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EVIDENCE THAT PHOSPHATIDYLSERINE AND INORGANIC PHOSPHATE MAY MEDIATE CALCIUM TRANSPORT DURING CALCIFICATION

Abraham M. Yaari[1], Irving M. Shapiro[1] and Charles Eric Brown[2]

[1]Departments of Biochemistry and Orthodontics/Pedodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pa. 19104

and

[2]Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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Phosphatidylserine, a major acidic phospholipid of calcifying matrix vesicles, transports Ca^{2+} across a lipid barrier via a mechanism that is stimulated by inorganic phosphate (Pi). This mechanism involves formation of phosphatidylserine-Ca and phosphatidylserine-Ca-Pi complexes. These complexes form aggregates with lipophilic exteriors in a non-polar environment. Complexes formed in this manner could serve to transport Ca^{2+} and Pi across the membrane bilayer of matrix vesicles.

INTRODUCTION

In the mineralizing epiphyseal growth plate, mineral is first seen within membranous structures termed matrix vesicles (1, 2). The vesicles are enriched in acidic phospholipids and have been shown to be formed by budding of chondrocyte processes (3). Mineralization of the vesicles is associated with an increase in the concentration of extracellular inorganic phosphate (Pi) (4) and the presence of specific phospholipids. It has been reported that a stable non-dissociable phospholipid complex isolated from calcifying tissue can nucleate calcium phosphates from metastable solutions (5). This complex is considered to be the site of formation of mineral during calcification (6). How Ca^{2+} and Pi are transported through the phospholipid complex and the process by which Pi modulates either the transport or the deposition step is not understood.

The intent of this investigation is first to demonstrate that phosphatidylserine (PS), a major acidic phospholipid of matrix vesicles, can mediate

Abbreviations used: PS, phosphatidylserine; NMR, nuclear magnetic resonance; Pi, inorganic phosphate.

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Ca^{2+} transport non-enzymatically and that this process is enhanced by Pi. Second, using ^{31}P and ^{1}H nuclear magnetic resonance (NMR) spectroscopy to show that complexes of PS, Ca^{2+} and Pi are formed which would permit: 1) transport of Ca^{2+} across membrane bilayers, and 2) enhancement of Ca^{2+} transport by Pi.

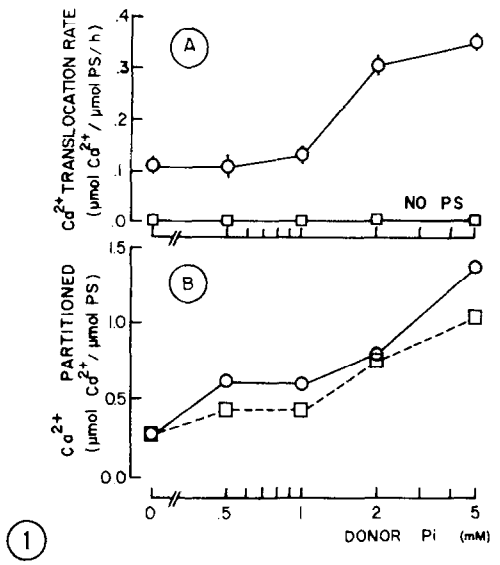
MATERIALS AND METHODS

1. Measurement of Ca^{2+} transport rate. The rate of Ca^{2+} translocation across a lipid barrier was measured in a three-compartment Pressman cell (7). The cell consists of a glass cylinder with a vertically welded partition. The partition was 4 mm short of the cylinder base, and served to create three compartments. The lower end of the vessel was filled with 200 μM PS dissolved in 2.0 ml presaturated chloroform/methanol/water (2.0:2.0:1.8, v/v/v). Aqueous buffer (1.0 ml) was placed in the two compartments over the lipid phase. The area of contact between the organic and aqueous interface was 73 mm^2. One of the aqueous buffers (donor) was tetramethylammonium-Pipes (25 mM at pH 6.4) which contained $CaCl_2$ (1.0 mM) labeled with tracer ^{45}Ca. The second aqueous buffer (receiver) was tetramethylammonium-citrate (25 mM) at pH 4.0. Inorganic phosphate as H_3PO_4 was added to the donor buffer in concentrations of 0 to 10 mM. Ca^{2+} translocation from the donor to the receiver through the lipid phase was determined by liquid scintillation counting in 20 μl samples of the buffers.

2. Measurement of Ca^{2+} partitioning between two phases. Ca^{2+} uptake and release by PS was measured in an aqueous-lipid partition system at equilibrium (8). For this procedure 0.5 ml aqueous donor buffer containing 1.0 mM Ca^{2+} labeled with ^{45}Ca was layered over 1.0 ml presaturated organic solvent containing 200 μM PS. The tube was then shaken vigorously for 15 s on a vortex mixer. The two phases were allowed to equilibrate at room temperature for 10 hr and then separated by centrifugation for 1 min at 2,000 x g. The aqueous layer was removed, and Ca^{2+} content was measured in 20 μl aliquots of the organic and aqueous phases.

Calcium release from PS was studied by a reversed extraction technique. In this procedure, Ca^{2+} was first partitioned into an organic phase containing PS as described above. The Ca^{2+} was then re-extracted from the organic phase by replacing the aqueous donor phase with 0.5 ml aqueous receiver buffer at pH 4.0. The organic and aqueous phases were vigorously mixed for 15 s, allowed to equilibrate for 1 hr and separated by centrifugation at 2,000 x g for 1 min. The buffer Ca^{2+} content was then determined. The compositions of the aqueous buffers and the organic phase were identical to those described earlier for the three-phase system.

3. ^{31}P and ^{1}H NMR spectroscopy. PS (0.5 mM) was dissolved in 20 ml of presaturated chloroform/methanol/water (10:10:9, v/v/v). This solution then was mixed vigorously with 10 ml of tetramethylammonium-Pipes buffer (25 mM, pH 6.4) that contained either no Ca^{2+}, 2.5 mM Ca^{2+} or 2.5 mM Ca^{2+} plus 12.5 mM Pi. The molar ratio of PS to Ca^{2+} was the same as in 2 above, whereas that of Pi to Ca^{2+} was 5:1. Following a 10 hr equilibration period, the phases were separated, and the lower lipid phase was dried in a stream of nitrogen. The samples were re-dissolved in either chloroform/methanol/D_2O (10:10:9, v/v/v) or deuterochloroform. The two layers of the chloroform/methanol/D_2O suspension were allowed to separate overnight, and then each layer (i.e., the upper, aqueous layer and the lower, organic layer) was transferred to a separate NMR sample tube. Such separation of layers obviously was not necessary when deuterochloroform was the solvent. ^{31}P and ^{1}H spectra were recorded at 60.7 MHz and 150.1 MHz, respectively, with a Nicolet NT-150 spectrometer. Chemical shifts are reported relative to 95% H_3PO_4 and tetramethylsilane.



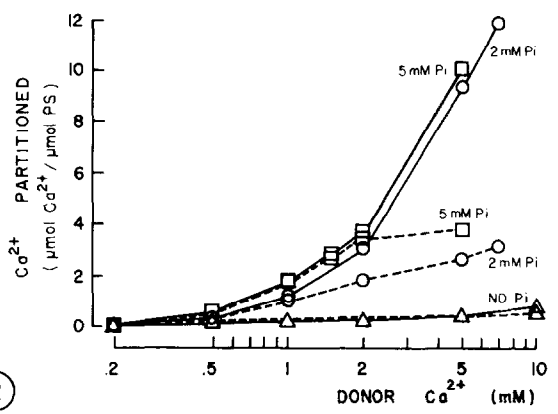


Fig. 1. The effect of Pi on PS-mediated Ca^{2+} (1.0 mM) transport across a lipid barrier.

Fig. 1A. The Ca^{2+} transport rate in the three-phase compartmental model is shown. Each data point represents the mean ± S.E.M. of six experiments.

Fig. 1B. The partitioning at equilibrium of 1.0 mM Ca^{2+} into and out of a lipid layer containing 0.2 mM PS is shown. Solid line indicates Ca^{2+} uptake into the lipid; dashed line shows Ca^{2+} repartitioned from the organic phase into the aqueous receiver buffer. Each data point represents the mean of three separate experiments. S.E.M. was less than 0.01 µmol.

Fig. 2. The effect of Ca^{2+} and Pi on partitioning of Ca^{2+} between aqueous and organic phases. The solid lines represent extraction of Ca^{2+} from the aqueous donor buffer into the organic phase. The dashed lines represent the repartitioning of Ca^{2+} into aqueous receiver buffer. Each data point is the mean of three experiments. S.E.M. was less than 0.10 µmol.

RESULTS

The rate of PS-mediated Ca^{2+} transport across a PS barrier is modulated by the donor Pi concentration. Using the three-phase system, between 1.0 and 2.0 mM Pi, there is a three-fold increase in the rate of Ca^{2+} transport from the donor into the receiver compartment (Fig. 1A). The partition assay, which permitted Ca^{2+} uptake into the lipid phase to be measured separately from Ca^{2+} release into the receiver buffer, indicates that the elevation in the transport rate in the presence of Pi is the result of an increase in Ca^{2+} binding.

At low concentrations of Ca^{2+} and in the presence of Pi partitioning of Ca^{2+} into the lipid phase is almost completely reversible (Fig. 1B and Fig. 2, dashed lines). However, as the concentration of Ca^{2+} is increased above 2 mM, and as the Pi level is raised, less Ca^{2+} partitions out of the organic phase

into the aqueous buffer (Fig. 2, dashed lines). Based on these experiments, it may be concluded that PS mediated Ca^{2+} uptake into the lipid phase is increased in the presence of Pi. Furthermore, at low Ca^{2+} concentrations, the process is reversible; however, at high Ca^{2+} levels (above 2 mM), Ca^{2+} uptake is increased, but release is inhibited. In this latter situation, Ca^{2+} accumulates in the lipid phase.

The effect of Ca^{2+} on the NMR spectra of PS and Pi, in solvents of differing polarity indicates that lipid complexes and larger lipid aggregates are formed. In both the organic and aqueous layers of the separated chloroform/methanol/D_2O solvent system, the addition of Ca^{2+} to PS causes the ^{31}P resonance of the PS to shift upfield about 1.5 ppm (Fig. 3A and B). When Pi is added to this complex, a single resonance (Fig. 3C) occurs at the same chemical shift as PS-Ca in the absence of Pi (Fig. 3B). This suggests that both Pi and the phosphate group of PS bind to the Ca^{2+}. Each sample exhibits the same line width for its ^{31}P resonance in both the organic and aqueous layers of the separated chloroform/methanol/D_2O solvent system, but the relative peak intensities from these two separated layers vary depending on whether Ca^{2+} and Pi are present. The extent of partitioning of ^{31}P between the two layers as measured by NMR compares quite well with the extent of partitioning of Ca^{2+} in parallel experiments (Fig. 2). These results suggest that PS-Ca and PS-Ca-Pi complexes are formed and that these complexes partition differently between the two layers of the chloroform/methanol/D_2O solvent system. Since the line widths of the ^{31}P resonances are relatively narrow (Fig. 3A-C), one can conclude that the PS-Ca and PS-Ca-Pi complexes exhibit little constraint on the thermal motion of the phosphate groups in the relatively polar chloroform/methanol/D_2O solvent system.

When deuterochloroform is the solvent, however, Ca^{2+} has a marked effect on the mobility of Pi and the head group of PS. In this solvent, PS yields a narrow ^{31}P resonance (Fig. 3D) similar to that observed in the two phases of chloroform/methanol/D_2O (Fig. 3A), but the ^{31}P resonances from PS-Ca and PS-Ca-Pi are broadened (Fig. 3E and F). This suggests that these complexes aggregate in a non-polar environment. However, the ^{1}H resonances of the fatty

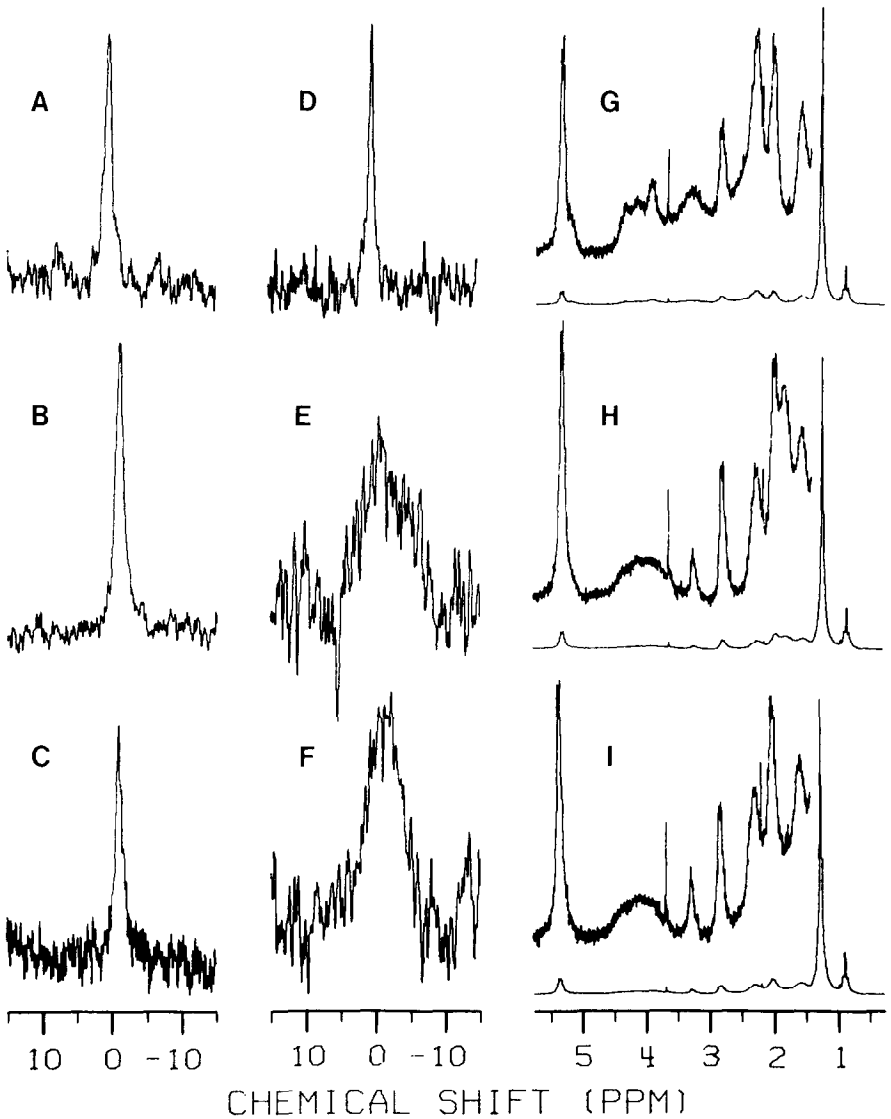


Fig. 3. NMR spectra of solutions that contain PS, Ca^{2+} and Pi. A-F are ^{31}P spectra, whereas G-I are ^{1}H spectra. The solvent in A-C is the organic layer from equilibrated chloroform/methanol/D_2O (10:10:9, v/v/v) and in D-I is deuterochloroform. (A, D, G) PS alone; (B, E, H) PS plus Ca^{2+}; (C, F, I) PS plus Ca^{2+} and Pi. Each upper aqueous layer from the separated chloroform/methanol/D_2O solvent system yields a ^{31}P resonance at the same chemical shift and with the same line width as its respective organic layer (A-C). Furthermore, the relative peak intensities from the aqueous and organic layers of each sample agree with the extent of partitioning of Ca^{2+} between the two layers in the same solvent system (Fig. 2). It should be noted that each sample (i.e., PS alone, PS plus Ca^{2+}, and PS plus Ca^{2+} and Pi) exhibits the ^{31}P peak maximum at about the same chemical shift regardless of whether the solvent is the organic layer of chloroform/methanol/D_2O (A-C), the aqueous layer of this same solvent system (not shown) or deuterochloroform (D-F).

acid side chains of these complexes in deuterochloroform (Fig. 3H and I) exhibit similar line widths as PS (Fig. 3G). Thus, the spectral characteristics of the PS-Ca and PS-Ca-Pi complexes in chloroform are the same as those of

phospholipid micelles and vesicles in water (9). It seems likely that in non-polar environments the PS-Ca and PS-Ca-Pi complexes form aggregates in which the PS phosphate head groups and Ca^{2+} and Pi are centrally sequestered while the fatty acid side chains interact with the solvent.

DISCUSSION

These experiments demonstrate that Ca^{2+} can be translocated across a lipid phase by PS and that Pi stimulates Ca^{2+} transport. The experimental data indicate that it is a two step process in which Ca^{2+} partitions from an aqueous phase into the phospholipid and then re-partitions from the phospholipid to the receiver aqueous phase. Pi increases the capacity of the phospholipid to accept Ca^{2+} and thereby elevates the rate at which Ca^{2+} can be transported. When the Pi concentration is raised (above 2 mM) the second step is inhibited and the maximum rate at which Ca^{2+} can be transported is limited.

Partitioning of Ca^{2+} into the phospholipid of a membrane bilayer might involve binding of Ca^{2+} to the phosphate head group of PS followed by aggregation of several of these complexes to form a "micelle" in the center of which Ca^{2+} ions are sequestered. This "micelle" would be lipophilic on its surface and thus able to traverse the core of a lipid bilayer. Combination of this "micelle" with the opposite head group region of the membrane bilayer would complete the transport of Ca^{2+} across the membrane. Pi would be expected to faculitate transport by neutralizing the charge on the Ca^{2+} thereby facilitating closure of the micelle and also possibly permitting transport of additional Ca^{2+}.

It is interesting to relate the findings of this model system to the mineralization of vesicles in vivo. The early accumulation of Ca^{2+} within matrix vesicles could be related to the presence of a proton gradient across the membrane. Such a gradient might exist as the intravesicular pH would be expected to reflect the intracellular pH and be greater than that of the extracellular cartilage lymph (10). Following the initial uptake of Ca^{2+}, subsequent mineralization is related to the appearance of Pi in the cartilage matrix (4). The findings of this study suggest that Pi may act to enhance both Ca^{2+}

uptake and accumulation in the vesicle. Elevated levels of Ca^{2+} in the vesicle interior, in the presence of Pi, could result in retention of Ca^{2+} in the lipid bilayer and subsequent nucleation of calcium phosphates in the vesicle membrane.

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