mu\text{g}/\text{ml}$) and Ca^{2+} (0.5 mM), at 15°C (○); 26°C (▲); 36°C (□); 45°C (●); and 60°C (△).

within 90 min at 60°C. The temperature dependence of the initial rate for transport reflects an Arrhenius activation energy of 12.8 kcal/mol. Thus, even with a significant reduction in the amount of ionophore, which could be of importance in *in vivo* applications, high cation uptake levels may still be rapidly achieved at elevated temperatures.

At the lower temperatures used (< 36°C) 100% entrapment was not achieved within the two hour time course. In this regard, it should be noted that under these experimental conditions there are only four A23187 molecules per vesicle. In order to attain high internal Ca^{2+} levels (approx. 150 mM) observed in the above experiments at 45 and 60°C, each ionophore must translocate a large number of Ca^{2+} ions. For example, at an ionophore concentration of 0.05 $\mu\text{g/ml}$, an initial Ca^{2+} concentration of 0.5 mM and 2.0 mM LUV phospholipid, it may be readily calculated that each ionophore molecule has to undergo approximately 12500 translocating events in order to achieve 100% entrapment.

Mauk and Gamble (1979) [16] have previously demonstrated that (in the absence of a transmembrane pH gradient) in the presence of a suitable chelator (for example nitrilotriacetic acid or ethylenediaminetetraacetic acid (EDTA)) in the intravesicular space, an increase in the A23187-mediated uptake of cations can be observed. The chelator provides a strong driving force for mediated cation uptake. In order to distinguish between entrapment by chelation and cation uptake due to membrane proton gradients detailed above, experiments were conducted in the absence of a pH gradient (pH 7.4 in and out) and the presence of 300 mM citrate (pH 7.4) or 300 mM EDTA (pH 7.4) in the vesicle interior. In each case the extravesicular buffer was 300 mM sucrose, 20 mM Hepes (pH 7.4). The data of Fig. 7 illustrate that citrate, unlike EDTA, lacks the ability to act as a driving force for the A23187-mediated

loading of Ca^{2+} into LUVs. This suggests that citrate is not a sufficiently strong chelating agent to drive A23187-mediated cation uptake.

As a final point it is important to note that throughout all the above experiments no change in light scattering, (measured at 450 nm), or increase in vesicle size (as detected by quasi elastic light scattering) was detected for the vesicle suspensions used. This indicates that no significant vesicle-vesicle fusion or aggregation was occurring during the time course of the experiments. Furthermore, no lipid degradation was apparent as detected by thin-layer chromatography [17]. This eliminates the possibility of fatty acid or lysophosphatidylcholine break down products acting as an additional source of ionophoretic activity.

Discussion

The results of this investigation clearly demonstrate that carboxylic ionophoretic molecules such as A23187 together with LUVs experiencing a transmembrane pH gradient (acidic interior) may be used to achieve a rapid and efficient entrapment of Ca^{2+} . Uptake levels corresponding to essentially complete sequestration of the extravesicular cation were readily attainable. Under the conditions employed here, this can result in intravesicular Ca^{2+} concentrations in excess of over 250 mM, equivalent to an inside-outside concentration gradient of more than 400-fold. Furthermore, the protocol may be easily optimized to achieve even higher levels of cation encapsulation.

A23187 facilitates diffusion of cations across the lipid bilayer by a carrier-mediated mechanism. The A23187- Ca^{2+} complex has been closely characterized, and it is generally accepted that Ca^{2+} crosses the membrane complexed with two molecules of A23187 [6–9]. The carboxylic group of the ionophore participates in the electrically neutral exchange of cations for protons. At pH values above the pK_a of the ionophore ($\text{pK}_a \approx 6.7$) [18], the protons dissociate from the carboxyl group, and formation of the cation carboxylate is favoured. At pH values below the pK_a , the carboxylate will reprotonate, resulting in the release of the cation. This is supported by the observations of Liu and Herman (1978) [19] who showed that essentially no complexing of Ca^{2+} by A23187 occurs below pH 4.0, whereas binding reaches a maximum at approximately pH 7.4 [20].

The mechanism of A23187-mediated Ca^{2+} accumulation in response to a transmembrane pH gradient is directly related to the mode of action of the ionophore. LUVs experiencing a transmembrane pH gradient (acidic interior), with the extravesicular aqueous phase at a pH of 7.4, favours formation of a 2:1 electroneutral complex of the anionic form of the ionophore with the cation. The acidic vesicle interior which possesses a

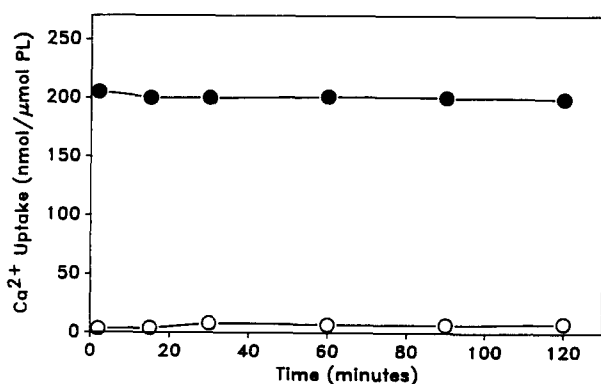


Fig. 7. The effect of internal buffer on A23187 (10 $\mu\text{g/ml}$) mediated accumulation of Ca^{2+} in EPC LUVs (100 nm) in the absence of transmembrane pH gradient. 300 mM citrate (pH 7.4) (○), and 300 mM ethylenediaminetetraacetic acid (pH 7.4) (●). In both cases the external buffer was 300 mM sucrose, 20 mM Hepes (pH 7.4) and the phospholipid concentration 2.0 mM.

high concentration of protons, facilitates protonation of the ionophore and cation release. Such a mechanism of action is consistent with the experimental results of this study, whereby increasing the initial buffering capacity, or the magnitude of the transmembrane pH gradient, results in an increase in the level of ionophore-mediated cation uptake. In each case the internal proton pool is the important variable. In addition the results clearly demonstrate that the predominant driving force for enhanced A23187-mediated cation entrapment is the presence of the transmembrane pH gradient. Chelation is not a major factor in the above experiments. This is because citrate is not a sufficiently strong chelator (see Fig. 7). It is likely, however, that the limited solubility of calcium citrate leads to formation of a precipitate in the LUV interior.

It is of interest to compare the transmembrane pH gradient procedure for loading Ca^{2+} into LUVs with other procedures. First passive entrapment during liposome formation results in low trapping efficiencies and the maximum interior concentrations is often limited by solubility constraints [21]. The limitations of this technique originates in part from the internal aqueous volume of the liposomes, which typically represents only a small fraction of the total suspension volume.

High encapsulated cation levels can be achieved by entrapping appropriate chelating agents (for example ethylenediaminetetraacetic acid, 8-hydroxyquinoline and nitrilotriacetic acid) in the intravesicular space [16,22–25]. The maximum amount of entrapped cation, however, is limited by the interior concentration of the chelating agent that can be achieved, and also by the binding constant of the metal ion to the chelating agent.

It may be concluded that the pH driven cation loading is a convenient procedure. As A23187 is capable of transporting a wide range of cations [14,26,27], it is likely that the technique for cation loading described here, may be applicable to any cationic species that can be complexed to the ionophore [6,23,28,29]. These cations include the alkaline earth series, Fe^{2+} , La^{3+} and trivalent radionucleotides.

The ability to load high concentration of cation in the aqueous compartment of vesicles has many potential applications. For example, mCi amounts of radionucleotides such as $^{153}\text{Gd}^{3+}$ or $^{67}\text{Ga}^{3+}$ may potentially be loaded into the interior of a small concentration of vesicles resulting in a high specific activity. LUVs containing Gd^{3+} or very radioactive vesicles may be useful for contrast enhanced nuclear magnetic resonance imaging, and for determining the biodistribution of vesicles in in vivo studies [30,31]. Furthermore, the encapsulation of a variety of cations including Fe^{2+} and La^{3+} could allow the preparation of 'heavy' liposomes, and the creation of electron dense LUVs. These liposomes have potential in cell separation protocols and as cell markers. These and other applications are currently

under active investigation. Finally, it should be noted that all carboxylic cation ionophores contain a carboxylic acid functional group which deprotonates upon complexation. Therefore, it is likely that other carboxylic acid antibiotic ionophores such as ionomycin and A-204, will respond to transmembrane pH gradients in the same way as A23187.

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