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Measurement of outward translocation of phospholipids across human erythrocyte membrane

(phospholipid asymmetry/lipid flip–flop/aminophospholipid translocase/erythrocytes/spin labels)

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ABSTRACT **Spin-labeled phospholipids have been used to study the outside → inside and inside → outside transport of phospholipids across the human erythrocyte membrane at 37°C. As already shown, inward transport is much faster for aminophospholipids than for phosphatidylcholine. In addition, we show here that outward transport of the phosphatidylserine and phosphatidylethanolamine analogues is three to four times faster than that of phosphatidylcholine. Magnesium depletion of the erythrocytes considerably decreases the outward rate of both aminophospholipids to values close to that of phosphatidylcholine. These results suggest that the outward aminophospholipid translocation is, at least partly, protein mediated. The protein involved could be identical to the inward Mg-ATP-dependent aminophospholipid carrier.**

Inward translocation of phosphatidylserine and phosphatidylethanolamine across human erythrocyte membrane is mediated by a specific ATP-dependent protein (1–5). This selective and active transport has been demonstrated also in other cells (6–8). To explain the observed phospholipid transbilayer asymmetry (9) in terms of a dynamic equilibrium, existence of an outward motion was postulated (1). Thus, the rate of the outward transport of a given phospholipid can be estimated from knowing both its rate of inward translocation and its asymptotic distribution. Such a calculation was made by Herrmann and Muller (1986) (10) and more recently by Williamson *et al.* (11).

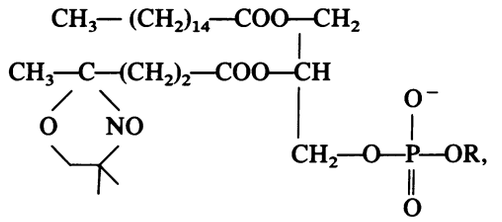
In the present paper we give direct experimental evidence for outward phospholipid motion across the human erythrocyte membrane, and we assess this diffusion rate by using spin-labeled analogues of phospholipids. This outward motion of aminophospholipids is shown to be more than passive diffusion through the bilayer. Evidence for the role of a selective protein carrier is presented.

MATERIALS AND METHODS

Buffers. The buffer used was 140 mM NaCl/10 mM Hepes, pH 7.4; in some experiments 1 or 2 mM EDTA was added.

Erythrocytes. Fresh human blood was obtained from healthy volunteers or from a local blood bank (Hôpital Cochin). Blood collected on EDTA was washed three times in 3 vol of buffer by centrifugation (1000 *g* at 4°C for 5 min). The washed erythrocytes were stored on ice and used within 6 hr. All samples contained 5 mM diisopropyl fluorophosphate (6) to minimize hydrolysis of spin-labeled phospholipids.

Spin-Labeling. The spin-labeled phospholipids used throughout this study can be represented by the structure:



where the residue R was either serine, ethanolamine, or choline. The molecules were 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine [designated (0,2)PtdSer], 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylethanolamine [designated (0,2)PtdEtn], or 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylcholine [designated as (0,2)PtdCho]. A few experiments were also done with the sphingomyelin derivative, *N*-(4-doxylpentanoyl-*trans*-sphingenyl-1-phosphocholine [(0,2)Sph*P*Cho]. Spin labels were synthesized as described (1, 12, 13). Suitable amounts of spin labels in chloroform or chloroform/methanol 2:1 (vol/vol) were dried under vacuum and resuspended in buffer. Two volumes of this aqueous dispersion of labels (ice-cold) were then added to one volume of ice-cold washed erythrocyte pellet. We verified, as in ref. 1, that the spin-labeled phospholipids incorporate in erythrocyte membranes within 3 min under such conditions. The spin-label concentration, unless specified, was 2 mol % of the endogenous phospholipids. Spontaneous reduction of the spin-labeled concentration was monitored after bovine serum albumin (BSA) extraction by following a specific ESR spectral peak intensity at 20°C. ESR spectra were recorded with a Varian E109 spectrometer equipped with a temperature-control device and connected to a Tektronix 4051 computer.

Kinetics of Inward and Outward Passages of Spin-Labeled Phospholipids Through the Erythrocyte Membrane. The labeled erythrocyte suspension was incubated at 37°C. During incubation 200-μl aliquots of the suspension were drawn at regular intervals and mixed with 60 μl of a 10% (wt/vol) fatty acid-free bovine serum albumin solution in buffer. The mixture was incubated for 1 min on ice and then centrifuged (1000 × *g* for 5 min), and then the supernatant was separated into two 90-μl aliquots. The ESR spectrum of the first aliquot was recorded directly, whereas 10 μl of 100 mM ferricyanide was added to the second aliquot to reoxidize the reduced fraction of the label before ESR measurement; reoxidation of the labels was completed within 3 min.

In a second series of experiments, the labeled erythrocyte suspension was first incubated at 4°C for 4 hr to allow inward translocation of the labeled phospholipids with minimal spin-label reduction and afterwards incubated at 37°C as

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Abbreviations: (0,2)PtdCho, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylcholine; (0,2)PtdSer, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine; (0,2)PtdEtn, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylethanolamine; (0,2)Sph*P*Cho, sphingomyelin derivative *N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine; BSA, bovine serum albumin.

above. Some samples of erythrocyte suspensions were Mg^{2+}-depleted at this stage by adding 10 μM ionophore A23187 and 2 mM EDTA in the suspension.

Data Analysis. The following quantities correspond each to a mole fraction of the total spin labels, either oxidized or reduced, present in a sample. $E_o(t)$ represents the fraction of oxidized spin label on the external leaflet at a given time t, and this is assessed by BSA extraction of the label at time t. $E(t)$ represents the total fraction of spin label on the external layer at time t and is measured by reoxidation of the BSA-extracted label by ferricyanide. Finally, $E_R(t)$ represents the fraction of reduced label on the external leaflet. Similarly, one can define the molar fractions: $I_R(t)$, $I_o(t)$, and $I(t)$ associated with the label on the inner leaflet. However, only the quantities associated with the labels on the external layer can be directly measured.

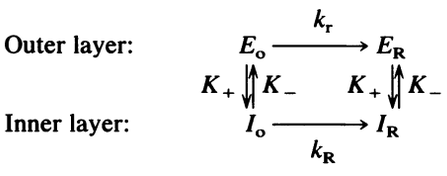
Two quantities are determined as a function of the incubation time for graphical representation: (*i*) the nonextractable fraction of the label:

$$I(t) = 1 - E(t),$$

and (*ii*) the reduced fraction of the label extracted from the membrane outer layer:

$$R(t) = E_R(t)/E(t) = [E(t) - E_o(t)]/E(t).$$

A simplified kinetic model, not intended to be comprehensive, is used to deduce the characteristic time constant of the outward translocation from the experimental data:



where K_+ and K_- are, respectively, the inward and outward translocation rate constants, and k_r and k_R are the rate constants of spin-label reduction, respectively, at the outer and inner face of the membrane. Values of k_r and k_R were directly measured in separate experiments.

The corresponding system of differential equations was numerically solved using a fourth-order Runge and Kutta method. I^* and R^* are the computed values of I and R; they were compared to the experimental quantities for each value of the discrete variable t. For these simulations, k_r and k_R were fixed within the range afforded by direct measurements; notice that we departed slightly from the average measured value whenever it was useful to get better fit. Values for K_+ were estimated from the initial slope of the experimental plot of $I(t)$. Only the *outward* translocation rate, K_-, was systematically varied until the sum of the squares:

$$\sigma^2 = \sum [R^*(t) - R(t)]^2 + \sum [I^*(t) - I(t)]^2$$

reached a minimum. The resultant value of K_- was taken as an estimate of the outward transport rate.

RESULTS

Measurement of the Rate Constants of Spontaneous Spin-Label Reduction by the Intracellular and Extracellular Medium. The intracellular medium was made accessible to the spin labels by sonication of pelleted cells. By this procedure, all spin labels were at a membrane interface and now exposed to the original *intracellular* medium. This procedure allowed us to estimate k_R. The following values were obtained: 3.5 ± 0.7 × 10^{-2} min^{-1}, 4.1 ± 0.7 × 10^{-2} min^{-1}, and 3.8 ± 0.7 × 10^{-2} min^{-1} for (0,2)PtdSer, (0,2)PtdEtn, and (0,2)PtdCho, respectively. Note that k_R values are very similar for the polar head groups of the three spin labels. As for the reducing power of the extracellular medium, we found a rate of 6 ± 2 × 10^{-3} min^{-1} for (0,2)PtdCho and (0,2)Sph*P*Cho when added to erythrocytes at 37°C. The latter value was used to characterize the reduction rate of the spin labels at the extracellular face of the membranes (k_r) because these spin labels have a very slow outside→inside diffusion constant through the erythrocyte membrane.

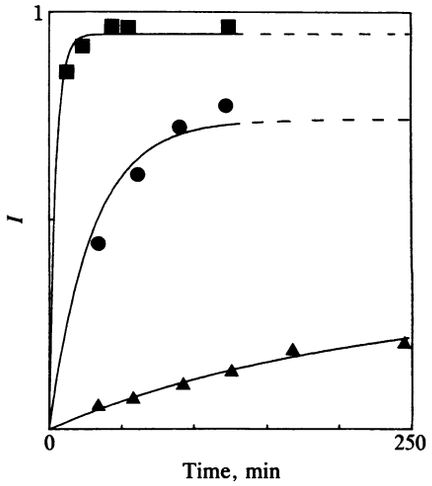


FIG. 1. Transmembrane inward transport kinetics at 37°C, for three spin-labeled phospholipids. ■, (0,2)PtdSer; ●, (0,2)PtdEtn; ▲, (0,2)PtdCho. I is the fraction of labels that is not extracted by BSA and thus corresponds to the fraction of the label located on the inner erythrocyte leaflet. The curves are calculated from a model (see text) that fits the data of this figure and those of Fig. 2. The fixed parameters in the curve fitting (namely, k_R, k_r, and K_+) were selected within the range determined by direct measurements (see Table 1). The value of K_- is the floating parameter used for minimization.

Kinetics of Inward and Outward Passages of Spin-Labeled Lipids Through the Erythrocyte Membrane. Fig. 1 is a plot of the quantity I versus time of incubation, where I represents the nonextractable fraction of label—i.e., the fraction of label that accumulates on the inner layer. The rates of inward transport (K_+) can be determined from the initial slopes of the curves in Fig. 1 and are listed in Table 1. Results show that the inward translocation rate of the spin-labeled phospholipids decrease according to the order PtdSer>PtdEtn>PtdCho. The theoretical curves (I^*) shown in Fig. 1 were determined as explained in *Materials and Methods*, and they lead to the extrapolated values of the asymptotic distribution

Table 1. Characteristic rate constants of inward and outward translocation of (0,2)PtdSer, (0,2)PtdEtn, and (0,2)PtdCho in human erythrocytes at 37°C

	(0,2)PtdSer	(0,2)PtdEtn	(0,2)PtdCho
K_+, 10^{-2} min^{-1}	21 ± 1	2.0 ± 0.5	0.15 ± 0.05
τ, min	3.29	34.6	461
K_-, 10^{-2} min^{-1}	1.2 ± 0.2	0.9 ± 0.2	0.3 ± 0.1
τ, min	57.7	77.0	230

K_+ and τ are, respectively, the rate and half time for outside → inside translocation, whereas K_- and τ correspond to the inside → outside movement. Values for K_+ (and τ) were determined from the initial slopes in Fig. 1. Values for K_- were obtained by fitting *simultaneously* $R(t)$ and $I(t)$ in Figs. 1 and 2 by the model indicated in *Materials and Methods*. The uncertainties indicated for K_- and K_+ are estimated from the fits of a single series of measurements of I and R, such as those shown in Figs. 1 and 2, and not by SDs of a series of different experiments. τ is deduced by a straightforward inversion from K_+ (and K_-).

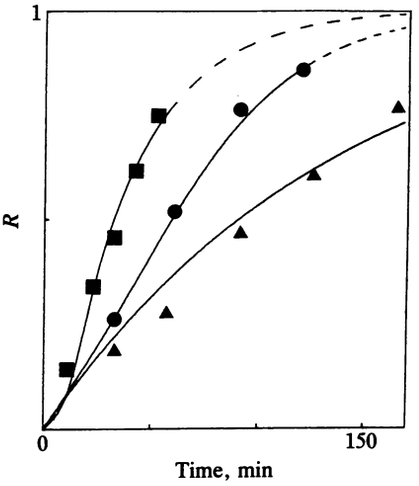


FIG. 2. Signal reduction kinetics at 37°C for three spin-labeled phospholipids. R is the fraction of reduced spin-labeled phospholipids on the membrane outer leafet. ■, (0,2)PtdSer; ●, (0,2)PtdEtn; ▲, (0,2)PtdCho. The curves are obtained as in Fig. 1; the same set of parameters (k_R, k_r, K_+ and K_-) describe the theoretical curves of Figs. 1 and 2.

of the spin labels of 95% (0,2)PtdSer on the inner membrane leaflet, 75% for (0,2)PtdEtn and 28% for (0,2)PtdCho.

Fig. 2 shows R, the fraction of reduced label on the outer layer, as a function of incubation time. When the external spontaneous reduction is neglected and when the internal reduction can be considered as fast (see *Discussion*), R should be associated with the fraction of labels on the outer layer, which has been, at least once, on the inner layer. Thus, the initial slopes in Fig. 2 indicate the rates of outward motion of the lipids. These rates decrease (as in Fig. 1) in the following order: PtdSer>PtdEtn>PtdCho. But the differences, as revealed by the difference in slopes, are smaller between the outward than between the inward translocation rates. The theoretical curves (R^*) result from the same minimization as I^* (see *Materials and Methods*) and thus correspond to the same set of parameters. In practice, the curve fitting in Fig. 1 is not only constrained by the data plotted in Fig. 1 but also by the data of Fig. 2 and vice versa. The calculated values of K_- (Table 1) confirm that the outward translocation rate is faster for the aminophospholipids, (0,2)PtdSer and (0,2)PtdEtn, than for the zwitterionic (0,2)PtdCho, although as indicated previously, the difference is not so large as for the inward translocation rate.

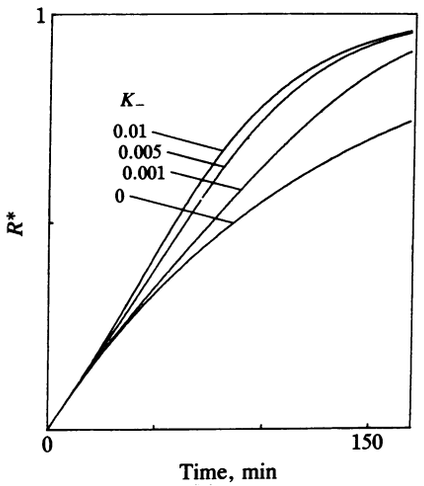


FIG. 3. Calculated R^* curves for various K_- values expressed in min^{-1}. The other parameters were fixed to the values used for (0,2)PtdEtn simulation in Fig. 2.

Table 2. Characteristic rate constants of inward and outward translocation of (0,2)PtdSer and (0,2)PtdEtn in Mg^{2+}-depleted human erythrocytes at 37°C from Fig. 4

	(0,2)PtdSer	(0,2)PtdEtn
K_+, 10^{-2} min^{-1}	1.0 ± 0.5	1.0 ± 0.5
τ, min	69.3	69.3
K_-, 10^{-2} min^{-1}	0.11 ± 0.10	0.14 ± 0.10
τ, min	630	495

The determination of these parameters is explained in the text and in Fig. 4 legend.

Fig. 3 shows the theoretical changes of R^* when the magnitude of K_- is varied, whereas K_+, k_r, and k_R are fixed to the value estimated for (0,2)PtdEtn. For example, the difference between the curves corresponding to $K_- = 0.01$ min^{-1} and $K_- = 0$ min^{-1} demonstrates how R is expected to vary from the existence of an outward transport of this lipid.

Effect of Magnesium Depletion. When erythocytes are Mg^{2+} depleted by incubation with A23187 and EDTA, the selective outside → inside translocation of aminophospholipids was considerably diminished (14). Values of K_+ for the spin-labeled aminophospholipids were determined at 37°C with Mg^{2+}-depleted cells from the initial slopes in plots of I versus time (see Table 2; the corresponding figures are not shown). Because of the reduced outside → inside translocation rates in Mg^{2+}-depleted cells, the determination of K_- from the results of the previous experiments is not appropriate. Thus, we have carried out a slightly different experiment in which samples were preincubated at 4°C to allow the labels to first accumulate on the inner layer at a temperature where spontaneous reduction by the cytosol is negligible (1). Half of the cells were Mg^{2+} depleted, and the other half were used as control cells. Then temperature was brought to 37°C, and I and R were determined as before. The results of such experiments done with (0,2)PtdSer are shown (Fig. 4). The discrepancy between the theoretical curve representing R and the experimental values at the beginning of the process

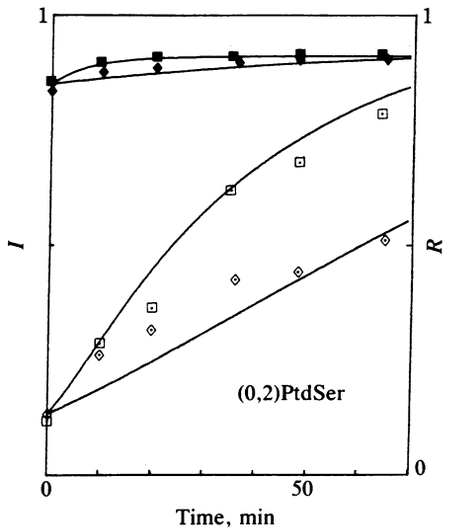


FIG. 4. Plots of experimental values of I and R and best theoretical fits for (0,2)PtdSer for Mg^{2+}-depleted cells and in control cells. The values of the parameters used are listed in Table 2. In these experiments the cells were first incubated 4 hr at 4°C and then Mg^{2+} depleted at 37°C. Time zero in this figure corresponds to the moment where the temperature was brought to 37°C. Control cells: I (■) and R (⊡). Mg^{2+}-depleted cells: I (♦) and R (◈).

is probably because the Mg^{2+} depletion is not instantaneous, taking ≈15 min at 37°C for completion (see ref. 14). Overall, the rate of appearance of reduced (0,2)PtdSer on the membrane outer layer is significantly lower in Mg^{2+}-depleted cells than in control cells. A similar result (data not shown) was obtained for (0,2)PtdEtn. For both (0,2)PtdSer and (0,2)PtdEtn, K_- is decreased by Mg^{2+} depletion (see Table 2) to values close to that for untreated cells labeled with (0,2)PtdCho.

DISCUSSION

In a previous paper (1), it was shown that the inward translocation rate of the spin-labeled analogues of aminophospholipids is much faster than that of phosphatidylcholine and that the equilibrium transbilayer distribution of these aminophospholipids is consistent with direct determinations on endogenous lipids (9). This result was found by incubating the erythrocytes at 4°C and by using selective reduction by ascorbate of the labels on the outer membrane leaflet. With our method (incubation at 37°C, BSA-extraction, and reoxidation of the extract by ferricyanide) a good agreement for translocation rates determined earlier (1) was obtained (Fig. 1), except for higher translocation rates at higher temperature. In addition, we have been able to extrapolate a value for the stationary distribution of the phosphatidylcholine analogue, which was not possible at 4°C due to slow (0,2)PtdCho diffusion. This value is also consistent with measurements of endogenous lipid transmembrane asymmetry (9). Thus, the BSA extraction technique at physiological temperature is a valuable method for determining transmembrane orientation of amphiphilic spin-labeled lipids.

The data that refer to the reduced form of the labels on the outer membrane face (Fig. 2) provide evidence for an outward transport of the spin-labeled aminophospholipids, taking some account of the intracellular and extracellular reducing power. To demonstrate, it is convenient to consider the limiting situation in which the reducing power of the extracellular medium is negligible when compared with that of the intracellular medium. Then, reduced spin-labeled phospholipids present on the outer membrane leaflet are explicable only if the labeled compounds had spent some time on the inner leaflet; in other words, the labels "recall" their passage to the inner leaflet. This is a specific feature of spin labels, which cannot be easily achieved with either radioactive or fluorescent probes.

However, the reducing power of the extracellular medium, even though lower than that of the intracellular medium, is not completely negligible. This fact can be verified by measuring the reduction rates of (0,2)Sph*P*Cho or (0,2)PtdCho incorporated into erythrocyte membrane outer leaflets. Such an experiment allows a good estimate of the reduction rate k_r of labels in the membrane outer leaflet because of the very slow translocation of these labels to the inner leaflet (1, 12). Thus, the outward transport rates must be reckoned by considering the extracellular reduction. In practice, an accurate value of K_- is obtained only when $K_- >> k_r$. This situation, however, is not so for (0,2)PtdCho. In Fig. 2, for example, the curve corresponding to (0,2)PtdCho could be explained only by external label reduction—i.e., $K_- \approx 0$. The small difference seen was used to place an upper limit to the (0,2)PtdCho outward diffusion rate (Table 1). On the other hand, the experimental R values for (0,2)PtdSer and (0,2)PtdEtn do not fit the theoretical curve corresponding to $K_- = 0$. This discrepancy proves that fast outward motion certainly occurs for the aminophospholipids.

An important point is the overall coherence of the data fit displayed in Figs. 1 and 2 by a kinetic model including outward lipid translocation. We emphasize that a satisfactory fit of both series of data (R and I) for each phospholipid analogue was achieved by using the same set of values for K_+, K_-, k_r, and k_R. This selection allowed us to draw unequivocal values for the outward diffusion rate of lipids from the experimental data.

It remains to interpret the difference seen between the outward translocation rates of the three phospholipid analogues used here. First, notice that when inward active translocation is inhibited, for instance by Mg^{2+} depletion, residual inward passage of (0,2)PtdSer or (0,2)PtdEtn is identical to that of (0,2)PtdCho (1, 12). No difference of passive transverse diffusion rates between the three phospholipid analogues can then be invoked to explain the outward rate difference between the aminophospholipid analogues and (0,2)PtdCho. This difference could indicate the activity of an outward aminophospholipid carrier protein. Although it is difficult in these experiments to test the role of ATP in outward passage, the partial inhibition of outward motion after Mg^{2+} depletion suggests that the outward carrier protein behaves under similar conditions as the inward carrier. Indeed, Mg^{2+} is known to be required (1, 14) with ATP for selective, protein-mediated inward aminophospholipid translocation. The same protein might be involved for the inward and outward aminophospholipid transport. The efficiency of the transport would not need to be the same in both directions. Thus, an asymmetric distribution of lipids could be generated by this "aminophospholipid translocase." A paradoxical situation has been suggested in which the aminophospholipid carrier would work equally well for inward as for outward transport, and the fact that apparent outward transport rate is lower than inward transport rate would be due to selective interaction of the aminophospholipids with intracellular spectrin (11). This explanation is paradoxical because ATP-driven pumps do not function in opposite directions. In addition, the influence of spectrin on aminophospholipid asymmetry is still debatable. Recently we have shown that heat-induced vesicles from human erythrocytes, which are severely spectrin depleted, can still selectively accumulate aminophospholipids on the inner layer (15).

The model used to analyze the experimental data throughout this paper is a simple diffusion model that is not rigorous for an active transport process. In particular, we have shown previously that the inward translocation rate is ATP and concentration dependent (1, 2). We recognize the simplifications introduced here and used by other investigators (10, 11). The diffusion model is considered, however, as a first approximation, and a more sophisticated model may be developed later with more experimental data. The present model has the advantage of minimizing the number of unknown properties. One consequence of having an active inward transport of phospholipids is that the K_+ value can vary significantly from one sample to another due to fluctuations in intracellular ATP content; we found fewer changes of K_- values between different preparations, which could indicate that K_- is less sensitive to ATP than K_+.

Protein-mediated inward motion of aminophospholipids discovered first in erythrocytes has been found also in other cells (6–8). Thus, the outward protein-mediated motion of aminophospholipids probably also exists in other cell membranes, which could have important physiological consequences. To illustrate, certain conditions, such as change of cytosolic Ca^{2+} concentration, will very rapidly affect the outward rate (unpublished results) and result in rapid redistribution of phosphatidylserine, which, in turn, could affect specific membrane enzymic activity; protein kinase C activity, for example, was shown to be influenced by phosphatidylserine concentration (16).

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