Ca^{2+} Induces Transbilayer Redistribution of All Major Phospholipids in Human Erythrocytes†

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ABSTRACT: Elevating cytoplasmic Ca^{2+} levels in erythrocytes activates a pathway for transbilayer diffusion of plasma membrane phospholipids. The use of spin-labeled and fluorescent phospholipid analogues revealed that the pathway permits diffusion of all the major classes of phospholipids and does not distinguish between the two types of probes. Diffusion was bidirectional, began immediately upon elevation of cytoplasmic [Ca^{2+}] above 50–100 μM, persisted as long as the [Ca^{2+}] remained elevated, and disappeared promptly when Ca^{2+} levels fell. Diffusion was unaffected by conditions which suppress shedding of vesicles, discounting this event as a requisite for phospholipid reorientation induced by Ca^{2+}.

The distribution of phospholipids is normally asymmetric across the plane of the plasma membrane of erythrocytes (Verkleij et al., 1973; Kahlenberg et al., 1974; Gordesky et al., 1975) and platelets (Schick et al., 1976; Chap et al., 1977; Bevers et al., 1982), with the aminophospholipids phosphatidylethanolamine (PE)[1] and PS mostly, or completely (respectively), concentrated in the inner leaflet and the neutral phospholipids PC and Sph mostly, or completely (respectively), concentrated in the external leaflet. Studies of the transbilayer movement of specific phospholipids (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Zachowski et al., 1986; Tilley et al., 1986; Connor & Schroit, 1987; Morrot et al., 1989) and their interaction with cytoskeletal proteins (Pradhan et al., 1989, 1991) have implicated an ATP-dependent aminophospholipid translocase in the maintenance of this asymmetric lipid distribution. This enzyme moves PS, and less efficiently PE, from the outer to the inner leaflet in an energy-dependent reaction. Its molecular identity remains a matter of current debate: Schroit and co-workers have argued that the Rh antigen is the molecule responsible for this activity (Schroit et al., 1987, 1990; Connor & Schroit, 1989), while comparative studies have suggested that the erythrocyte membrane Mg-ATPase is the enzyme (Morrot et al., 1990).

While translocase-mediated movement of PS and PE may be critical to the maintenance of lipid asymmetry, it is unclear whether this activity plays any role in the disruption of lipid asymmetry which occurs during platelet activation (Bevers et al., 1982, 1983) or when Ca^{2+} ions are admitted to the cytoplasm of erythrocytes, either by incubating the cells with ionophore and Ca^{2+} (Williamson et al., 1985; Chandra et al., 1987) or by lysing and resealing them in the presence of Ca^{2+} (Williamson et al., 1985; Connor et al., 1990: Verhoven et al., 1992). Although the translocase activity may be essential for reversing this loss of asymmetry (Comfurius et al., 1990; Verhoven et al., 1992), the pathway by which phospholipids initially equilibrate across the bilayer in response to Ca^{2+} is still largely uncharacterized. As a first step in delineating this equilibration process, we have used spin-labeled and fluorescent phospholipid probes to monitor lipid movement in human erythrocytes treated with ionophore and Ca^{2+}.

Materials and Methods

Erythrocytes were obtained by venipuncture from healthy volunteers, and used within 1 h. Cells were pelleted at 7600*g* for 5 min, the buffy coat was removed, and the erythrocyte pellet was washed 3 times with and then suspended in buffer B (120 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 5 mM adenosine, 1 mM inosine, 0.1 mM EGTA, and 20 mM sodium phosphate, pH 7.4) or HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 mM inosine, 0.1 mM EGTA, and 5 mM Na-HEPES, pH 7.5). As indicated in some cases, high-potassium versions of these buffers (buffer BK) containing 90 mM KCl, and correspondingly less NaCl were used. Because of their relatively high K^+ content, these buffers minimize Ca^{2+}-induced loss of K^+ and resulting cell shrinkage (Gardos, 1958). After being washed, cells were incubated for 15 min at 0 °C with 5 mM freshly prepared diisopropyl fluorophosphate (DFP, Sigma) to prevent degradation of internalized spin-label (Morrot et al., 1989); control experiments indicated that treatment had no effect on any of the lipid movements, or lack thereof, reported herein. For measurements of vesicle release, this step was omitted. Cells were then diluted to a final hematocrit of 30% and, where indicated, were treated with 1 mM sodium vanadate (Sigma) for 15 min at 0 °C before transfer to a 37 °C water bath for measurement of lipid movement.

Spin-labeled [1-palmitoyl, 2-(4-doxylpentanoyl)] phospholipids were prepared as described previously (Morrot et al., 1988). Fluorescent NBD-PC (acyl chain derivative) was purchased from Avanti Polar Lipids (Pelham, AL); NBD-PS was prepared from NBD-PC by transphosphatidylation as

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SL, spin labeled; Sph, sphingomyelin.

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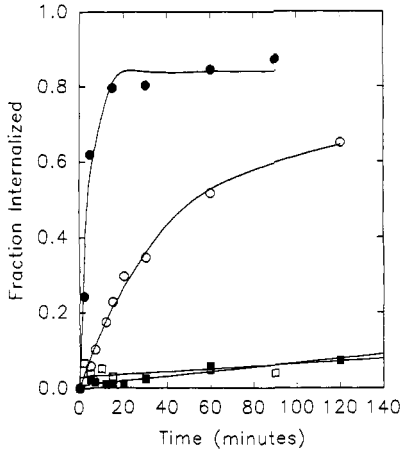

FIGURE 1: Internalization of fluorescent and spin-labeled PS and PC by normal human erythrocytes. Spin-labeled (SL) and NBD-labeled phospholipids were introduced into the external leaflet of erythrocytes, and the fraction of the added probe which was internalized was measured as label which could not longer be extracted with BSA. (●) SL-PS; (■) SL-PC; (○) NBD-PS; (□) NBD-PC.

described by Juneja et al. (1988) and purified by preparative thin-layer chromatography. To label cells, spin-labeled phospholipids, resuspended in buffer B by vigorous vortexing after evaporation of solvent ($CHCl_3$ or $CHCl_3$/methanol) with argon, were added to prewarmed cells to a final concentration of about 1% of the endogenous phospholipids, followed by addition of 20 μM A23187 (Sigma, from a 5 mM stock in DMSO/ethanol) and 1 mM $CaCl_2$, unless otherwise noted. The concentrations of Ca^{2+} noted in the text are the final excess over the 0.1 mM EGTA present in BK buffer. Under these conditions, cell lysis was less than 2%, as measured by hemoglobin release. Measurement of the amount of probe in the external leaflet was made by removing 200-μL aliquots to prechilled tubes containing 15 μL of 100 mg/mL lipid-free BSA (Sigma) in buffer. After 2 min at 0 °C, tubes were centrifuged for 2.5 min in an Eppendorf tabletop centrifuge (7600*g*); 100 μL of the supernatant was removed and assayed for probe. Spin-labeled samples were mixed with 10 μL of 0.1 M potassium ferricyanide to oxidize any label which had been reduced during the incubation, and the amount of label present was quantitated as described previously (Morrot et al., 1989). Fluorescent probes were used similarly, except that probe was added to cells at 0 °C, incubated for 10 min on ice to allow complete incorporation, and after addition of ionophore and $CaCl_2$, the cells were transferred to prewarmed tubes at 37 °C to initiate movements. From these incubations, 70-μL aliquots were removed to 10 μL of 120 mg/mL BSA, and 40-μL aliquots of the BSA extract were transferred to Triton X-100 buffer for quantitation as described previously (Colleau et al., 1991).

Results

In the absence of Ca^{2+}, the transbilayer movement of phospholipids in erythrocytes is controlled by the aminophospholipid translocase. To monitor such movement, phospholipid probes are incorporated into the outer leaflet by incubating cells and probe together. Cells are then incubated at 37 °C for various times, and the amount of label remaining in the outer leaflet was determined by washing cells with BSA-containing buffer to remove probe still on the cell exterior. Figure 1 illustrates the specificity of the translocase pathway in erythrocytes for some of the probes used in this study. The translocase rapidly moves spin-labeled PS, but not PC, from the outer to the inner leaflet. This same specificity

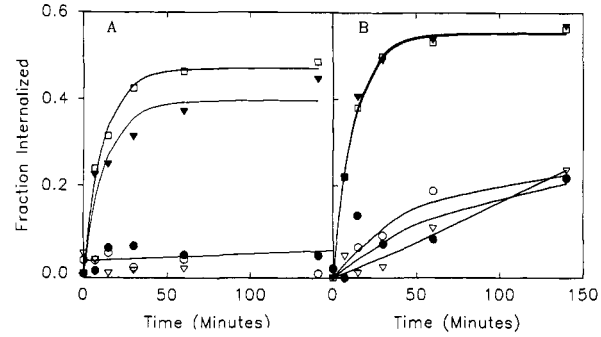

FIGURE 2: Ca^{2+}-induced internalization of PC. Internalization of spin-labeled PC was measured as in Figure 1. (A) Cells not pretreated with vanadate. (B) Cells pretreated with vanadate. (●) Controls with no Ca^{2+} or ionophore. (○) Controls in buffer containing Ca^{2+}, but no ionophore. (▽) Controls treated with ionophore in buffer lacking Ca^{2+}. (▼) Cells treated with ionophore in buffer containing 400 μM free Ca^{2+}. (□) Cells treated with ionophore in buffer containing 900 μM free Ca^{2+}.

is apparent with fluorescent NBD-labeled PS and PC, but the transport of NBD-PS is markedly slower than transport of its spin-labeled counterpart (Colleau et al., 1991).

This pattern of phospholipid movement to the cell interior is markedly altered when cytoplasmic [Ca^{2+}] is elevated. As shown in Figure 2A, the normally slow inward movement of spin-labeled PC was unaffected by either Ca^{2+} or ionophore alone. When both were added, however, PC was rapidly internalized, reaching a plateau in less than 30 min where roughly equal amounts of probe were present in each leaflet. Enhanced diffusion of PC induced by Ca^{2+} was observed as rapidly as measurements could be made after cells were warmed to 37 °C, i.e., within 3 min; no movement was observed during preincubation on ice (data not shown). Kinetic analysis indicated that the rate of PC movement from the outer to the inner leaflet was increased 20–40-fold over its normal value (to about 0.03 min^{-1}); this rate is about 25% of that observed for the ATP-dependent inward movement of PS catalyzed by the aminophospholipid translocase in these same cells (0.13 min^{-1}).

To make a fair comparison of the rate of Ca^{2+}-induced redistribution of spin-labeled PC with the rate of spin-labeled PS and PE movement, vanadate was used to inhibit the ATP-dependent inward movement of the latter aminophospholipids. As shown in Figure 2B, when Ca^{2+} and ionophore were added to vanadate-treated cells, PC was rapidly internalized, reaching a plateau in roughly 30 min, just as in untreated cells. Thus, inhibition of the translocase had no effect on this process. Besides inhibiting the aminophospholipid translocase, vanadate also inhibits the erythrocyte Ca-ATPase (Rossi et al., 1981), thereby eliminating any effects resulting from alteration in Ca^{2+} levels by the enzyme. As also seen in Figure 2B, pretreatment with vanadate eliminated the small but reproducible difference in rates between 400 and 900 μM free Ca^{2+} seen in untreated cells, suggesting that Ca-ATPase activity accounted for this difference. In contrast to translocase-mediated lipid movement, the Ca^{2+}-induced pathway for lipid redistribution was insensitive to the nature of the reporter group affixed to the probe. As shown in Figure 3, the rate of movement of spin-labeled and NBD-labeled fluorescent PC was similar in Ca^{2+}-treated cells.

Also apparent in these experiments was an increased rate of PC internalization in vanadate-treated cells in the absence of cytoplasmic Ca^{2+}, suggesting that a pathway for transbilayer lipid diffusion was opened in the membrane by vanadate treatment alone. To avoid this effect, other inhibitors of the translocase (NEM or vanadyl ions) were tested as alternatives

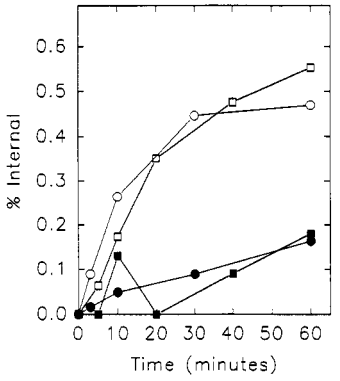

FIGURE 3: Ca^{2+}-induced internalization of spin-labeled PC and NBD-PC. Internalization of spin-labeled PC or NBD-PC by erythrocytes pretreated with vanadate was measured in Figure 1. Solid symbols: Controls without Ca^{2+}. Open symbols: Experimental samples. (■, □) SL-PC. (●, ○) NBD-PC.

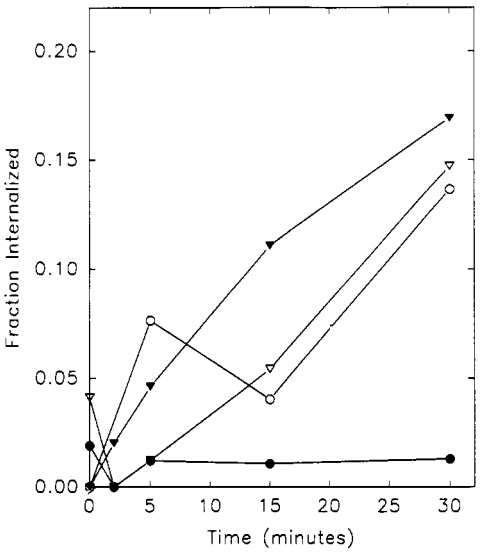

FIGURE 4: Headgroup specificity of vanadate-induced lipid internalization. Internalization of spin-labeled phospholipids by vanadate-treated cells was measured as in Figure 1. (●) Sph; (○) PS; (▼) PC; (▽) PE.

to vanadate, but could not be used because these treatments did not completely eliminate transport of PE and PS by the translocase. The rates of spin-labeled PC, PE, PS, and Sph internalization by this vanadate-induced pathway were compared in cells incubated in the absence of Ca^{2+}. As shown in Figure 4 for one set of such experiments, PC, PE, and PS moved at similar rates, while Sph remained completely extractable from the outer leaflet. Since the aminophospholipid translocase is specific for PS and PE, and in addition normally transports PS into the internal leaflet at a rate 20–100-fold higher than it transports PE, the finding that PS, PE, and PC were internalized at equivalent rates shows that the enzyme is strongly inhibited by the pretreatment with vanadate, and also suggests that the translocase does not participate in the pathway opened by vanadate.

Plateau values of roughly 50% redistribution of PC as seen in Figures 2 and 3 might represent an approach to equilibrium, with internalization balanced by an equivalent rate of diffusion from the internal to the external leaflet, i.e., a cessation only in net movement. Alternatively, these values might result from the gradual closing of the pathway for diffusion opened by Ca^{2+}, i.e., an actual cessation in all movement. These possibilities were distinguished by incubating unlabeled cells with Ca^{2+} and ionophore for 60 min at 37 °C (at which time internalization would normally have reached a plateau) and then

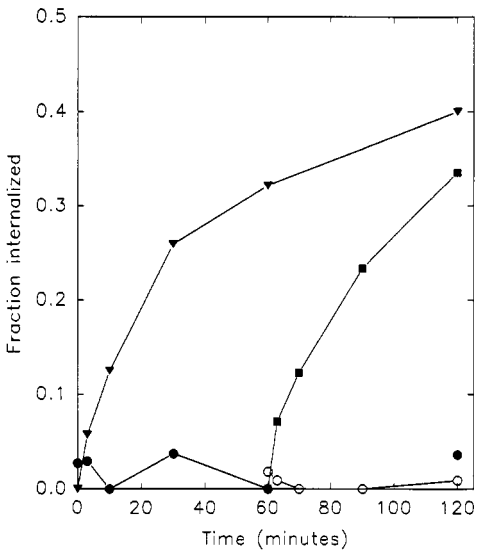

FIGURE 5: Ca^{2+}-induced internalization of NBD-PC after preincubation with Ca^{2+} and ionophore. Vanadate-treated cells were divided into three aliquots, and NBD-PC was added to one pair of aliquots. After addition of Ca^{2+} and ionophore to one labeled and one unlabeled aliquots, all were incubated at 37 °C, with samples of labeled cells taken to measure internalization. At 60 min, all aliquots were chilled briefly, and label was added to the two remaining aliquots. All aliquots were then rewarmed and samples were taken as indicated. (▼) NBD-PC and Ca^{2+} added at 0 time. (●) NBD-PC at 0 time with no added Ca^{2+}. (■) Ca^{2+} added at 0 time; NBD-PC added at 60 min. (○) NBD-PC added at 60 min, no Ca^{2+}.

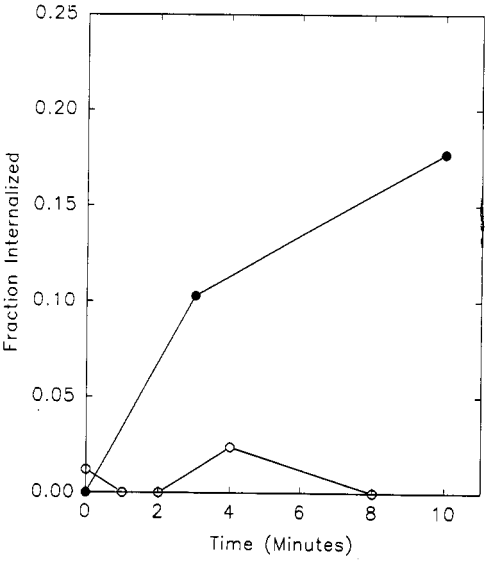

FIGURE 6: Shutdown of the Ca^{2+}-induced pathway. Ca^{2+} and ionophore were added to vanadate-treated erythrocytes, and the mixture was incubated for 60 min. The sample was then chilled and divided in two, NBD-PC was added to both aliquots, and one was made 1 mM in EGTA. Both were then rewarmed and samples taken as indicated. (●) Label only added at 60 min. (○) Label and EGTA added at 60 min.

adding probe. As shown in Figure 5, internalization of NBD-PC added at 60 min proceeds with essentially the same kinetics as probe added at the beginning of the incubation with Ca^{2+}, indicating that the pathway remains patent for at least 2 h. To determine whether the continued presence of Ca^{2+} is required for the maintenance of this pathway, a similar experiment was performed, except that EGTA was added at 60 min along with the probe. As shown in Figure 6, movement is halted by this treatment, indicating that the continued presence of Ca^{2+} is required for the maintenance of the pathway.

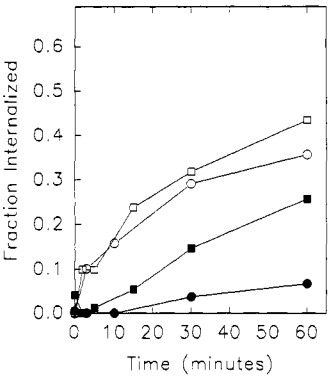

FIGURE 7: Ca^{2+}-induced internalization of spin-labeled PS and NBD-PS. Vanadate-treated cells were labeled with spin-labeled PS or NBD-PS, and internalization was measured as in Figure 1. Filled symbols: no Ca^{2+} added. Open symbols: Ca^{2+} added. (■, □) SL-PS. (●, ○) NBD-PS.

Table I: Rates[a] of Ca^{2+}-Induced Movement of Phospholipid Probes in Erythrocytes

	headgroup			
label	PS	PC	PE	Sph
NBD	2.7 ± 0.5 (7)	2.4 ± 0.9 (7)	ND[b]	ND
SL	3.8 ● 1.6 (3)	2.9 ± 1.1 (11)	2.9 ± 2.2 (9)	0.89 ± 0.2 (3)

[a] Rates (min^{-1}) are means ± standard deviation for the number of experiments given in parentheses. [b] Not determined.

To determine the specificity of the pathway for lipid movement induced by Ca^{2+}, PS, PE, and Sph were examined. The results with PS probes are shown in Figure 7. In addition to indicating that the pathway supports the redistribution of PS, the results demonstrate that both fluorescent PS and spin-labeled PS were internalized at similar rates, as was the case for Ca^{2+}-induced PC movement, and in contrast to translocase-mediated movement of PS. Data for the rate of Ca^{2+}-induced probe movement of all probes examined are collected in Table I, and indicate that PC, PS, PE, and Sph are all capable of redistributing through the Ca^{2+}-induced pathway.

The fact that the pathway induced by Ca^{2+} remains open for at least 2 h at 37 °C (Figure 5, above) suggested that the approach to a plateau observed for all the probes represented an approach to equilibrium with probe internalization being balanced by probe externalization. Externalization of probe can be demonstrated directly by allowing the translocase to internalize PS, followed by washing to remove any residual probe on the external surface and addition of Ca^{2+} and ionophore. Such an experiment is shown in Figure 8. Addition of Ca^{2+} results in prompt reappearance of the previously internalized probe on the external surface, while in the absence of added Ca^{2+}, the probe remains on the inside over the time scale of the experiment. The rate of reappearance of probe is comparable to the rate of internalization of probe from the external leaflet, providing direct evidence that the pathway permits lipid movement in both directions across the bilayer.

Elevation of cytoplasmic Ca^{2+} levels in erythrocytes results in the release of small vesicles from the plasma membrane; if these vesicles were not removed by centrifugation in BSA-containing buffers, probe associated with them, whether external or not, would be found in the supernatant and be considered as external. However, because the data are corrected for probe which remains in the supernatant in the absence of BSA, release of vesicles cannot be misconstrued as externalized probe. Nevertheless, since formation of vesicles has been suggested as an essential step in the reorientation of phospholipids during platelet activation (Sims et al., 1989; Wiedmer et al., 1990), a role of vesicle release was tested by comparing probe movement in the presence of high and low levels of K^+ in the suspending buffer, since high K^+ suppresses vesicle release (Allan & Thomas, 1981). Direct assay of vesicle release by measuring released acetylcholinesterase confirmed that high [K^+] reduced vesicle release at least 2-fold, from 43% to 18% of the total activity (data not shown). Yet, as shown in Figure 8, the rate of PS reorientation was no different in high- and low-K^+ buffers, suggesting that vesicle release is not required for PS reorientation in erythrocytes. Similar results were obtained for influx of PS, PC, PE, and Sph probes (data not shown), indicating that the properties of the Ca^{2+}-induced pathway are similar whether or not vesicle release is inhibited.

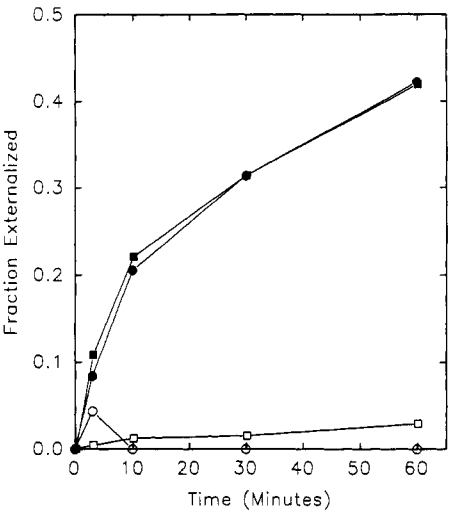

FIGURE 8: Ca^{2+}-induced externalization of NBD-PS at high and low [K^+]. Erythrocytes were incubated with NBD-PS for 60 min at 37 °C in either high or low K^+-containing saline. The cells were then chilled, washed with BSA-containing buffers to remove any remaining external probe, treated with vanadate, and then divided into two aliquots. To one was added ionophore and Ca^{2+}, while the other received no Ca^{2+}. The aliquots were then rewarmed, and samples were taken at intervals and washed with BSA-containing buffer to extract NBD-PS which had returned to the external surface. (●) + Ca^{2+}, low [K^+]; (○) −Ca^{2+}, low [K^+]; (■) +Ca^{2+}, high [K^+]; (□) −Ca^{2+}, high [K^+].

To determine the minimal concentration of Ca^{2+} at which an increase in the internalization rate could be observed, probe movement was assessed in cells treated with differing concentrations of Ca^{2+}. As shown in Figure 9 for PE, an increase in rate was readily discernible at a free Ca^{2+} concentration as low as 100 μM. In experiments with the other phospholipids, the dependence on Ca^{2+} concentrations was similar (data not shown).

Discussion

Previous investigations of the effects of elevating cytoplasmic [Ca^{2+}] on the transbilayer distribution of phospholipids in erythrocytes delineated the final distribution of endogenous lipids (Williamson et al., 1985) or fluorescent tracer lipid analogues (Connor et al., 1990) following treatment, and the changes in lipid packing which normally accompany rearrangements of the bulk endogenous lipid distribution (Verhoven et al., 1992). However, none of these studies, nor comparable ones in platelets (Bevers et al., 1982, 1983), have shed any light on the kinetics of movement of the various phospholipid species. Using spin-labeled and fluorescent phospholipid analogues, we have shown here that introduction of Ca^{2+} into intact cells induces an increase in the rate of transbilayer movement of all four of the major phospholipid

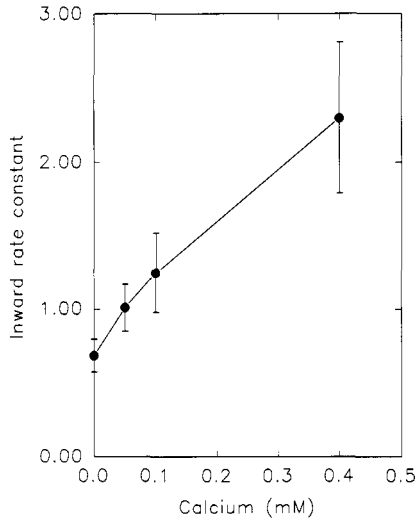

FIGURE 9: $[Ca^{2+}]$ dependence of internalization of PE. Spin-labeled PE was introduced into the external leaflet of vanadate-treated erythrocytes, and the internalization was measured as in Figure 1 in the presence of ionophore and various levels of Ca^{2+}. Data from two separate series of reactions (eight experiments) were averaged, the apparent internalization rate constants were determined for Ca^{2+} concentration, and these constants are plotted versus the Ca^{2+} concentration. The error bars represent the uncertainty in the estimation of the rate constant from the actual kinetic data. The differences between the data for all the points except 0.05 mM Ca^{2+} are statistically significant ($p < 0.05$; paired Wilcoxon signed rank test).

types. Moreover, the rates of movement of PC, PE, and PS do not differ significantly (whether spin- or NBD-labeled). This bidirectional movement is temperature and Ca^{2+} content dependent.

Several possible mechanisms for this enhanced transbilayer diffusion can be critically evaluated in light of the data provided in this study. Ca^{2+} can inactivate the aminophospholipid translocase (Bitbol et al., 1987). If Ca^{2+} acts by uncoupling lipid movement from ATP hydrolysis, the enzyme might remain a pathway for lipid movement, but be incapable of influencing the direction of that movement. In this case, the known specificity of the translocase predicts that the specificity of movement would be biased strongly toward PS, with a lesser prediliction for PE and none at all for PC or Sph. The data clearly speak against this model.

Another possibility is that Ca^{2+} operates by inducing the formation of inverted micelles at sites where membrane fusion releases vesicles (Sims et al., 1989; Wiedman et al., 1990). If phospholipids redistributed through these sites, headgroup specificity should be biased toward those lipids which can easily enter such a nonbilayer phase, and in particular PE should reorient more readily than PC (Ellens et al., 1989). Again, our observation that the pathway does not preferentially facilitate redistribution of PE or PS does not lend support to this mechanism. This conclusion is consistent with the observation that suppression of vesicle release by use of high-K^+ buffers has no effect on phospholipid distribution.

This study also delineates several properties of the pathway. It can be opened by as little as 5×10^{-5} M Ca^{2+}. Within the resolution of the techniques used here, the pathway opens immediately, remains open with unchanged properties for at least 2 h, and closes promptly when the level of cytoplasmic Ca^{2+} falls. These results suggest that the pathway is not simply membrane damage, a conclusion supported by the fact that cell lysis under the conditions used here is not progressive. The pathway is insensitive to inhibition by DFP (used here to prevent lipid breakdown in the presence of Ca^{2+}) and NEM (data not shown).

The broad specificity of movement suggests that the pathway may be the same one by which Ca^{2+} induces accelerated transbilayer movements of such diverse species as palmitoylcarnitine (Henseleit et al., 1990) and platelet activating factor (Bratton et al., 1991). Given that the effective Ca^{2+} concentration for opening the pathway is well above that normally experienced within the red cell cytoplasm, the pathway is probably activated rarely, if ever, during the normal lifespan of erythrocytes, although redistribution of phospholipids has been suggested to target senescent erythrocytes for removal from the circulation (Schroit et al., 1985; McEvoy et al., 1986). However, Ca^{2+} is released into the cytoplasm at such levels during activation of platelets (Hallam et al., 1984), suggesting that this mechanism might account for the overall lipid randomization which occurs in these close relatives of erythrocytes (Bevers et al., 1983, 1990). Substantial internal reservoirs of vesicular Ca^{2+} are also present in sickle erythrocytes (Lew et al., 1985; Rubin et al., 1986), suggesting that this mechanism may contribute to the elevated nonspecific lipid movements which can occur in these cells (Blumenthal et al., 1991), predisposing them to activation of a pathway for transbilayer redistribution of phospholipids which could contribute to the pathology of sickle cell disease.

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CORRECTION

Fluorescence, CD, Attenuated Total Reflectance (ATR) FTIR, and ^{13}C NMR Characterization of the Structure and Dynamics of Synthetic Melittin and Melittin Analogues in Lipid Environments, by Arthur J. Weaver, Marvin D. Kemple,* Joseph W. Brauner, Richard Mendelsohn, and Franklyn G. Prendergast*, Volume 31, Number 5, February 11, 1992, pages 1301–1313.

Page 1303. Equation 3 should read

$$\bar{r}/r_0 = \frac{1 - S^2}{1 + \tau_f(\tau_e^{-1} + \tau_m^{-1})} + \frac{S^2}{1 + (\tau_f/\tau_m)} \qquad (3)$$

Calculated results presented in the paper were made with the correct version of eq 3.