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Rapid loss and restoration of lipid asymmetry by different pathways in resealed erythrocyte ghosts

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The normal asymmetric distribution of phospholipids across the plasma membrane of erythrocytes can be abolished by lysing and resealing cells in the presence of Ca^{2+}. In the present study, using flow cytometric analysis of the binding of merocyanine 540 to monitor transbilayer phospholipid distribution, Ca^{2+}-induced loss of asymmetry is shown to be independent from the aminophospholipid translocase which catalyzes movement of normally internal phospholipids from the outer to the inner leaflet of the membrane. Loss of asymmetry is rapid, temperature-sensitive, and occurs in an uninterrupted, intact bilayer, rather than by diffusion of lipids through the hemolytic pore. Addition of ATP during lysis reverses loss of asymmetry, and this restoration can be blocked by inhibitors of the aminophospholipid translocase. These results suggest that the ATP-dependent translocase is essential for recovery of asymmetry, in turn suggesting that separate mechanisms mediate the loss and the recovery of lipid asymmetry in erythrocytes.

Introduction

Phospholipid distribution across the erythrocyte plasma membrane is asymmetric, with the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) concentrated in the inner monolayer, and the two choline phospholipids sphingomyelin and phosphatidylcholine located predominantly in the outer monolayer [1]. Vesicles reconstituted from purified erythrocyte lipids do not spontaneously adopt this asymmetric arrangement [2], indicating that establishing and maintaining transbilayer asymmetry requires a mechanism supplied by the cell.

Early analyses of lipid asymmetry depended on the use of reagents which assessed the distribution of endogenous lipids across the bilayer. The results of these investigations suggested that lipid asymmetry is a consequence of a selective interaction between aminophospholipids and cytoskeletal proteins [2–6]. More recently, however, reagents which measure the inward movement of trace amounts of phospholipid analogs introduced into the outer leaflet of the plasma membrane have revealed that PS and PE are translocated by an ATP-dependent process [7–10]. The translocation mechanism is specific for aminophospholipids [7,9,11,12] and sensitive to sulfhydryl reagents [11–13], vanadate [14], temperature [12] and intracellular Ca^{2+} concentrations [14], implicating a specific enzyme activity, the aminophospholipid translocase, in the maintenance of lipid asymmetry. Following its discovery in erythrocytes, a similar activity has been found in the plasma membranes of platelets [15], lymphocytes [16], fibroblasts [17] and in chromaffin granule membranes [18]. While the existence of this activity is not disputed, direct evidence that it can generate an asymmetric distribution of phospholipids starting from a symmetric distribution remains unavailable [19,20].

Interwoven with the question of how lipid asymmetry is established and maintained is the issue of how it is lost when Ca^{2+} is introduced into the cytoplasm of the cell. In erythrocytes, phospholipase digestion of the lipids in the external leaflet [21–23], the prothrombinase complex assay [24,25] and 'back-exchange' of NBD-lipids into acceptor membranes [26] have all been used to document Ca^{2+}-induced loss of asymmetry. In platelets as well, lipid asymmetry is lost during activation by collagen plus thrombin or by treatment with A23187 and Ca^{2+} [27,28]. The specific mechanism re-

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sponsible for this disruption of lipid asymmetry remains to be articulated. Introduction of Ca^{2+} into erythrocyte ghosts by lysis and resealing is a potentially useful system for analyzing this mechanism, particularly because it permits manipulation of the cytoplasmic milieu for investigation of the cytoplasmic factors involved in regulation of lipid asymmetry.

Binding of the fluorescent probe merocyanine 540 (MC540) to the outer leaflet of normal and pathologic intact erythrocytes [29–32], ghosts [21], and membrane vesicles derived from normal, aged and sickle erythrocytes [29,33,34] has been shown to reflect their endogenous lipid distribution, determined by the more direct phospholipase methods. Increased binding of the dye upon loss of asymmetry is likely a consequence of the probe's property of binding more strongly to loosely packed lipid bilayers than to those more tightly packed [35]. The inner leaflet of the plasma membrane is normally more fluid than the outer leaflet [36], so that equilibration of inner leaflet lipids between the two leaflets upon loss of lipid asymmetry results in the external lipid leaflet becoming less ordered [37], enhancing its affinity for MC540. Although indirect, this method has the advantage over other methods of assessing endogenous phospholipid distribution that it is rapid and nondisruptive.

In this report we use flow cytometric analysis of MC540 binding to erythrocyte ghosts to characterize Ca^{2+}-induced loss of lipid asymmetry. We also show that loss of asymmetry can be reversed, providing the first evidence that an asymmetric bilayer can be generated from a symmetric one by a mechanism with properties identical to those of the aminophospholipid translocase.

Materials and Methods

Chemicals

Phosphate-buffered saline contained 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4, 8.5 mM KH_2PO_4, and 1 mM Mg^{2+} (PBS/Mg). Lysis buffer contained $1/50 \times$ PBS, 1 mM Mg^{2+}, and Ca^{2+} at various concentrations as described in the text. ATP, adenylyl imidodiphosphate, BSA, lucifer yellow, MC540, *N*-ethylmaleimide (NEM), sodium fluoride, sodium vanadate and the ATP estimation kit were purchased from Sigma Chemical Co. (St. Louis, MO). The MC540 quencher carbocyanine D-1389 was obtained from Molecular Probes, Inc. (Eugene, OR). MC540 and lucifer yellow were used from a 1 mg/ml stock solution in water.

Preparation of ghosts

Blood was collected in heparinized tubes and washed three times in PBS/Mg, carefully removing the buffy coat. Washed erythrocytes were kept on ice and used within 6 h.

Lysis was performed by addition of ice-cold lysis buffer to an aliquot of approximately 10^8 washed erythrocytes at a ratio of buffer volume to aliquot volume as indicated in the text. In experiments using ATP, fluoride or NEM, these compounds were dissolved directly in lysis buffer immediately before cell lysis. After 5 min at 0°C, lysed cells were resealed by restoring isotonicity with the appropriate amount of $10 \times$ PBS followed by incubation at 37°C for 30 min unless otherwise mentioned in the text. Resealed ghosts were collected by centrifugation at 5000 rpm for 4 min in a Sorval HB-4 rotor at 4°C and washed twice with 1 ml ice-cold PBS/Mg.

MC540 staining

Two slightly different MC540 staining methods were used, described as staining method I or II in the figures.

Method I. Approximately 10^8 washed ghosts were resuspended in 1 ml of ice cold PBS/Mg containing 0.1% BSA and MC540 at 1 μg/ml, and then transferred to a dry bath at 37°C. After 4 min at 37°C, stained ghosts were collected by centrifugation at 4°C and washed twice in 1 ml of ice-cold PBS/Mg containing 0.1% BSA.

Method II. Approximately 10^8 washed ghosts were resuspended in 1 ml of PBS/Mg containing 0.1% BSA and MC540 at 1 μg/ml, which had been preheated to 37°C. Immediately after resuspension in this staining solution, the samples were collected by centrifugation at 4°C and washed once in 1 ml of ice-cold PBS/Mg containing 0.1% BSA.

Flow cytometry

Stained ghosts were held on ice in 0.5 ml PBS/Mg containing 0.1% BSA and analyzed within 30 min on a Beckton-Dickinson FACScan flow cytometer. The excitation source was a 15 milliwatt, 488 nm, argon-ion laser. Fluorescence emission was detected using a 585 nm band pass filter.

Leakiness testing

Ghosts were prepared as described above using lysis buffer containing 5 μg/ml lucifer yellow. The experimental sample was prepared in the presence of 0.4 mM Ca^{2+} and was incubated at 37°C for 15 min after restoring isotonicity as usual. The leaky control was prepared by lysing in the absence of Ca^{2+}, and never raising the temperature after restoration of isotonicity. Both samples were washed two times with ice cold PBS/Mg. Suspensions were examined by brightfield and fluorescence microscopy.

ATP measurements

ATP concentration in ghosts was determined using the Sigma enzymatic diagnostic test kit No. 366-UV,

with ghosts being used in the assay instead of whole blood.

Results

Ghosts prepared by lysis and resealing in the presence of Mg^{2+} as the sole divalent cation bind quantities of MC540 much the same as untreated whole cells, indicating retention of the normal plasma membrane lipid arrangement. Inclusion of Ca^{2+} along with Mg^{2+} in the lysis buffer, however, results in ghosts which bind increased amounts of MC540, indicative of a perturbed lipid arrangement [38]. Analysis of phospholipid distribution by phospholipase digestion indicates that the ghosts prepared in the absence of Ca^{2+} retain an asymmetric distribution of phospholipids, whereas those prepared in its presence have a symmetric distribution [21]. To determine the level of Ca^{2+} required to abolish the normal arrangement of phospholipids, erythrocytes were hypotonically lysed and resealed in the presence of various concentrations of Ca^{2+}, stained with MC540, and analyzed by flow cytometry. As shown in Fig 1, ghosts prepared with only Mg^{2+} (curve b) exhibited essentially the same affinity for MC540 as untreated whole cells (curve a), while addition of Ca^{2+} to the lysis buffer resulted in ghosts which were about 10-fold more fluorescent (curves c–h). This effect was half-maximal at about 0.3 mM Ca^{2+} (curve f) under the conditions of lysis used (1 : 10 ratio of cell pellet to lysis buffer volume), with an abrupt disappearance of normally staining ghosts between 0.25 mM and 0.3 mM Ca^{2+} (curves e vs. f).

The increased fluorescence of ghosts prepared with Ca^{2+} might be a consequence of leakiness, which would increase staining by allowing MC540 access to the inner leaflet. To test this possibility, lucifer yellow was loaded into ghosts by including it in the lysis buffer.

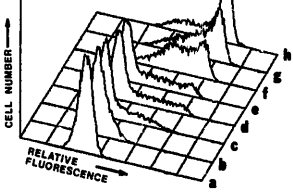

Fig. 1. MC540 staining of whole cells and 1:10 dilution ghosts made at various concentrations of Ca^{2+}. Staining method II. (a) Whole cells; (b) 0.0 mM Ca^{2+}; (c) 0.1 mM Ca^{2+}; (d) 0.2 mM Ca^{2+}; (e) 0.25 mM Ca^{2+}; (f) 0.3 mM Ca^{2+}; (g) 0.35 mM Ca^{2+}; (h) 0.4 mM Ca^{2+}.

This water-soluble fluorescent probe is about the same size as MC540, and like MC540, depends on sulfonate residues for its membrane impermeability. Leaky ghosts can therefore be detected by their inability to retain loaded lucifer yellow during washing, as shown in Figs. 2a and 2b. As shown in Figs. 2c and 2d, ghosts lysed and resealed in the presence of 0.5 mM Ca^{2+} uniformly retained lucifer yellow during washing, indicating their non-leakiness. Additionally, the MC540 fluorescence of ghosts is abolished by external application of the aqueous quencher carbocyanine D-1389 (data not shown and [21]), indicating by its accessibility to the dye that MC540 resides in the outer leaflet and has not translocated or otherwise gained access to the inner leaflet of the plasma membrane.

The existence of an endogenous phospholipase A_2-like activity in erythrocytes represents a possible alternative explanation for these data: Ca^{2+} activation of the enzyme might generate sufficient lysophospholipids to increase MC540 staining in the absence of any change in lipid asymmetry. In order to test this possibility, cells were pre-treated with and ghosts prepared in the presence of diisopropylfluorophosphate, an inhibitor of phospholipid hydrolysis activity [39]: the kinetics, magnitude and Ca^{2+}-sensitivity of MC540 staining was not affected (data not shown). Additionally, in previous studies where exogenous phospholipase A_2 was used as a probe for lipid location [21], controls without added phospholipase produced a similar yield of lysophospholipids whether ghosts were prepared with or without Ca^{2+}. These results do not support the view that endogenous phospholipase A_2 has a role in the increase of MC540 staining induced by Ca^{2+}.

Ghosts can be protected from Ca^{2+}-induced loss of lipid asymmetry by decreasing the volume of buffer used to lyse the cell pellet [21]. When ghosts were prepared at a 1:5 dilution, rather than at the 1:10 dilution used in Fig 1, 0.4 mM Ca^{2+} could not induce staining (data not shown). Conversely, when ghosts were prepared at a 1:20 dilution, a Ca^{2+} concentration 2-fold lower than that effective at a 1:10 dilution was sufficient to induce maximal staining in half the ghosts, as shown in Fig. 3. These results suggest that some limiting cytoplasmic component which compensates for the effect of increased Ca^{2+} is lost upon dilution.

The existence in erythrocytes of an ATP-dependent inward transport of exogenously added PS raises the possibility that ATP is the cytoplasmic component whose dilution is critical in modulating the Ca^{2+}-promoted loss of lipid asymmetry. This possibility was tested by asking whether addition of ATP to the lysis buffer could prevent loss of asymmetry at a Ca^{2+} concentration and dilution otherwise sufficient to abolish it. As shown in Fig. 4, inclusion of ATP during lysis at a 1:20 dilution in the presence of 0.4 mM Ca^{2+} did

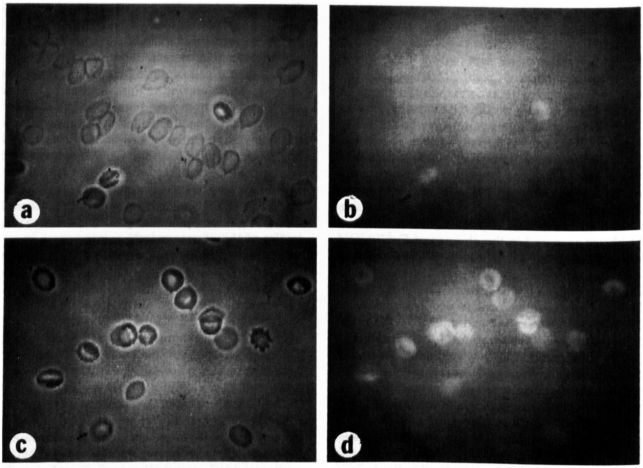

Fig. 2. Leaky and resealed ghosts loaded with lucifer yellow at lysis. (a and b) Ghosts lysed in the absence of Ca^{2+} and returned to isotonicity but not incubated at 37°C (leaky). (c and d) Ghosts lysed in the presence of Ca^{2+}, returned to istonicity and incubated 15 min. at 37°C (resealed). (a and c) Bright-field micrographs. (b and d) Fluorescence micrographs of the same field.

prevent loss of asymmetry, with a half maximal effect at about 0.15 mM ATP. It should be noted that chelation of Ca^{2+} by ATP is unlikely to account for this effect, since the Ca^{2+} concentration (0.4 mM) exceeds the final calculated ATP concentration (0.225 mM; ATP added plus remaining endogenous ATP) by an amount sufficient to induce complete loss of asymmetry in ghosts prepared at this dilution (see Fig. 3).

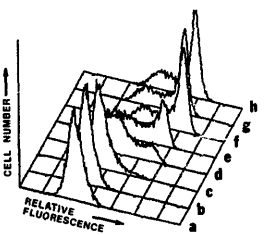

Fig. 3. Ca^{2+} concentration-dependent loss of lipid asymmetry in 1:20 dilution ghosts. Staining method I. (a) 0.0 mM Ca^{2+}; (b) 0.05 mM Ca^{2+}; (c) 0.1 mM Ca^{2+}; (d) 0.15 mM Ca^{2+}; (e) 0.175 mM Ca^{2+}; (f) 0.2 mM Ca^{2+}; (g) 0.3 mM Ca^{2+}; (h) 0.5 mM Ca^{2+}.

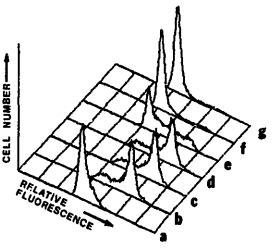

Fig. 4. ATP-dependent prevention of Ca^{2+}-induced loss of lipid asymmetry. 1:20 dilution ghosts were prepared in the presence of 0.4 mM Ca^{2+}. Staining method I. (a) control, no Ca^{2+} present; (b) 0.0 mM ATP; (c) 0.05 mM ATP; (d) 0.1 mM ATP; (e) 0.2 mM ATP; (f) 0.3 mM ATP; (g) 0.4 mM ATP.

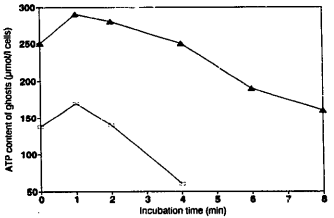

Fig. 5. ATP levels in ghosts during incubation at 37°C. 1:10 dilution ghosts were prepared in the presence of 0.4 mM Ca^{2+}; with 0.1 mM added ATP (▲) and without added ATP (□). Data of a single experiment, representative of three so-performed.

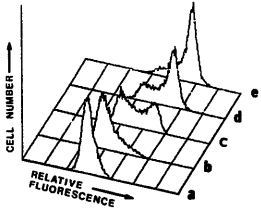

Fig. 6. Minimun Ca^{2+} concentration requirements of Ca^{2+}-induced loss of lipid asymmetry. Cells were pretreated 15 min at 37°C with 2.0 mM vanadate then made into 1:26 dilution ghosts in the presence of 2.0 mM vanadate and various concentrations of Ca^{2+}. Staining method I. (a) 0.0 mM Ca^{2+}; (b) 0.05 mM Ca^{2+}; (c) 0.1 mM Ca^{2+}; (d) 0.15 mM Ca^{2+}; (e) 0.2 mM Ca^{2+}.

To determine the effect of the added ATP on available levels of ATP, the actual concentration of ATP present was measured. As shown in Fig. 5, the intracellular ATP concentration in ghosts prepared with Ca^{2+} made at a 1:10 dilution ratio without added ATP increased slightly from 0.140 mM to 0.170 mM at 1 min (probably due to the activities of endogenous adenylate kinase [40]) and then declined to below 0.050 mM within 4 min into the 37° resealing step. Ghosts prepared with 0.10 mM added ATP (the minimal amount required for restoration of lipid asymmetry in 1:10 ghosts) demonstrated a similar initial rise in total ATP from 0.250 mM to 0.290 mM in the first min, followed by a decline to 0.190 mM after 8 min. Calculations using reported binding constants for the formation of complexes of ATP with Mg^{2+} and Ca^{2+} [41] indicated that at the start of the incubation, 0.194 mM of the 0.250 mM total ATP present is available in the physiologically useful $MgATP^{-}$ complex. Furthermore, this value is expected to rise significantly by 1 min into the resealing step due to the measured increase of total ATP present and the extrusion of Ca^{2+} via the Ca^{2+}-ATPase. These data, combined with the inability of the nonhydrolyzable ATP analog, adenylyl imidodiphosphate, to prevent loss of asymmetry (data not shown), strongly suggest that ATP is acting as an energy source.

Identification of ATP as the cytoplasmic component responsible for modulating Ca^{2+}-induced loss of lipid asymmetry permits quantification of the minimum intracellular Ca^{2+} concentration needed to activate inside-outside phospholipid translocation. Cells were pretreated with and ghosts maded in the presence of vanadate as an ATP antagonist. As demonstrated in Fig. 6, half maximal loss of lipid asymmetry was seen at 0.10 mM Ca^{2+}, which represents the minimum intracellular Ca^{2+} concentration required, in the absence of ATP, to activate Ca^{2+}-induced loss of asymmetry and which is in agreement with observations made using 'back-exchange' of NBD-lipids [26].

Since preparation of ghosts involves both a lysis step, carried out at 0°C at low ionic strength, and a resealing step at 37°C at physiological ionic strength, a simple model might explain the effect of ATP: lysis in the presence of Ca^{2+} permits rapid equilibration of lipids across the bilayer, perhaps at the periphery of the hole(s) in the membrane; after resealing, inward transport of the externalized aminophospholipids restores asymmetry if ATP is available. However, contrary to this model, when cells were lysed in the presence of Ca^{2+}, and then resealed and analysed without incubation at 37°C (save about 30 s during staining to allow efficient MC540 binding), no increase in MC540 fluorescence was seen (Fig. 7, curve c), indicating that loss of asymmetry had not occurred during lysis. To determine when asymmetry was subsequently lost, lysed

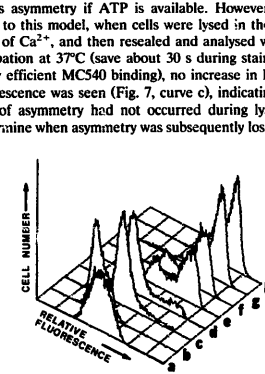

Fig. 7. Time-dependent loss of lipid asymmetry in 1:10 dilution ghosts. Samples c–h were lysed with 0.4 mM Ca^{2+} present. All samples were returned to isotonicity at 0°C followed by a 37°C incubation for the length of time indicated. Staining method II. (a) No Ca^{2+}, 0.5 min; (b) no Ca^{2+}, 10 min; (c) 0.5 min; (d) 1 min; (e) 1.5 min; (f) 2 min; (g) 3.5 min; (h) 10.5 min.

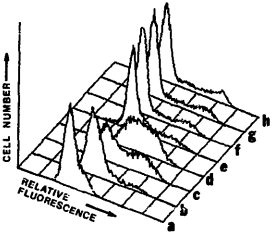

Fig. 8. ATP-dependent reversal of Ca^{2+}-induced loss of lipid asymmetry in 1:10 dilution ghosts. Samples b–h were lysed with 0.4 mM Ca^{2+} and 0.1 mM ATP present, returned to isotonicity at 0°C, and incubated at 37°C for length of time indicated. Staining method II. (a) No Ca^{2+}, 16 min; (b) 0.5 min; (c) 1 min; (d) 1.5 min; (e) 2.5 min; (f) 4.5 min; (g) 8.5 min; (h) 16 min.

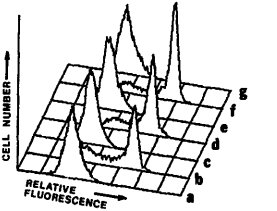

Fig. 9. Inhibition of ATP-dependent reversal of Ca^{2+}-induced loss of asymmetry in 1:10 dilution ghosts. Staining method II. (a) Control: no Ca^{2+} or ATP; (b) control: 0.4 mM Ca^{2+}, no ATP; Samples c–g had 0.12 mM ATP included at lysis. (c) 0.4 mM Ca^{2+}; (d) 0.4 mM Ca^{2+}, 5 mM NEM; (e) no Ca^{2+}, 5 mM NEM; (f) 0.4 mM Ca^{2+}, 25 mM fluoride; (g) no Ca^{2+}, 25mM fluoride.

and resealed ghosts were shifted to 37°C for increasing lengths of time, then chilled back to 0°C, washed and analyzed. As shown by curve d of Fig. 7, after a total of 1 min at 37°C (including the 0.5 min staining period), the fluorescence of ghosts prepared in the presence of Ca^{2+} was not increased. Membrane integrity was apparently efficiently reestablished at the lower temperature, since a leaky membrane would have resulted in bright staining of the cell interior. Indeed, resealing may be more effective in the presence of Ca^{2+} (cf curves a and c), a result consistent with previous studies of the effects of divalent cations upon erythrocyte resealing [42,43]. Continued incubation of the ghosts at 37°C resulted in the appearance of the staining phenotype at 1.5 min, with most of the cells showing this phenotype after 3.5 min (curves e–h). These results imply that lipid asymmetry is lost *after* the membrane has regained its integrity as a diffusion barrier, and that loss is rapid, temperature dependent and requires Ca^{2+}.

ATP could be acting by either preventing loss of asymmetry induced by Ca^{2+} or by restoring it once it was lost. To distinguish these possibilities, the previous experiment was repeated with ATP present in the lysis buffer. As shown in Fig. 8, ATP did not block the initial increase in MC540 staining (curves c and d). However, in the presence of ATP, the progressive increase in MC540 binding was halted, then reversed, resulting in the reappearance of the normal staining pattern by about 3 min after the onset of lipid reorientation (curves e–h). These results demonstrate that the rapid loss of lipid asymmetry can be equally rapidly reversed. They also raise the possibility that the ATP-dependent aminophospholipid translocase acts to restore lipid asymmetry following Ca^{2+}-induced outward translocation of internal phospholipids.

If the aminophospholipid translocase activity is involved in reestablishing lipid asymmetry, NEM, an inhibitor of the translocase, should prevent restoration of asymmetry, even in the presence of ATP. As shown in Fig. 9, curves c–e, such was the case. While inclusion of NEM in the lysis buffer had no effect on the retention of asymmetry in the absence of added Ca^{2+}, or on its loss in the presence of Ca^{2+}, it completely prevented the return of asymmetry in the presence of Ca^{2+} and added ATP. As shown in Fig. 9, curves f and g, similar results were observed with fluoride, an inhibitor of the ATPase II activity from adrenal chromaf-

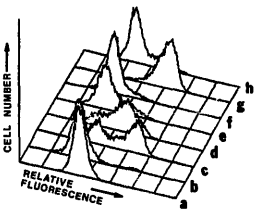

Fig. 10. Vanadate inhibition of ATP-dependent reversal of Ca^{2+}-induced loss of lipid asymmetry in 1:10 dilution ghosts made in the presence of 0.4 mM Ca^{2+} and 0.12 mM ATP. Samples were incubated at 37° for the length of time indicated. Staining method II. (a) Control: no Ca^{2+}, 8 min; (b) 0.5 min; (c) 1 min; (d) 1.5 min; (e) 2.5 min; (f) 4.5 min; (g) 3 mM vanadate added 1 min into the 37°C incubation, followed by an additional 4 min at 37°C; (h) 3 mM vanadate added 4 min into the 37°C incubation, followed by an additional 4 min at 37°C.

fin granules [44] that has been suggested to correspond to the translocase [18].

Vanadate is a potent, membrane permeable [45] inhibitor of the translocase, and was thus useful in inhibiting the activity at various points along the reaction pathway. As shown in Fig. 10, curve g, addition of vanadate at 1 min of incubation at 37°C completely prevented the restoration of asymmetry by ghosts prepared with Ca^{2+} and ATP (curves b–f). If, however, vanadate addition was postponed until 4 min of incubation, lipid asymmetry was uniformly regained (curve h). These results indicate that by 4 min, the Ca^{2+}-induced mechanism responsible for loss of lipid asymmetry is no longer operating. They also demonstrate that once asymmetry has been reestablished in Ca^{2+}-loaded ghosts, it is stable and cannot be abolished simply by inhibiting the translocase.

Discussion

In this report, we have exploited MC540 in a rapid and quantitative flow cytometric assay for transbilayer lipid asymmetry to investigate in greater detail the mechanisms controlling lipid asymmetry in erythrocytes. We find that loss of lipid asymmetry induced in ghosts prepared with Ca^{2+} is complete within 4 min of incubation at 37°C and is half-maximal at 0.10 mM Ca^{2+} in the absence of ATP. Moreover, loss can be reversed by a process which requires hydrolyzable ATP, and is sensitive to vanadate, NEM, and fluoride. These characteristics are all consistent with participation of the translocase in the process and are, in fact, all the characteristics currently available for identifying translocase-dependent lipid movement. Given that the translocase is ATP saturable with a reported K_m in the range of 220–260 μM [46], our data indicate that suffient ATP is present, in 1:10 ghosts with 100 uM added ATP, to drive the translocase at half V_{max} during the time that Ca^{2+}-induced loss of asymmetry is being reversed. If maximal MC540 staining is equated with complete loss of lipid asymmetry, restoration of PS asymmetry in the time observed here would require roughly 60–80 molecules of enzyme per cell calculated from the turnover rate of the enzyme [44,47], assuming one lipid molecule is translocated per ATP hydrolyzed.

Upon inactivation of the aminophospholipid translocase, phospholipid asymmetry might be expected to decay spontaneously by unopposed passive diffusion of phospholipids between the two leaflets of the bilayer. Remarkably, however, at 0°C, such diffusion does not take place, even in the presence of an obvious path for diffusion, the lytic pore. Yet at 37°C, lipid randomization in resealed ghosts prepared in the presence of Ca^{2+} is complete in minutes, despite the fact that passive phospholipid flip-flop in erythrocyte membranes occurs with a $t_{1/2}$ of hours at 37°C [48]. This rapid inside to outside movement of aminophospholipids suggests that erythrocytes contain a Ca^{2+}-dependent, facilitated transport mechanism for membrane phospholipids. Although loss is inhibited at 0°C, it proceeds normally in the presence of vanadate, fluoride, or NEM, suggesting that it is not mediated by the ATP-dependent aminophospholipid translocase operating in reverse [19,49].

Ca^{2+} concentrations above 50 μM are sufficient to activate calpain under the conditions used here to prepare erythrocyte ghosts (data not shown). In platelets, calpain activation has been linked to the rapid movement of PS to the outer leaflet which occurs upon activation [27,28]. However, calpain is strongly inhibited by NEM [50], while the data presented here show that Ca^{2+}-induced loss of asymmetry is not sensitive to this reagent. Several other potential mediators of a Ca^{2+} signal are present in erythrocytes, including calmodulin [51], phospholipase C [52], protein kinase C [53], and a member of the annexin family of Ca^{2+}-dependent membrane binding proteins [54]. Further experiments will be required, however, to implicate or rule out these proteins as a part of the mechanism responsible for loss of asymmetry.

It has been reported that the ATP-dependent translocase is inhibited by Ca^{2+} concentrations over 1 μM [12], suggesting that the translocase should be shut off upon exposure to the Ca^{2+} concentrations used here in preparing ghosts. This fact alone, however, is insufficient to account for the loss of lipid asymmetry which Ca^{2+} induces, since inhibition of the translocase by vanadate, fluoride, or NEM in ghosts without added Ca^{2+} does not result in loss of lipid asymmetry. However, if the translocase is Ca^{2+}-sensitive, then Ca^{2+} must be extruded from the ghosts before the Ca^{2+}-dependent loss of asymmetry can be reversed. Reduction in intracellular Ca^{2+} concentration can be accomplished by the plasma membrane Ca^{2+}-pump (Ca^{2+}-ATPase), thus permitting reactivation of the translocase: erythrocytes containing ATP in the physiological range have been reported to be able to lower cytoplasmic Ca^{2+} concentrations from 500 μM to 0 μM in less than two minutes [55]. That cytoplasmic Ca^{2+} concentrations may be changing during the course of incubation at 37°C is supported by the observation that arrest of the translocase (and presumably the Ca^{2+} pump) by vanadate at 4 min of incubation after resealing does not result in reexpression of the Ca^{2+}-induced loss of symmetry (Fig. 10). Direct Ca^{2+} measurements will be required, however, to determine what fluctuations in Ca^{2+} concentration actually occur.

The overall working scenario which emerges from these experiments is that upon elevation of cytoplasmic Ca^{2+}, the translocase is inactivated and Ca^{2+}-promoted transverse equilibration of phospholipids begins. In the absence of sufficient ATP, this equilibration goes

to completion; in its presence reduction in Ca^{2+} concentration by the Ca^{2+} pump ensues, reactivating the phospholipid translocase and inactivating the Ca^{2+}-induced equilibration process, allowing asymmetry to be restored.

It should be noted that the plasma membrane Ca^{2+}-pump has the same sensitivity to NEM, vanadate and fluoride as the translocase, raising the possibility that inhibition of the reversal of MC540 staining by these agents is due to the inability of the cell to remove Ca^{2+}. This possibility raises the question of whether Ca^{2+} acts directly to induce MC540 staining, so that its removal by the Ca^{2+}-pump is all that is needed to reverse staining. However, this model is not supported by the data in Fig. 7, curve c, where an internal concentration of 0.4 mM Ca^{2+} is not sufficient, by itself, to induce staining. In any event, direct distinction between the contribution of the translocase and the Ca^{2+} pump will require inhibitors specific to each of these enzymes. Regardless, the major conclusion that the translocase is not involved in *loss* of asymmetry is unaffected.

The data presented here do not directly contribute to the debate over the relative importance of lipid–cytoskeleton interactions and the translocase in maintaining phospholipid asymmetry. Cytoskeletal interactions may explain how lipid asymmetry is maintained during lysis of erythrocytes when the translocase is inhibited, and the translocase may reverse Ca^{2+}-induced loss of lipid asymmetry by facilitating a rearrangement whose final state is dictated by cytoskeletal interactions. The recent identification in chromaffin granules of a molecule which may correspond to the translocase, an identification supported by evidence presented here, may provide an avenue for resolving this long-standing issue.

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