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# PHOSPHATIDIC ACID AS A CALCIUM IONOPHORE IN LARGE UNILAMELLAR VESICLE SYSTEMS

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**The ionophoretic capabilities of dioleoylphosphatidic acid (DOPA) for transporting calcium across phospholipid bilayers have been investigated. Calcium uptake by large unilamellar vesicles is shown to depend on the presence of DOPA. This uptake is sensitive to the nature and concentration of calcium chelators in the vesicle interior, indicating that accumulation results from DOPA-mediated translocation of calcium across the membrane. Further, it is shown that characteristics of DOPA-mediated $Ca^{2+}$ uptake are similar to those observed for the fungal calcium ionophore, A23187.**

Considerable controversy surrounds the possibility [1] that phosphatidic acid can act as a $Ca^{2+}$ ionophore in transmembrane signalling events. Initially, it was demonstrated that phosphatidic acid can facilitate $Ca^{2+}$ transport between two aqueous compartments separated by organic solvent [2] and across the bilayers of multilamellar liposomal systems [3]. In biological membrane systems it has been shown that an increase in phosphatidic acid content due either to addition of exogenous phosphatidic acid [4–6] or phospholipase D treatment [7] results in physiological effects associated with an influx of $Ca^{2+}$. However, a recent report [8] indicates that phosphatidic acid (derived from egg PC) is unable to facilitate $Ca^{2+}$ transport across bilayer phosphatidylcholine (PC) systems. In this work we characterize the ability of dioleoylphosphatidic acid (DOPA) to act as a $Ca^{2+}$ ionophore in large unilamellar vesicle systems. We show that the presence of DOPA results in $Ca^{2+}$ uptake which is sensitive to the nature and concentrations of $Ca^{2+}$ chelators trapped inside the large unilamellar vesicle systems.

1,2-Dioleoyl-*sn*-glycero-3-phosphorylcholine (DOPC) was synthesized employing oleic acid as described elsewhere [9]. Dioleoylphosphatidic acid (DOPA) was prepared from DOPC employing standard procedures utilizing phospholipase D [10] and was purified by carboxymethyl cellulose chromatography [11]. No lysophosphatidic acid or other contaminants were detectable by two-dimensional thin-layer chromatography (2D-TLC) and the ionophoretic properties observed were insensitive to extended storage in $CHCl_3$ under $N_2$ (4 weeks). Very similar results were observed for four different preparations of DOPA. Phosphatidylserine (PS) was prepared from egg phosphatidylcholine by similar methods. The negatively charged phospholipids were converted to their sodium salt form. All phospholipids were greater than 99% pure as indicated by 2D-TLC and were stored under $N_2$.

Large unilamellar vesicles were prepared by repetitive extrusion of multilamellar vesicles through a polycarbonate (Nucleopore) filter (0.1 $\mu$m pore size) as described elsewhere [12]. These vesicles exhibited a size distribution of $700 \pm 100$ Å as detected by freeze-fracture procedures [12]. Experiments employing entrapped $^{22}Na$ indicated trapped volumes of $1.0 \pm 0.05$ $\mu$l/$\mu$mol phospholipid. The untrapped buffer was replaced with

the desired external buffer by passage of the vesicle preparation over a 1.5 × 15 cm Sephadex G-50 column equilibrated with the appropriate buffer. In all cases, internal and external media of equal osmolarity were used. No detectable leakage of entrapped $^{22}Na$ occurred under all conditions used over the experimental time-course (4 h).

Calcium uptake was monitored by incubating the vesicles (3–5 μmol phospholipid/ml) in a buffer containing 2 mM $CaCl_2$ and $^{45}Ca$ (1 μCi/μmol $Ca^{2+}$). Aliquots (100 μl) were removed over a 4-h time course and the external $^{45}Ca^{2+}$ was removed by passage over a 1 ml Sephadex G-50 column. The vesicles were subsequently analyzed for $^{45}Ca$ on a Philips PW-4700 liquid scintillation counter and for lipid phosphorus [13]. In some cases dual-label experiments were used to monitor $^{45}Ca$ and phospholipid simultaneously by employing trace amounts of [$^{3}H$]dipalmitoylphosphorylcholine (NEN, Canada) and a $^{3}H/^{45}Ca$ dual label channel.

Initial experiments were designed to determine whether DOPA-containing vesicles could accumulate exogenous calcium. Potassium phosphate (20 mM) was included in the vesicle interior to sequester $Ca^{2+}$ in the event of a membrane transport process. As shown in Fig. 1, the presence of DOPA does result in increased levels of vesicle associated $Ca^{2+}$ which is dependent on the amount of DOPA present. In particular, increasing the DOPA content from 5 to 20 percent increases the amount of vesicle associated $Ca^{2+}$ from 1.8 to 10.0 nmol $Ca^{2+}$/μmol phospholipid. The presence of 20 mol percent egg PS did not result in detectable $Ca^{2+}$ uptake (results not shown).

In order to determine whether this calcium uptake was due to binding to phosphatidic acid on the vesicle exterior, or reflected actual translocation of $Ca^{2+}$ across the membrane, various concentrations and types of calcium chelators or 'sinks' were entrapped in the vesicle interior. As shown in Fig. 2, calcium uptake increases as the amount of phosphate in the vesicle interior is increased. In addition, different trapping efficiencies of the calcium sinks employed are observed, with EGTA being more effective than oxalate or phosphate. The amount of $Ca^{2+}$ uptake observed employing EGTA indicates that > 90% of the entrapped EGTA is complexed to $Ca^{2+}$ at 4 h.

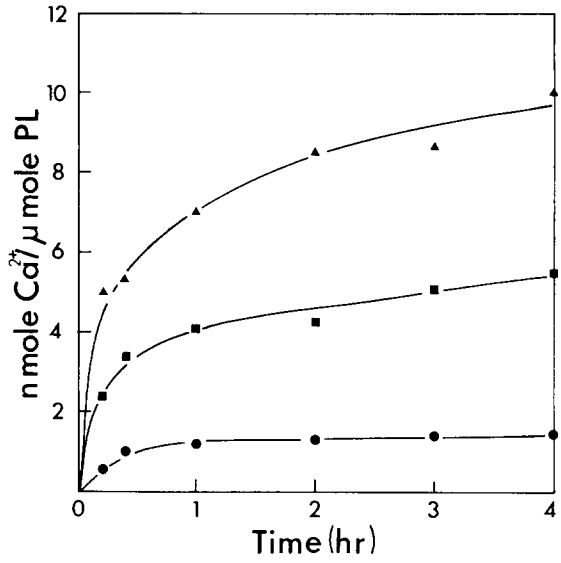


Fig. 1. Uptake of $Ca^{2+}$ into large unilamellar vesicle systems containing: (●) 5 mol% DOPA, 15 mol% egg PS and 80 mol% DOPC; (■) 15 mol% DOPA, 5 mol% egg PS and 80 mol% DOPC; (▲) 20 mol% DOPA, 80 mol% DOPC. The large unilamellar vesicle systems were prepared by an extrusion technique (see text) in the presence of a buffer containing 125 mM KCl, 20 mM $KH_2PO_4$ (pH = 7.4) and the untrapped buffer was exchanged for a buffer containing 125 mM KCl, 20 mM Hepes (pH = 7.4) by gel filtration employing Sephadex G-50. These vesicles were incubated (20 °C) in the presence of 2 mM $CaCl_2$ (1 μCi/μmol $^{45}Ca$), aliquots removed at various times and exterior (non-sequestered) $Ca^{2+}$ removed by gel filtration. The eluate was then assayed for $^{45}Ca^{2+}$ and lipid phosphorus.

Such a sensitivity of $Ca^{2+}$ 'uptake' to vesicle inner contents is clearly consistent with a DOPA-dependent transport of calcium to the vesicle interior, rather than binding of $Ca^{2+}$ to outer monolayer lipids. This conclusion was corroborated by passing aliquots of vesicles (containing sequestered $Ca^{2+}$) over 1 ml gel filtration columns which were pre-equilibrated with cold calcium (2 mM). Any $^{45}Ca$ bound to the vesicle exterior would be expected to exchange with non-radioactive calcium during this procedure. This would result in a decrease of vesicle-associated $^{45}Ca^{2+}$ if the uptake process reflects $Ca^{2+}$ binding to the vesicle exterior. In contrast, such a procedure consistently resulted in a vesicle-associated level of $^{45}Ca^{2+}$ that was 20% higher than $^{45}Ca^{2+}$ associated with vesicles that were passed down columns that had

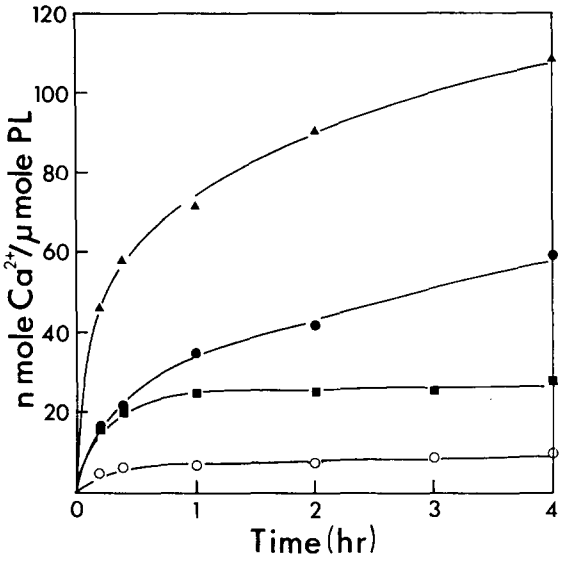


Fig. 2. Uptake of $Ca^{2+}$ into large unilamellar vesicle systems composed of 20 mol% DOPA and 80 mol% DOPC. The interior of the vesicles contained a variety of concentrations and types of $Ca^{2+}$ chelators: (○) 125 mM KCl, 20 mM $KH_2PO_4$ (pH = 7.4); (■) 120 mM KCl, 40 mM $KH_2PO_4$ (pH = 7.4); (●) 100 mM potassium oxalate, 50 mM KCl, 20 mM Hepes (pH = 7.4); (▲) 100 mM EGTA, 50 mM KCl, 20 mM Hepes (pH = 7.4). The vesicle were prepared in the presence of these various buffers and the untrapped buffer exchanged for KCl, 20 mM Hepes buffer (pH = 7.4) of equal osmolarity by gel filtration. For further details, see Fig. 1 legend and text.

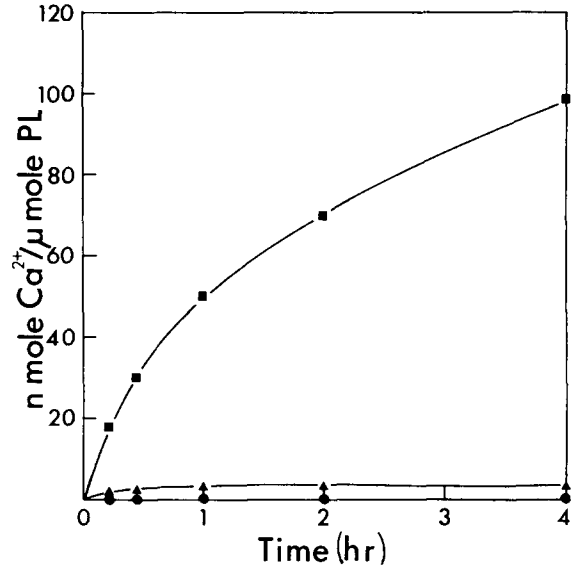


Fig. 3. Uptake of $Ca^{2+}$ into large unilamellar vesicle systems composed of DOPC; (●) DOPC vesicles containing 100 mM potassium oxalate, 50 mM KCl, 20 mM Hepes (pH = 7.4); (▲) DOPC vesicles containing 175 mM KCl, 20 mM Hepes (pH = 7.4) where the $Ca^{2+}$ ionophore A23187 was added (in ethanol) prior to incubation with $Ca^{2+}$ to achieve a 1:500 (mol/mol) A23187 to DOPC ratio; (■) DOPC vesicles containing 100 mM potassium oxalate, 50 mM KCl, 20 mM Hepes (pH = 7.4) where A23187 (1:500) was present during the incubation with $Ca^{2+}$. The vesicles were prepared in the presence of the various buffers, and untrapped buffer was exchanged for 175 mM KCl, 20 mM Hepes (pH = 7.4) by gel filtration. Uptake of $Ca^{2+}$ at subsequent times was determined as indicated in the legend to Fig. 1 and text.

not been pre-equilibrated with unlabelled $Ca^{2+}$. This suggests that when $Ca^{2+}$ free columns are employed, some of the sequestered $Ca^{2+}$ is transported out of the vesicles during the gel filtration procedure.

The preceding results demonstrate that the presence of 20 mol% DOPA results in uptake of $Ca^{2+}$ into the interior of the vesicle systems employed here. It is of interest to compare the rate and extent of such uptake with that observed for the fungal $Ca^{2+}$ ionophore A23187 in vesicle systems which do not contain DOPA. As shown in Fig. 3, no significant $Ca^{2+}$ uptake into DOPC vesicle systems is obtained in the absence of A23187 or internal oxalate. In the presence of 100 mM oxalate-containing vesicles, the rate and amount of $Ca^{2+}$ uptake is similar to that observed in DOPA-containing systems (see Fig. 2). Again, pre-equilibration of the gel filtration columns with unlabelled $Ca^{2+}$ resulted in an increase of vesicle-associated $^{45}Ca^{2+}$ levels similar to that observed for DOPA-containing vesicles.

In summary, these studies provide strong evidence that DOPA can act as a $Ca^{2+}$ ionophore in large unilamellar vesicle systems. Calcium can be accumulated when DOPA is present, whereas no such uptake is observed in the absence of DOPA. Maximum calcium uptake levels obtained here yielded $Ca^{2+}$/phosphatidic acid ratios of approximately 0.5. While this value could be achieved if $Ca^{2+}$ uptake were the result of binding to exterior phosphatidic acid, experiments performed with unlabelled $Ca^{2+}$ in the gel filtration columns indicate that uptake is the result of active $Ca^{2+}$ accumulation and not phospholipid binding. Furthermore, the amount of $Ca^{2+}$ accumulated is depen-

dent on the amount and type of the $Ca^{2+}$ chelator employed. Clearly, in order for $Ca^{2+}$ to be sensitive to material trapped inside the vesicle, it must be transported to the vesicle interior. This observation also indicates that sequestered calcium is predominantly associated with the trapped chelator and not with phosphatidic acid in the vesicle inner monolayer. Finally, the characteristics of $Ca^{2+}$ uptake observed in DOPA-containing vesicles are similar to those observed for pure DOPC vesicles in the presence of the $Ca^{2+}$ ionophore A23187. The discrepancy between these results and previous observations for egg phosphatidic acid [8] could reflect the more saturated nature of egg phosphatidic acid, the concentrations of phosphatidic acid employed or the presence of 10% diacetylphosphate in the previous study [8].

It should be noted, however, that these studies do not necessarily prove a role for phosphatidic acid as a $Ca^{2+}$ ionophore in vivo. In particular, the levels of DOPA employed here greatly exceed phosphatidic acid levels which may be expected to occur in biological systems. It remains to be determined whether variables such as phosphatidic acid unsaturation or localization can reduce the levels required to biologically relevant concentrations.

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