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EFFECT OF FLUORIDE ON PHOSPHATIDYLSERINE-MEDIATED CALCIUM TRANSPORT

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Experiments to study the effect of F^- on phosphatidylserine-mediated Ca^{2+} transport were performed utilizing two and three compartment lipid-aqueous phase model systems. Using the three compartment model, it was shown that F^- modulated the rate of Ca^{2+} transport in a biphasic manner. Low concentrations of F^- enhanced the Ca^{2+} translocation rate and high levels of F^- inhibited the rate of Ca^{2+} transport. To determine whether the enhancement or inhibition of Ca^{2+} transport rate by F^- was due to an increase in the uptake of Ca^{2+} into the lipid phase, or to an increase in the ability of the lipid phase to release Ca^{2+}, a two compartment model was used. It was found that the ability of the phosphatidylserine phase to take up Ca^{2+} increased as the F^- concentration of the aqueous donor phase was raised. In addition, Ca^{2+} release from the phosphatide was also modulated by F^-. High F^- levels inhibited the release of Ca^{2+} from the lipid, while low levels of F^- did not retain Ca^{2+} in the lipid phase. Thus, it was concluded that F^- enhanced the interaction between Ca^{2+} and phosphatidylserine, possibly by forming a phosphatidylserine-Ca-F complex. Once this interaction had taken place, Ca^{2+} release into the aqueous receiver compartment was dependent on the F^- concentration. Thus, low F^- levels induced a net increase in Ca^{2+} transport, while high F^- levels inhibited Ca^{2+} translocation rates.

Introduction

Previous studies have shown that F^- has a profound effect on cell activity and mineral formation. At low concentrations, F^- changes the solubility, crystallinity and structural characteristics of bone mineral. At higher concentrations, F^- modulates a number of Ca-dependent enzymatic, biosynthetic and transport processes. In addition, chronic exposure to F^- frequently leads to an aberrant accumulation of both Ca^{2+} and F^-. However, despite the known effect of both high and low levels of F^-, the mechanism by which Ca^{2+} and F^- interact is poorly understood. This investigation addresses itself to this question by studying the relationship between Ca^{2+}, F^- and phosphatidylserine (PS), a membrane phospholipid frequently associated with Ca^{2+} binding [1–3].

The interaction between PS and mono- and divalent ions is complex and difficult to study in vivo. However, it can be conveniently examined using lipid-aqueous phase compartmental models [4,5]. Using bi- and triphasic models this study examined the effect of F^- on PS-mediated Ca^{2+} transport. The results of the investigation clearly show that F^- modulates Ca^{2+} translocation by regulating the ion uptake and release characteristics of the phospholipid.

Materials and Methods

Measurement of phosphatidylserine-mediated Ca^{2+} and F^- transport

The rate of Ca^{2+} and F^- transport was mea-

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Pipes, piperazine-*N*,*N*′-bis(ethanesulfonic acid); PS, L-phosphatidyl-L-serine.

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sured in a cylindrical glass reaction chamber [6]. The vessel was designated to accommodate three immiscible liquid compartments. Two of the compartments contained aqueous buffers which were separated from each other by a glass partition. The partition was welded into the center of the glass vial and extended 4 mm above the floor of the chamber. A third compartment was formed by an organic solvent that was placed at the bottom of the vial, below the two aqueous phases. The organic solvent formed two separate interfaces with each of the aqueous buffers. The volume of the organic phase was 2.0 ml; the volume of the individual aqueous buffers was 1.0 ml; the area of each interface surface was 0.73 cm^2. Calcium transport between the compartments was facilitated by stirring the organic phase with a magnetic stirrer.

The aqueous and organic solutions were prepared from a chloroform/methanol mixture, presaturated with water by the method of Bligh and Dyer [7]. A single phase mixture of chloroform/methanol/water (1.0:2.0:0.8, v/v) was separated into two phases by the addition of an equal volume of chloroform and water to give a final volume ratio of 2.0:2.0:1.8 (v/v). The upper phase was used for the preparation of all the aqueous solutions, and the lower hydrophobic phase was used to dissolve 0–200 μM PS. The Ca^{2+} donor buffer was 25 mM tetramethylammonium-Pipes at pH 6.4 or tetramethylammonium-Tricine at pH 8.3. Calcium chloride, 1.0 to 10 mM, labeled with ^{45}Ca to give about 6000 cpm/mmol was added to the buffer. Fluoride as NaF was added to the donor buffer in concentrations of 0 to 10 mM in the tetramethylammonium-Pipes buffer. The receiver compartment contained 25 mM tetramethylammonium-citrate buffer at pH 4.0 or pH 5.4. The rate of translocation of Ca^{2+} from the donor buffer to the receiver was ascertained by sampling the donor and receiver compartments and measuring the ^{45}Ca content of each phase by liquid scintillation counting in a Packard Tri-Carb spectrometer.

A F^- specific electrode (Orion Research, Inc., Cambridge, MA) was used for the measurement of F^- in 200 μl samples of donor and receiver buffer [8]. For this procedure, to remove all traces of organic solvents, 20 μl of both samples and standards were evaporated to dryness at room temperature and the residue was dissolved in 200 μl total ionic strength activity buffer. The F^- determinations were performed at 25°C and at pH 5.5.

At least four reaction chambers were used for each experiment. The stirrer speed of each chamber was periodically synchronized with a tachometer. This adjustment minimized variations between the different chambers to about ± 10%. Linear values for translocation rates were consistently obtained when the receiver compartment was stirred prior to sampling. All experiments were carried out at 25°C.

Ca^{2+}- and F^--extraction experiments

Ca^{2+} or F^- binding to PS was studied in a two-phase partition system according to the method described by Feinstein [9]. Using this procedure 0.5 ml of the aqueous buffer containing 0–10 mM $CaCl_2$ labeled with ^{45}Ca and 0–10 mM F^- was layered over 1.0 ml of the organic phase in which 0–200 μM PS was dissolved. The tube was then shaken vigorously for 15 s on a Vortex mixer. The two phases were separated by centrifugation for one min at 2000 × *g* and allowed to equilibrate at room temperature. The aqueous layer was removed and the level of Ca^{2+} or F^- in the organic phase was measured.

A reverse extraction procedure was used to examine the release of Ca^{2+} or F^- from the lipid phase. In this procedure Ca^{2+} and F^- were first extracted from the donor buffer into the phospholipid phase (see above). The aqueous donor phase was then removed and receiver buffer (0.5 ml) was layered over the organic phase. After vigorous mixing for 15 s the phases were separated by centrifugation and allowed to equilibrate for 1 h at 25°C. The aqueous phase was then removed and the concentration of Ca^{2+} or F^- transported into the receiver buffer was ascertained.

Materials

Tetramethylammonium; Tricine, *N*-tris-(hydroxymethyl)methylglycine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) and L-phosphatidyl-L-serine (bovine brain), were purchased from Sigma Chemical Co. Thin-layer chromatography revealed that the phospholipid was 98–99% pure and it was used directly without any further purification. ^{45}Ca was obtained from

Amersham Corp. as $CaCl_2$ in aqueous solution (10 mCi/mg Ca). Total ionic strength activity buffer and NaF standard solution were obtained from Orion Research, Inc.

Results

The kinetics of Ca^{2+} transport in the three-phase system is shown in Fig. 1. The figure clearly shows that in the presence of PS, Ca^{2+} was translocated from the donor into the receiver compartment at a linear rate of 0.176 μmol/h. The figure also shows that the phospholipid was necessary for Ca^{2+} translocation. Thus, when PS was absent, the Ca^{2+} transport rate was very slow.

The effect of different concentrations of F^- on Ca^{2+} transport is seen in Fig. 2. Low concentrations of F^- (0–0.05 mM) did not change the rate of Ca^{2+} transport. However, when the donor F^- concentration was between 0.1 and 0.5 mM there was an increase in the rate of Ca^{2+} transport into the receiver compartment. The highest transport rate was 30% above the control (no F^-) values. This rate was achieved when the $F^-:Ca^{2+}$ molar ratio was 0.5:1.0. When the F^- concentration of the donor compartment was above 1.0 mM there was a marked decrease in the Ca^{2+} translocation rate. The rate continued to decline as the F^- concentration was increased to 10 mM. At equimolar concentrations of Ca^{2+} and F^- (1.0 mM), Ca^{2+} transport declined to 9% above that of the control. A 78% reduction in the Ca^{2+} translocation rate was achieved when the $F^-:Ca^{2+}$ ratio was 5:1. A further elevation of the $F^-:Ca^{2+}$ molar ratio to 10:1 almost totally inhibited Ca^{2+} transport.

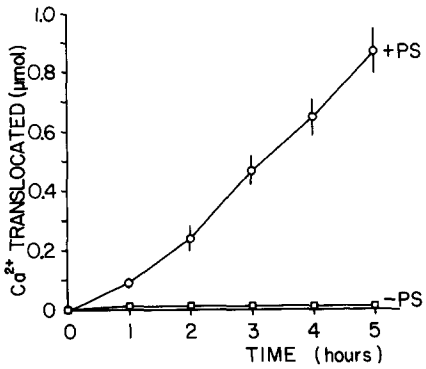

Fig. 1. Phosphatidylserine(PS)-mediated Ca^{2+} translocation in the three-phase system. ^{45}Ca (10 mM) was placed in the donor compartment and its appearance in the receiver compartment was determined at various time intervals. The buffer used in the donor compartment was 25 mM tetramethylammonium-Tricine at pH 8.3 and the receiver buffer was 25 mM tetramethylammonium-citrate at pH 5.4. The organic phase contained 200 μM PS dissolved in pre-saturated chloroform/methanol/water (2.0:2.0:1.8, v/v). Values shown represent the mean of six experiments ± S.E. ○——○, Ca^{2+} in receiver phase in the presence of PS. □——□, Ca^{2+} in receiver phase in the absence of PS.

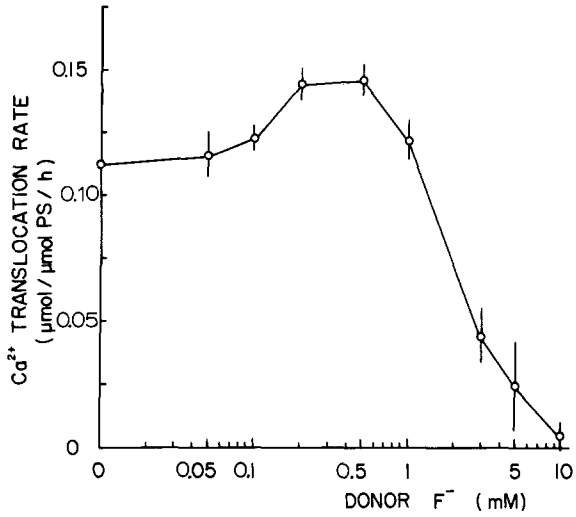

Fig. 2. The effect of F^- on PS-mediated Ca^{2+} transport in the three-phase system. The F^- concentration of the donor compartment was varied from 0 to 10 mM and the rate of Ca^{2+} translocation into the receiver compartment was measured. The PS concentration in the organic phase was 200 μM. The donor buffer was 25 mM tetramethylammonium-Pipes at pH 6.4 and the receiver was 25 mM tetramethylammonium-citrate at pH 4.0. Data shown are the mean of six experiments ± S.E.

The three-phase system described above demonstrates net Ca^{2+} flux. It could not be used to differentiate between the rate at which Ca^{2+} was taken up by the organic phase or released into the receiver buffer. In order to separate out these two effects the two-phase system was employed. This method permitted separate measurements of the kinetics of Ca^{2+} binding and release, expressed as a function of the F^- concentration.

Fig. 3 shows the equilibrium measurements of Ca^{2+} uptake and release by the phospholipid in a two-phase system. Increasing concentrations of F^- served to facilitate the extraction of Ca^{2+} from the donor phase into the organic phase (Fig. 3A and

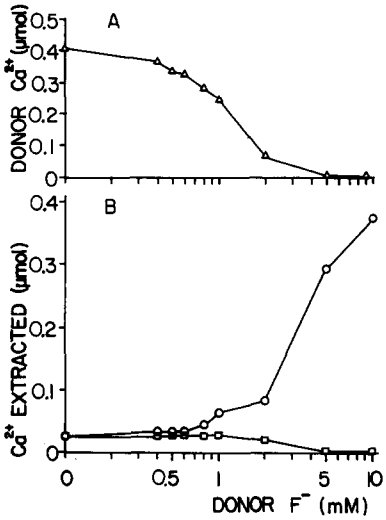

Fig. 3. The effect of F^- on Ca^{2+} extraction in a two-phase system. The two-phase procedure was used to measure Ca^{2+} uptake and release from the phospholipid. Ca^{2+} uptake was measured by extracting 1 mM Ca^{2+} from the donor compartment into the organic phase in the presence of 0–10 mM F^-. Ca^{2+} release was determined by re-extraction of Ca^{2+} from the organic phase into the receiver buffer. The composition of the aqueous and organic phases in this experiment were the same as those described in Fig. 2. The values shown are the mean of six experiments; the S.E. was less than ±0.01 μmol. (A) △——△, Ca^{2+} in the donor. (B) ○——○, Ca^{2+} in the organic phase; □——□, Ca^{2+} in the receiver.

B). When the release of Ca^{2+} into the receiver buffer was measured at 0 mM F^-, Ca^{2+} was completely re-extracted into the aqueous receiver buffer (Fig. 3B). This result indicated that Ca^{2+} in the organic phase was available for transportation into the aqueous phase. At F^- levels up to 0.6 mM, only 80% of the Ca^{2+} was re-extracted. When the F^- level was between 0.6 and 2.0 mM, while the Ca^{2+} level in the organic phase markedly increased, the amount of Ca^{2+} that was transported into the receiver phase declined. At very high levels of F^- (10 mM) about 0.37 μmol Ca^{2+} was extracted into the organic phase, however, less than 0.003 μmol Ca^{2+} was transported. In summary, at low F^- concentrations, a high percentage of Ca^{2+} in the organic phase was transportable and could be released into the aqueous receiver buffer. However, when the F^- concentration was high, only a small percentage of the Ca^{2+} in the organic phase was released and transported into the receiver.

To determine whether PS was required for Ca^{2+} extraction and translocation, the experiment was also performed in the absence of phospholipid (not shown). In this experiment, very low Ca^{2+} concentrations were extracted into the organic phase and hence only small amounts of Ca^{2+} were available for transportation into the receiver. In the absence of PS, F^- did not promote Ca^{2+} extraction or transport; further, while Ca^{2+} was rapidly removed from the donor buffer at high F^- concentrations, there was no visible precipitation in any of the phases and measurable amounts of Ca^{2+} were not detected in the organic phase.

Since Ca^{2+} transport was modulated by F^- it was of interest to determine whether Ca^{2+} modulated the transport of F^-. Fluoride translocation in the three-phase system is shown in Fig. 4. In the presence of PS, the rate of F^- transport through the organic solvent was linear with time, and the translocation rate was 0.164 μmol/h. In the absence of PS, F^- was translocated at a reduced rate. Thus, in the first 4 h, the F^- transport rate was 0.064 μmol/h. The presence of 10 mM Ca^{2+} resulted in almost total inhibition of F^- transport.

The two-phase system was used to ascertain which of the two possible F^- translocation steps were affected by Ca^{2+}. In the presence of PS an increase in the Ca^{2+} concentration resulted in a

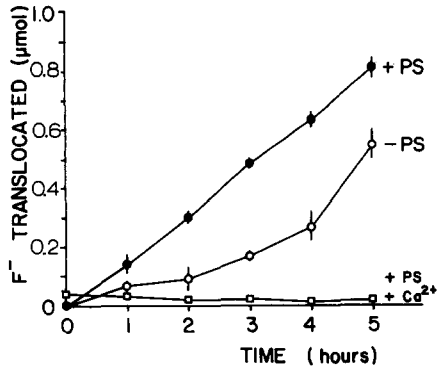

Fig. 4. PS-mediated F^- transport in the three-phase system. 10 mM F^- was placed in the donor compartment and its appearance in the receiver was monitored by sampling. The composition of the aqueous and organic phases in this experiment were the same as in Fig. 1. Values shown represent the mean of six experiments ± S.E. ●——●, F^- in the receiver phase in the presence of PS; ○——○, F^- in the receiver phase in the absence of PS; □——□, F^- in the receiver phase in the presence of PS and 10 mM Ca^{2+}.

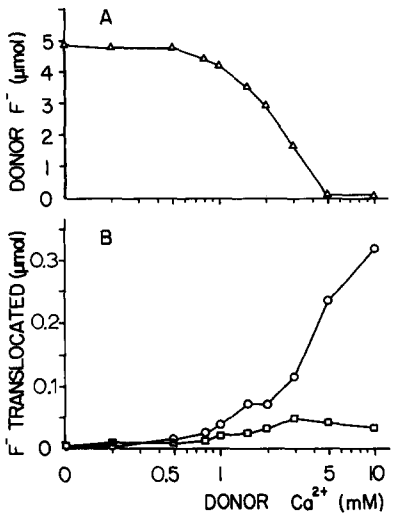

Fig. 5. The effect of Ca^{2+} on extraction of F^- in the two-phase system. The two-phase procedure was utilized to determine F^- uptake and release from the phospholipid. F^- uptake was measured by extracting 10 mM F^- from the donor compartment into the organic phase in the presence of 0–10 mM Ca^{2+}. F^- release was determined by re-extracting Ca^{2+} from the organic phase into the receiver buffer. The composition of the aqueous and organic phases in this experiment were the same as those described in Fig. 2. Values shown represent the mean of six experiments; the S.E. was less than 0.01 μmol. (A) △——△, F^- in the donor. (B) ○——○, F^- in the organic phase; □——□, F^- in the receiver.

progressive loss of F^- from the donor compartment and an increase in the amount of extractable F^- in the organic phase (Fig. 5A and B). To study the release of F^- from the phospholipid, the organic phase was re-extracted with aqueous buffer. At low Ca^{2+} concentrations (0–1.0 mM), a high percentage of F^- was re-extracted. When the donor Ca^{2+} concentration was 2.0 mM, less than 50% of the total extractable F^- in the organic phase, was transported into the receiver phase. When the donor Ca^{2+} was 5.0 mM, although the uptake of F^- in the lipid phase was 0.24 μmol, only 0.045 μmol F^- was transported into the receiver phase.

In the absence of phospholipid (not shown), F^- was not extracted into the organic phase in measurable amounts.

Discussion

Experiments described in this report were aimed at studying F^- modulation of phospholipid-mediated Ca^{2+} transport. The experiments clearly show that low F^- concentrations accelerated Ca^{2+} transport whereas elevated F^- levels inhibited Ca^{2+} translocation. In addition, it was found that when the F^- concentration was high, Ca^{2+} translocation was inhibited and there was an accumulation of Ca^{2+} and F^- in the hydrophobic phase.

To understand the non-linear biphasic modulation of Ca^{2+} translocation by F^- the two-phase systems were utilized. This latter approach permitted independent determination of Ca^{2+} uptake into the organic phase and Ca^{2+} release into the receiver buffer, respectively. By separating the two steps it was shown that increased levels of F^- stimulated the passage of Ca^{2+} from the donor compartment into the organic phase, but its subsequent release into the receiver buffer was also regulated by the concentration of F^-. Thus, at low F^- levels (up to 0.6 mM), almost all of the extracted Ca^{2+} was transportable. Indeed, in the three-phase system, between 0–0.05 mM F^-, a slight but statistically significant increase in the Ca^{2+} translocation rate was noted. However, above 0.6 mM, the release step was rate limiting and there was a progressive decrease in the rate of Ca^{2+} transport into the receiver phase. It is concluded that F^- modulates the Ca^{2+} translocation rate by increasing the extent of Ca^{2+} extraction into the lipid phase and by inhibiting Ca^{2+} release from the bulk lipid phase.

Previous studies indicated that the mechanism by which anions modulate PS-mediated Ca^{2+} translocation depends on the formation of a dissociable lipid-soluble ion complex that would release bound ions at an aqueous-lipid interface [5]. In the presence of F^-, if a similar type of complex is formed, then the observed increase in the amount of Ca^{2+} in the hydrophobic phase could be due to F^- enhancing PS-Ca-F complex formation; F^- could also decrease the dissociability of the complex and thereby lower the amount of Ca^{2+} that could be re-extracted from the lipid. Although the mechanism of Ca^{2+} and F^- binding to PS has not been studied, the results suggest that dissociation of the complex is also a function of the F^- concentration.

While the experimental conditions were not physiological, the range of Ca^{2+} and F^- con-

centrations included values considered to be physiological or therapeutic. The study shows that when the donor Ca^{2+} and F^- concentrations were 1.25 mM and 0.05 mM respectively, some enhancement of non-enzymatic Ca^{2+} transport across lipid membranes might be expected. However, at high F^- concentrations, a non-transportable PS-Ca-F complex could accumulate in the hydrophobic surroundings of the membrane. The presence of complexed F^- could result in marked changes in lipid crystallinity and enzymatic activity. Together, these effects could have a profound effect on cell function and metabolism.

The results of the kinetic analysis indicated that F^- modulated Ca^{2+} translocation. To ascertain whether Ca^{2+} controlled F^- transport, the kinetics of F^- translocation were also explored. These experiments indicated that F^- translocation was modulated in a similar way to Ca^{2+} transport.

Experiments were performed in the absence of the phospholipid to evaluate the importance of PS in F^- transport. It was found that while some F^- was transported, PS increased the rate 3-fold. It is concluded that while Ca^{2+} transport is totally PS dependent, F transport across a bulk lipid phase has lipid-dependent and lipid-independent components. As far as lipid-independent transport is concerned, extraction experiments conducted with the two-phase method, in the absence of PS, indicated that Ca^{2+} and F^- concentrations in the organic phase were low. This observation would account for the decreased rate of ion transfer into the receiver compartment. Thus, in this model system, the phospholipid served to extract Ca^{2+} and F^- from the aqueous donor phase into the organic compartment and facilitated ion release into the receiver aqueous phase.

The model system described in this investigation also draws attention to the importance of hydrophobic environments in tissue mineralization. While the observation that mineral deposition occurs in membranes is not new, the mechanisms by which the hydrophobic environment promotes mineral deposition have received little study. This investigation indicated that both Ca^{2+} and F^- bind to phospholipids and only a part of the total bound mineral is available for transport. Further, high levels of external F^- increase Ca^{2+} uptake by the lipid and depress Ca^{2+} translocation. In this situation mineral formation would be stimulated. The type of mineral that is formed at different Ca^{2+} and F^- concentrations in hydrophobic environments, is presently under investigation.

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References

1 Joos, R.W. and Carr, C.W. (1967) Proc. Soc. Exp. Biol. Med. 124, 1268–1272
2 Houser, H., Drake, A. and Phillips, M.C. (1976) Eur. J. Biochem. 62, 335–344
3 Cotmore, J.M., Nichols, G., Jr. and Wuthier, R.E. (1971) Science 172, 1339–1341
4 Green, D.E., Fry, M. and Blondin, G.A. (1980) Proc. Natl. Acad. Sci. USA 77, 257–261
5 Yaari, A.M. and Shapiro, I.M. (1982) Calcif. Tissue Int. 34, 43–48
6 Pressman, B.C. (1973) Fed. Proc. 32, 1698–1703
7 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
8 Ekstrand, J. (1977) Calcif. Tissue Res. 23, 225–228
9 Feinstein, M.B. (1964) J. Gen. Physiol. 48, 357–374