Continuous Analysis of the Mechanism of Activated Transbilayer Lipid Movement in Platelets†

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ABSTRACT: Dithionite reduction of fluorescent (NBD) phospholipids was used as the basis of a continuous assay of transbilayer lipid movement to the cell surface during platelet activation. This assay reveals that virtually all previously internalized phosphatidylserine passes through the external leaflet of the membrane within 90 s after activation with Ca^{2+} and ionophore or with thrombin and thapsigargin. We demonstrate that this lipid scrambling is reversible, bidirectional, and insensitive to the lipid headgroup. Prolonged activation gradually results in inactivation of the scramblase. The assay also reveals that activation of the scrambling activity is sensitive to the sulfhydryl reagent pyridyldithioethylamine, suggesting the involvement of a protein in the process of activated transbilayer lipid scrambling.

In erythrocytes, lymphocytes, and platelets, the two leaflets of the plasma membrane bilayer differ in composition, with the aminophospholipids phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) concentrated in the inner leaflet and the neutral phospholipids sphingomyelin (Sph) and phosphatidylcholine (PC) concentrated in the outer leaflet [for reviews, see Schroit and Zwaal (1991), Williamson and Schlegel (1994), Devaux and Zachowski (1994)]. This asymmetric transbilayer lipid distribution is regulated by two opposing processes. Under normal conditions an ATP-dependent aminophospholipid transporter (Seigneuret & Devaux, 1984) transports PS and PE to the inner leaflet, maintaining asymmetry. This asymmetry can be abolished by a second mechanism that increases the rate of both inward and outward transbilayer movement of all of the preceding phospholipids. This scrambling is pronounced during platelet activation, where it was first detected (Bevers et al., 1982, 1983), but it also occurs in erythrocytes (Williamson et al., 1985, 1992; Schwartz et al.,1985; Chandra et al., 1987) and lymphocytes (Fadok et al., 1992; Schlegel et al., 1993). The process is induced by increased cytosolic Ca^{2+} (Williamson et al., 1985; Verhoven et al., 1991; Verhallen et al., 1988); although the aminophospholipid translocase is inhibited under these conditions (Bitbol et al., 1987), inhibition of this transporter by itself does not result in rapid lipid rearrangement (Haest, 1982; Comfurius et al., 1990; Connor & Schroit, 1990; Henseleit et al., 1990). While the aminophospholipid translocase activity has been tentatively assigned to a 115 kDa protein, the Mg-ATPase of the erythrocyte membrane (Morrot et al., 1990) or ATPase II of chromaffin granule membranes (Moriyama & Nelson, 1988), the nature of the scramblase activity remains unknown.

The identification and characterization of both of these activities have depended heavily on the use of phospholipid analogs, particularly spin- and fluorescent-labeled probes, whose distribution between inside and outside can be determined because external probe is susceptible to extraction. While the behavior of these two classes of lipid probes is similar, but not precisely equivalent in aminophospholipid translocase assays (Colleau et al., 1991; Williamson et al., 1992), they behave identically in assays of the erythrocyte scramblase (Williamson et al., 1992). Such probes, in combination with assays of membrane procoagulant activity (as a measure of surface-exposed endogenous PS) and merocyanine 540 binding assays (as an indicator of the decrease in lipid packing density accompanying the loss of asymmetry), have allowed the identification and characterization of the two activities in a variety of cell types (Williamson & Schlegel, 1994). The paradigm is platelets and erythrocytes treated with Ca^{2+}/ionophore where the scramblase is clearly distinguished from the translocase by its bidirectionality, energy independence, and opposite response to the elevation of intracellular Ca^{2+} (Comfurius et al., 1990; Williamson et al., 1992; Smeets et al., 1994). Equivalent scrambling of all types of phospholipids has been observed (Williamson et al., 1992; Smeets et al., 1994), although one report has suggested that the activation of platelets results in preferential externalization of PS and PE (Bassé et al., 1993). The Ca^{2+}-induced increased lipid movement in erythrocytes is much slower than that in platelets (Smeets et al., 1994), but the existence of a bleeding disorder in which lipid scrambling in both platelets and erythrocytes is impaired (Rosing et al., 1985a; Sims et al., 1989; Bevers et al., 1992) suggests that the scrambling mechanism is similar or related in both cell types.

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[1] Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; NBD-PC, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PS, 1-oleoyl-2-[12-[(7-nitro2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoserine; NBD-Sph, *N*-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]sphingosylphosphocholine; PDA, pyridyldithioethylamine; BSA, bovine serum albumin.

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The ability of sodium dithionite to rapidly abolish the fluorescence of NBD has recently been used to determine the location of NBD-labeled phospholipid probes in Chinese hamster ovary cells (McIntyre & Sleight, 1991), DIDS-treated erythrocytes (Pomorski et al., 1994), and lipid vesicles (Gruber & Schindler, 1994; Balch et al., 1994). Here we demonstrate the use of dithionite to assess the transbilayer distribution of NBD-labeled phospholipids in human platelets. In particular, dithionite offers the unique capability of being able to assay inside to outside probe movement *continuously*, thus providing a powerful tool for studying the scramblase in platelets. Using this assay, we have found evidence for the reversibility, bidirectionality, and nonspecificity of the platelet scramblase. Importantly, we provide a first clue as to the nature of the scramblase by showing that the scramblase itself, or the mechanism by which it is activated, is sensitive to the sulfhydryl-oxidizing agent pyridyldithioethylamine (PDA).

EXPERIMENTAL PROCEDURES

Materials. Calcium ionophore ionomycin, thapsigargin, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). 1-Oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and the corresponding phosphatidylserine analog (NBD-PS) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]hexanoyl]sphingosylphosphocholine (NBD-Sph) was obtained from Molecular Probes (Eugene, OR). Thrombin was purified from bovine blood as previously described (Rosing et al., 1985b). Pyridyldithioethylamine (PDA) was synthesized according to Connor and Schroit (1988). All other reagents were of the highest grade commercially available.

Isolation of Platelets. Washed human platelets were isolated by differential centrifugation of freshly drawn human blood, according to Bevers et al. (1983) with minor modifications. Briefly, platelets were pelleted and washed in Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, and 5 mM glucose (pH 6.6). Because the subsequent labeling of platelets with NBD-phospholipids is blocked by the presence of albumin, this component was omitted from both the washing buffer and the buffer in which the platelets were finally resuspended (Hepes buffer, pH 7.5). Phenylmethane sulfonyl fluoride (PMSF) appears to prevent the degradation of internalized NBD-lipids by an unknown mechanism; therefore, the platelet suspension was incubated with 500 μM PMSF for 30 min at 37 °C prior to labeling with the lipid probe.

Labeling of Platelets with NBD-Phospholipids. PMSF-treated platelets resuspended in Hepes buffer to a cell concentration of 2×10^8/mL were labeled with 1−2 μM NBD-phospholipid (corresponding to approximately 1.25−2.5% of the endogenous lipids) by adding probe from a concentrated stock solution in dimethyl sulfoxide or ethanol (final concentration of solvent was kept below 0.5%). To study the outward movement of NBD-PS, platelets were labeled for 45 min at 37 °C, which resulted in 85−90% internalization of the probe. Incorporation of these lipid analogs, in which the fluorescent NBD group is linked via an acyl chain to the glycerol backbone, did not affect platelet reactivity, unlike the N-substituted NBD-PS, which has been reported to cause platelet activation (Martin et al., 1985).

Back-Exchange of Fluorescent Lipid Probes. Transbilayer movement of NBD-phospholipids was measured using the BSA back-exchange procedure described by Connor et al. (1992). Aliquots of cells were removed at the indicated time intervals and placed on ice in the presence and absence of 1% BSA. Pellets obtained after 3 min of centrifugation at 12000*g* were solubilized in 2 mL of 1% (w/v) Triton X-100, and the amount of internalized lipid was determined by comparing the fluorescence intensity associated with the cells before and after back-exchange. Fluorescence of NBD-phospholipids was measured at a wavelength of 534 nm (λ_{ex} = 472 nm) on a Shimadzu RF-5001 PC spectrofluorimeter (Shimadzu Europe, Duisburg, Germany).

Dithionite Assays. Detection of NBD-lipid present in the platelet outer leaflet was based on the reduction of the NBD group by the dithionite ion, resulting in a loss of fluorescence intensity, as described previously (McIntyre & Sleight, 1991). A discontinuous procedure was followed to compare the dithionite assay with the BSA extraction assay, as depicted in Figure 1: 50 μL of the samples taken at the time intervals for the BSA extraction assay (see earlier) was diluted in 2 mL of Hepes buffer, and fluorescence intensity was measured before and 1 min after the addition of sodium dithionite (from a freshly prepared 1.0 M stock solution in 1.0 M Tris, pH 10) to a final concentration of 5 mM.

A continuous assay based on reduction of the NBD probe by dithionite was carried out as follows: 50−100 μL of NBD-phospholipid-labeled platelets was diluted in 2 mL of Hepes buffer and placed in the fluorometer at 37 °C under continuous stirring. Dithionite was added to a final concentration of 5 mM, either before or after activation of the platelets as indicated in the text. Fluorescence intensity was sampled at 534 nm (λ_{ex} = 472 nm) at 0.5 s intervals. Blank values were obtained after the addition of Triton X-100 to a final concentration of 1%, making all NBD label accessible to dithionite.

Curve Fitting. Data were fit to the equation:

$$I(t) = (I_0 - \mathrm{Blk})(A e^{-k_1(t-t_0)} + B e^{-k_2(t-t_0)}) + \mathrm{Blk}$$

where $I(t)$ is the fluorescence intensity as a function of time (t), I_0 is the intensity at time 0 (t_0), Blk is the residual fluorescence after the addition of Triton X-100, and A and B are the fractions of the label in the pools whose intensity decays at rates k_1 and k_2. Fits were carried out using the curve-fitting packages of Sigmaplot (Jandel Scientific) on MS-DOS machines or Deltagraph (Delta Point, Inc.) on Macintosh machines, with similar results. Simple exponential fits were obtained by setting $k_2 = 0$. Addition of a nondecaying fraction did not improve the quality of the fits.

Miscellaneous. Platelet function after labeling with NBD-lipid was tested by measuring aggregation and release of ATP using a lumiaggregometer (Chronolog Corp.). Degradation of NBD-lipids was detected after extraction according to Reed et al. (1960), followed by separation on HPTLC plates [solvent system: chloroform/methanol/acetic acid/water, 90:40:12:2 (v/v)].

RESULTS

Commonly, translocation of phospholipids is measured by introducing a marked phospholipid into the exterior leaflet of the plasma membrane, allowing time for translocation,

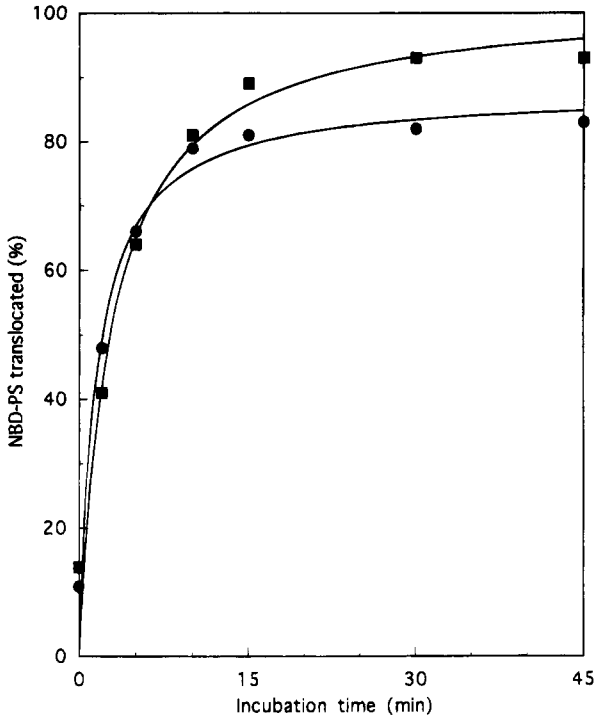

FIGURE 1: Uptake of NBD-PS in unstimulated platelets: comparison between back-exchange and discontinuous dithionite assay. 2×10^8 platelets/mL were incubated with 2 μM NBD-PS, and at intervals samples were taken to determine the amount of NBD-PS present in the outer leaflet either by extraction with BSA (■) or reduction by dithionite (●), as described in Experimental Procedures.

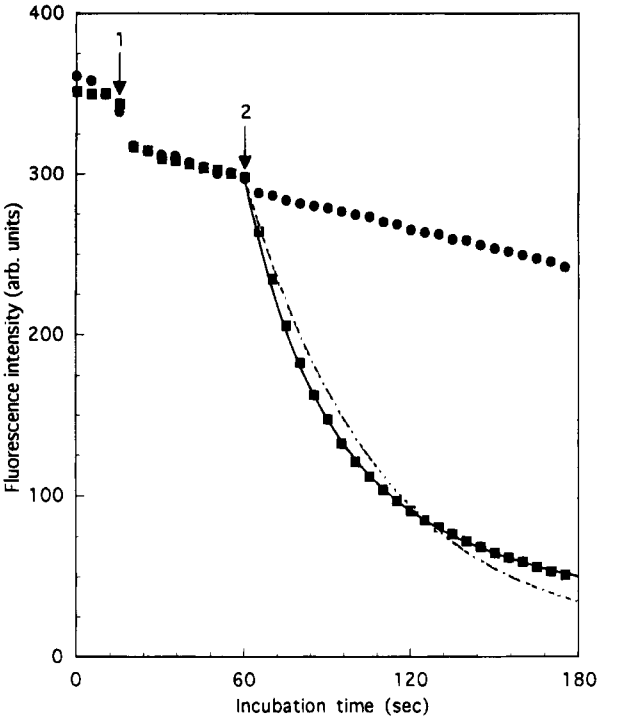

FIGURE 2: Continuous assay for outward movement of NBD-PS in unstimulated platelets and in platelets treated with ionomycin in the presence of extracellular Ca^{2+}, detected as a decay of the fluorescence signal in the presence of dithionite in the medium. Platelets were incubated with NBD-PS for 45 min to allow probe internalization. Samples were diluted in 2 mL of Hepes buffer containing 0.1 mM EGTA, and fluorescence recording was started. 5 mM dithionite was added at arrow 1, followed by ionomycin (1 μM) either without (●) or with (■) 0.5 mM Ca^{2+} at arrow 2. Lines represent curve fits using a monoexponential (dashed line) or a double-exponential equation (solid line). For reasons of clarity the actual data points are shown at 5 s intervals.

and then determining the fraction that remains extractable from the exterior leaflet. An NBD-labeled lipid offers the alternative of measuring the fraction of the probe that is accessible to reduction to a nonfluorescent form by sodium dithionite. To determine whether these assays were equivalent when applied to platelets, uptake of NBD-PS by the aminophospholipid translocase was measured using both assays. As shown in Figure 1, NBD-PS rapidly becomes inaccessible both to extraction with BSA-containing buffers and to reduction with sodium dithionite. The kinetics are similar in the two cases, with greater than 80% of the PS probe internalized by 30 min. Over a similar time period, NBD-PC remains accessible to both reagents (data not shown), in agreement with previous studies of the specificity of the translocase. In sum, these two methods can be applied to platelets for the determination of transbilayer lipid distribution and for the assay of aminophospholipid activity with equivalent results.

Internalization of NBD-PS brings the probe into contact with the intracellular milieu, where it becomes accessible to the metabolic machinery of the cell. In platelets, internalized NBD-PS is rapidly metabolized, as evidenced by a dramatic decrease in cell-associated fluorescence with time (data not shown). This degradation can be blocked, however, by pretreating the platelets with 500 μM PMSF. Even 24 h after internalization, thin-layer chromatography indicated that >95% of the fluorescence was recovered as NBD-PS (data not shown) from platelets treated in this way. Also, no appreciable degradation of NBD-PC occurred under the same experimental conditions. Incorporation of the NBD probes did not affect platelet function; no appreciable difference in thrombin-induced shape change, aggregation, and release of granule contents (ATP) was observed after 1 h of incubation of the platelets with NBD-PS or NBD-PC.

In contrast to erythrocytes (Pomorski et al., 1994), dithionite does not readily penetrate the plasma membrane of platelets. As shown in Figure 2, the addition of dithionite to platelets that have internalized NBD-PS causes an initial 10% reduction in fluorescence, followed by a decay of 5−8%/min. Measurements of this decay as a function of dithionite concentration suggest that it represents roughly equal contributions from the basal "flop" rate of NBD-PS to the cell surface and from the penetration of dithionite to the cell interior (data not shown). Measurements of the decay as a function of illumination showed that photobleaching of the NBD moiety during continuous monitoring was negligible (data not shown), presumably because dissolved O_2 in the medium is eliminated by the addition of dithionite.

Upon activation, the normal asymmetric lipid distribution in the platelet plasma membrane is randomized, allowing NBD-PS to appear on the cell surface (Smeets et al., 1994). To determine whether this apparent increase in the rate of PS "flop" could be observed directly as an increase in the rate of dithionite reduction of previously internalized NBD-PS, platelets were treated with Ca^{2+} and the Ca^{2+} ionophore ionomycin and fluorescence was monitored. As shown in Figure 2, the addition of ionomycin alone had no effect on lipid movement, as long as EGTA was present in the external medium. Also the addition of Ca^{2+}, by itself, to the medium had little or no effect on the rate of PS "flop". Addition of

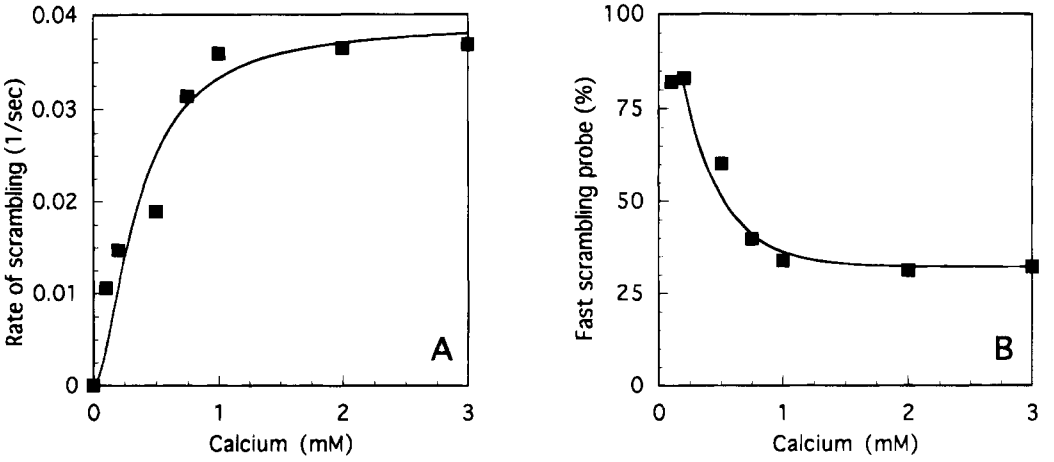

FIGURE 3: Ca^{2+} dependence of the scrambling of NBD-PS in human platelets activated with ionomycin. Outward movement of NBD-PS in platelets treated with ionomycin in the presence of varying concentrations of extracellular Ca^{2+} was measured continuously using the dithionite assay. Fluorescence decay curves were analyzed by double-exponential fit: (A) decay constant of the fast NBD-PS pool as a function of Ca^{2+} concentration; (B) size of the fast NBD-PS pool (as a percentage of the total NBD-PS) as a function of Ca^{2+} concentration.

the two agents together, however, resulted in a dramatic increase in the rate of NBD reduction. Since the treatment of platelets with ionophore and Ca^{2+} will cause the activation of endogenous phospholipases, we have analyzed by HPTLC the probe reisolated from platelets after treatment with Ca^{2+} and ionophore for 30 min. More than 95% of the recovered fluorescence was found to comigrate with intact NBD-PS, indicating that phospholipase activation is not a significant contributor to the observed increase in rate of NBD reduction. The same result was obtained when NBD-PC was used instead of NBD-PS.

Detailed analysis of the decay curve revealed several interesting features. As shown in Figure 2, the decay was not adequately modeled by a simple exponential decay curve, as would be expected if all the internalized probe had equal access to the "flop" sites. Adequate fits were obtained using a model in which the decay includes a fast component, which results in externalization of one fraction of the label, and a slower component, which affects the remainder. Fits to the curve using this two-part model indicated that the fast component represents a roughly 40-fold increase in PS "flop" to the external surface (from 0.0018 to 0.078 s^{-1}), with about 60% of the probe participating in this fast exchange. The slow component, in contrast, had an apparent rate constant that was slightly higher (0.0076 vs 0.0018 s^{-1}) than that of the decay observed before activation with Ca^{2+}/ionophore.

To determine in more detail how cytoplasmic Ca^{2+} affects NBD-PS movement, probe externalization was measured as a function of Ca^{2+} concentration (Figure 3). When analyzed by using the two-component model described earlier, the data showed that increasing Ca^{2+} concentrations from 50 μM to 3 mM induced a monotonic increase in the apparent rate constant for the fast component, with a plateau after about 1 mM (Figure 3A). Surprisingly, the size of the pool of label with access to this fast pathway was maximal at the lowest Ca^{2+} concentrations and fell to 30% of the total NBD-PS at Ca^{2+} concentrations above 1 mM (Figure 3B).

Activation of platelets with Ca^{2+}/ionophore is accompanied by the secretion of granular contents, known as the platelet release reaction (White, 1983; Holmsen & Weiss, 1979), a process that involves rapid fusion of internal membranes with plasma membranes. To determine whether this process contributes to the increased rate of NBD-PS reduction, platelets were activated with thrombin, which results in the same extent of granule release, but little increase in platelet procoagulant activity in comparison to activation with Ca^{2+} and ionophore (Bevers et al., 1983; Thiagarajan & Tait, 1991; Dachary-Prigent et al., 1993). As shown in Figure 4, thrombin by itself had little effect on the rate of NBD-PS externalization, indicating that secretion and its associated fusion events do not contribute to any appreciable extent to surface exposure of PS. When platelets are preincubated with thapsigargin (an inhibitor of the endoplasmic reticulum Ca-ATPase) prior to activation with thrombin, an elevated rate of probe externalization was observed, in agreement with previous findings demonstrating the loss of lipid asymmetry under these conditions (Smeets et al., 1993). These results argue strongly that the assay is directly measuring the activation of the phospholipid scramblase in platelets.

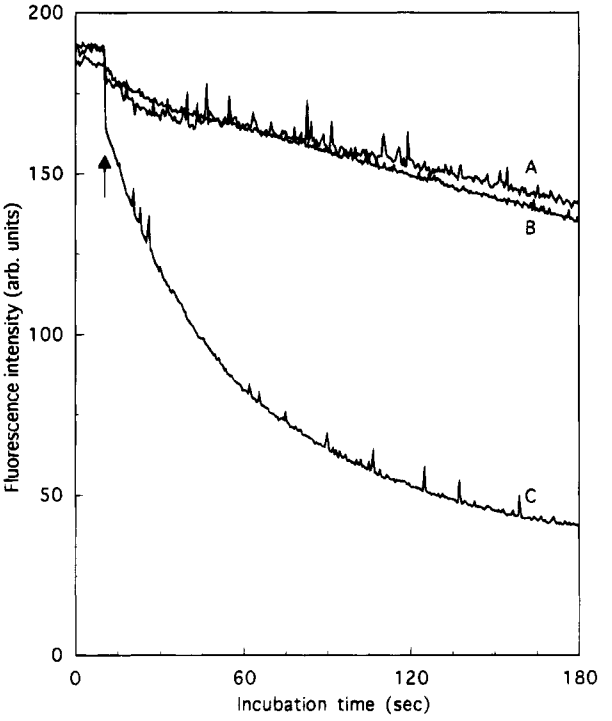

FIGURE 4: Outward movement of NBD-PS in platelets activated for 3 min with thrombin (8 nM) (curve A), thapsigargin (50 nM) (curve B), or a combination of thrombin and thapsigargin (curve C) measured by the dithionite assay (dithionite added at arrow).

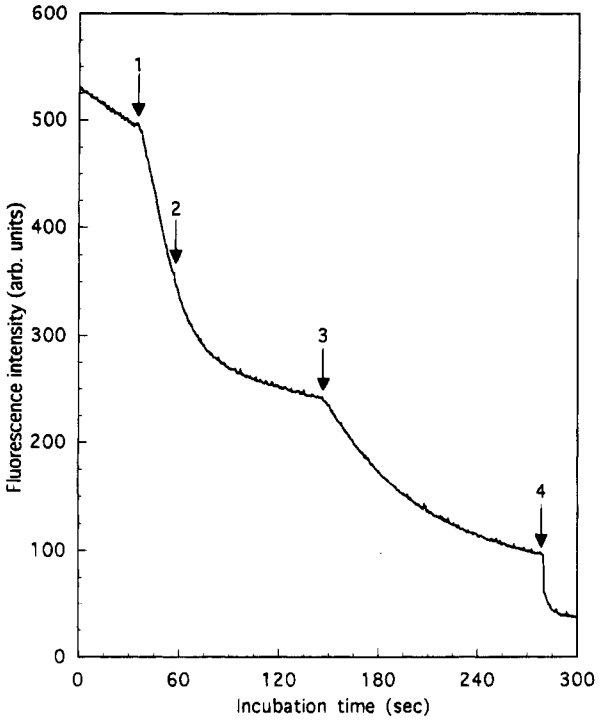

FIGURE 5: Inhibition and reactivation of the lipid scrambling process in platelets. NBD-PS labeled platelets were diluted in Hepes buffer containing 0.1 mM EGTA; dithionite (5 mM) and ionomycin (1 μM) were added, and 1 min later fluorescence recording was started. At arrow 1, Ca^{2+} was added to a final concentration of 1 mM, causing initiation of the scrambling process. At arrow 2, addition of 2 mM EGTA caused arrest of the scrambling process, which could be reactivated at arrow 3 by the renewed addition of Ca^{2+} (5 mM). Addition of detergent (arrow 4) led to complete abolition of the fluorescence signal.

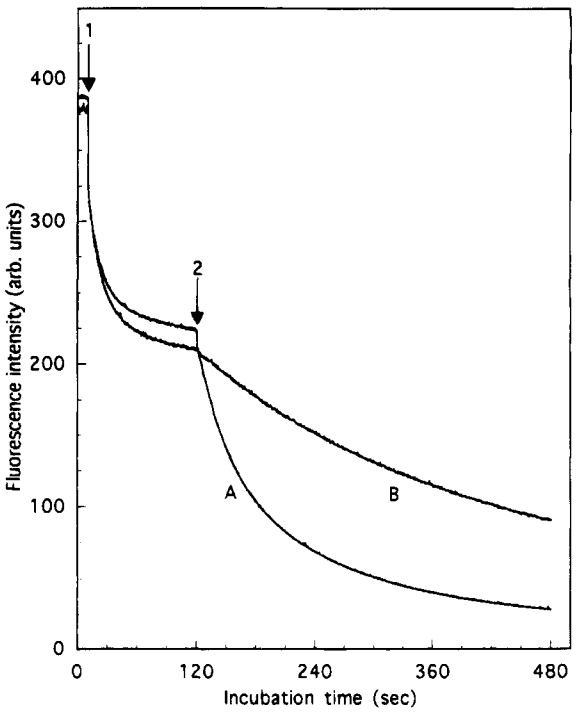

FIGURE 6: Reactivation of NBD-PS scrambling after an initial phase of Ca^{2+}-induced scrambling. Lipid scrambling was induced for 30 s or 30 min by ionomycin and Ca^{2+} (1 μM and 0.25 mM, respectively) followed by 0.5 mM EGTA. Fluorescence recording was started and dithionite (5 mM) was added at arrow 1; lipid scrambling was reactivated upon readdition of Ca^{2+} to a final concentration of 1 mM (arrow 2). Curve A: 30 s of preactivation with ionomycin and Ca^{2+}. Curve B: 30 min of preactivation.

Within the resolution of the assay (several seconds), the activation of the scramblase by Ca^{2+}/ionophore is immediate (Figure 2). To determine whether scramblase activity can be inactivated, Ca^{2+} was removed from the cells by the addition of EGTA to the medium with ionophore still present. As shown in Figure 5, the slope of the reduction curve begins to flatten within about 10 s after the addition of EGTA, finally reaching a level comparable to that observed prior to the initial activation (0.006 vs 0.002 s^{-1} for the reaction shown). Upon readdition of Ca^{2+} to the cells, increased lipid movement was reactivated, indicating that activation/inactivation of the scramblase is reversible and dependent on the continued presence or absence of Ca^{2+}. Indeed, although only limited time is available before all of the probe is externalized, other experiments indicated that the scramblase continues to respond through at least two full cycles of activation and inactivation (data not shown).

The data in Figures 2 and 5 indicate that the vast majority of internalized NBD-PS becomes accessible to dithionite within 90 s of platelet activation by Ca^{2+}/ionophore. By its nature, however, this experiment cannot reveal the net exposure of PS on the cell surface, since reinternalization of probe, by whatever means, goes undetected once the probe reaches the surface and is rendered nonfluorescent; in fact, the net exposure is determined by the balance of the rates of the internalization and externalization processes. Additional information on this balance can be obtained by activation in the absence of dithionite, so that externalized probe remains fluorescent, inactivation of the scramblase, and then addition of dithionite to measure externalized probe.

As shown in Figure 6, the addition of EGTA 30 s after activation with Ca^{2+}/ionophore (long enough for >90% of the label to move to the external leaflet and become reduced when dithionite is present) resulted in the net exposure of about 40% of internalized NBD-PS, indicating that the rate of NBD-PS flip to the interior during activation must have roughly equaled the rate of NBD-PS flop to the surface. This experiment also rules out the possibility that the accessibility of NBD-PS to reduction by dithionite was due to Ca^{2+}/ionophore-induced cell lysis, since access of dithionite to the cell interior would have rendered all probe nonfluorescent. Consistent with the data in Figure 5, the scrambling process can be reactivated upon the reelevation of Ca^{2+}, as indicated by the rapid reduction of the resistant NBD-PS. However, as illustrated in Figure 6, the rate at which NBD-PS becomes externalized upon readdition of Ca^{2+} decreases when the time of the first scrambling process is extended from 30 s to 30 min. In fact, curve analysis shows that the second scrambling process after 30 s of prescrambling (curve A in Figure 6) involves a pool of 35% with a rate constant of 0.033 s^{-1} ($t_{1/2}$ = 21 s), while the remaining NBD-PS is externalized at a rate of 0.007 s^{-1} ($t_{1/2}$ = 100 s). When the second scrambling is initiated after 30 min of prescrambling (curve B in Figure 6), decay can be described by only one pool with a rate constant of 0.004–0.005 s^{-1} ($t_{1/2}$ =138–175 s).

It should be noted that the high rate of outward movement of NBD-PS indicated by these experiments is unlikely to be due to the well-known sensitivity of the aminophospholipid translocase to inactivation by elevated Ca^{2+} (Bitbol et al., 1987). This point can be addressed more directly, however, by using as probes NBD-labeled derivatives of PC and Sph,

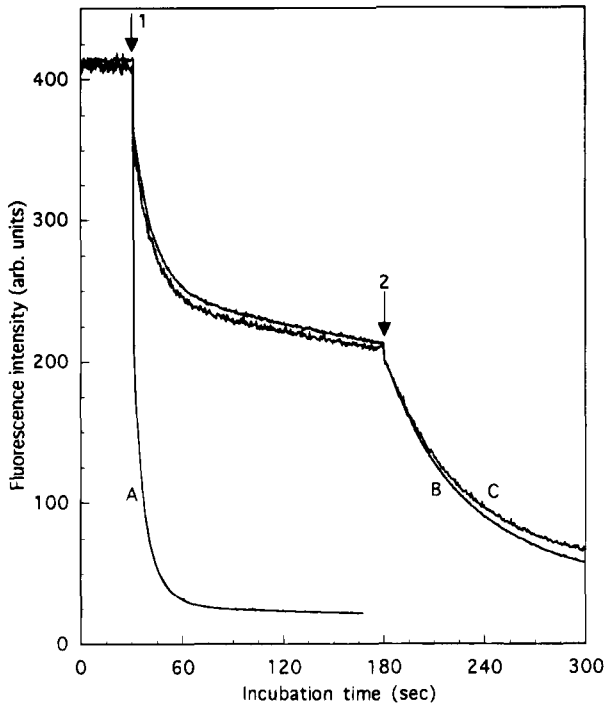

FIGURE 7: Ionophore/Ca^{2+}-induced movement of NBD-PC as monitored by continuous assay with dithionite. 2×10^8 platelets/mL were labeled with NBD-PC (2 μM) for 60 min. Aliquots of 50 μL were diluted in 2 mL of Hepes buffer, and Ca^{2+} (0.5 mM) with or without ionomycin (1 μM) was added. After 1 min, 1 mM EGTA was added to arrest lipid scrambling; 3 min later, fluorescence recording was started and 5 mM dithionite was added at arrow 1. Curve A: Ca^{2+} but no ionomycin. Curve B: ionomycin and Ca^{2+}. The resistant fraction of NBD-PC could be externalized upon readdition of Ca^{2+} (arrow 2). Curve C: Same experiment as described for curve B using NBD-Sph as a probe.

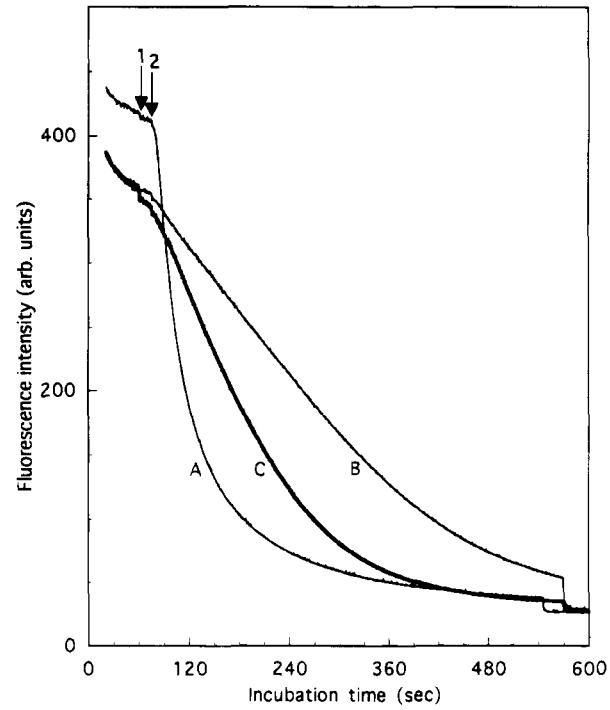

FIGURE 8: Inhibition of Ca^{2+} -induced scrambling of NBD-PS by PDA. Platelets were labeled with 2 μM NBD-PS for 1 h. After the addition of 5 mM dithionite, lipid scrambling was induced by ionomycin (1 μM, arrow 1) and Ca^{2+} (1 mM, arrow 2): curve A, control platelets; curve B, platelets pretreated with 0.5 mM PDA for 2 min; curve C, platelets pretreated with 0.5 mM PDA for 2 min followed by 20 mM DTT for 10 min.

lipids that are not internalized by the translocase. The experiment shown in Figure 6 was repeated with platelets whose outer leaflet was labeled with NBD-PC, except that ionophore was omitted during incubation with Ca^{2+}. After the addition of EGTA, the NBD-PC probe remained confined to the cell surface, as evidenced by its complete sensitivity to dithionite reduction (Figure 7), which is consistent with the well-known low rate of spontaneous PC flip in platelets. In contrast, when ionophore was included during the incubation with Ca^{2+}, a large fraction of NBD-PC or NBD-Sph became inaccessible to dithionite added after the addition of EGTA, indicating that both are flipped to the cell interior. The size of the resistant fraction was comparable in both cases to that observed in the same experiment with cells prelabeled with NBD-PS, confirming that the activation of lipid movement is bidirectional and suggesting that Ca^{2+}/ionophore-induced PC and Sph equilibration is essentially complete by 2 min of incubation, as it is for PS. The rate of inward movement of Sph, however, was found to be slower than that for PC (data not shown). It is unlikely that this different rate is a consequence of a preference for PC of the scramblase, because both internalized NBD-PC and NBD-Sph reappeared on the surface after the readdition of Ca^{2+} to reactivate the scramblase, with the same kinetics that was observed for NBD-PS (Figure 7), confirming that the activation-induced lipid movement is bidirectional and nonspecific. The slower inward movement of Sph, therefore is more likely to be due to its interactions with components in the external leaflet of the platelet, for instance, cholesterol (Demel et al., 1977).

The nature of the scrambling mechanism is presently unknown, although several mechanisms have been proposed and refuted (Comfurius et al., 1985, 1989, 1990 Sims et al., 1989; Wiedmer et al., 1990; Williamson et al., 1992; Bratton, 1994; Sulpice et al., 1994). To address the issue of whether protein(s) is involved, the effect of the permeant sulfhydryl-oxidizing agent PDA on activation-induced scrambling was investigated. As shown in Figure 8, pretreatment of NBD-PS-labeled platelets with 1 mM PDA for 2 min dramatically inhibited Ca^{2+}/ionophore-induced lipid movement. Inhibition was both concentration-dependent, reaching a maximum at about 0.5 mM PDA, and time-dependent, being complete by 2−3 min (data not shown). At longer times of incubation with PDA, an increase in Ca^{2+}/ionophore-independent probe externalization was observed (data not shown), which may reflect membrane damage and help account for the development of procoagulant activity previously observed in PDA-treated platelets (Comfurius et al., 1990; Bevers et al., 1989). Inhibition could be reversed by the addition of dithiothreitol (DTT), albeit at rather a high concentration (20 mM), resulting in partial restoration of the scrambling process. Concentrations of DTT below 5 mM were ineffective in restoring scramblase activity, suggesting that the sulfhydryl-reactive site in the scrambling mechanism is not as accessible to the highly water-soluble DTT as it is to the more hydrophobic PDA.

DISCUSSION

Reduction of NBD by sodium dithionite has previously been shown to be a rapid, accurate, and reliable method for determining the transbilayer distribution of NBD-phospholipid analogs in lipid bilayers (McIntyre & Sleight, 1991;

Pomorski et al., 1994; Gruber & Schindler, 1994; Balch et al., 1994). Application of the method to living cells, however, can be problematic, for example, if the membrane is permeable to the anion as is the case for erythrocytes (Pomorski et al., 1994). The evidence presented here shows that such limitations do not restrict the application of dithionite to platelets: in discontinuous assays, dithionite reduction reports the same distribution of NBD-phospholipids as the more conventional BSA extraction assays.

After about 30 min of incubation, about 90% of the NBD-PS probe added to the external leaflet of the platelet plasma membrane becomes inaccessible to both BSA and dithionite, reflecting the action of the aminophospholipid translocase in these cells. The most sensitive assays for PS, detection of prothrombinase activity (Rosing et al., 1985b) and binding of annexin (five) (Thiagarajan & Tait, 1991; Dachary-Prigent et al., 1993), indicate that the plasma membrane surface is virtually free of PS. The small fraction of fluorescent probe that remains accessible may reflect the lower efficiency of its inward movement compared to endogenous or spin-labeled phospholipids (Colleau et al., 1991; Williamson et al., 1992) or to the presence of nontransportable impurities in the probe preparation. The ability to label platelets with a stable pool of internal NBD-phospholipid is the key to developing an assay for continuous measurements of phospholipid flop and thereby phospholipid scrambling activity of platelets. As suggested by previous studies carried out at lower time resolution (Smeets et al., 1994; Bassé et al., 1993), the scramblase activity is very prominent in platelets: after induction by Ca^{2+}/ionophore, the activity develops immediately and, as demonstrated in the present study, exposes at least transiently 90% or more of the previously internalized probe in a headgroup nonspecific manner, suggesting that virtually all of the endogenous plasma membrane lipids reach the surface over this time scale. The capacity for this high rate of transbilayer lipid movement, at least 20-fold higher than that observed in maximally stimulated erythrocytes (i.e., with Ca^{2+}/ionophore), is consistent with the functional importance of this movement in platelets for the formation of a procoagulant surface, as opposed to erythrocytes where surface exposure of PS is likely pathological and to be avoided (Schroit & Zwaal, 1991; Williamson & Schlegel, 1994; Devaux & Zachowski, 1994).

Although more active, in other respects the scramblase in platelets appears to closely resemble its erythrocyte counterpart. The acceleration of transbilayer diffusion is bidirectional and affects neutral as well as aminophospholipids similarly, implying that the increase in procoagulant activity is not the consequence of specific movement of aminophospholipids from the cell interior (Bassé et al., 1993). The mechanism appears to be dependent on the continued presence of Ca^{2+} and is reversibly inactivated when Ca^{2+} is removed. Even within the higher time resolution of the dithionite assay, activation is virtually immediate, being complete within seconds after the elevation of cytoplasmic Ca^{2+}.

The actual kinetics of PS externalization is more complex than the simple exponential decay, which would characterize the basic flop from the inside to the outside of the cell. In fact, the actual kinetics might be expected to include several complicating components, including competition between reduction and flip back to the cell interior and the presence in the population of unresponsive or slowly responsive cells. The fast and slow components could reflect the diffusion of probe from (residual) internal membranes to the plasma membrane or from different pools of the probe in the plasma membrane itself. The decrease in the pool size at very high internal Ca^{2+} concentrations lends some support to the latter view (Figure 3B).

Reinitiation of the scrambling process after prolonged preincubation with Ca^{2+}/ionophore (Figure 6) results in only a small increase in the rate of fluorescence decay, which can be described by a monoexponential function, in contrast to reinitiation after a preincubation of 30 s, where the decay process is still best described by a double-exponential function. Although the reason is at present unclear, the difference could reflect the gradual inactivation of the scramblase over the longer time period of elevation of Ca^{2+}, especially since the rate of decay after long preactivation is actually lower than that of the slow component found after 30 s of preincubation with Ca^{2+}/ionophore. Inactivation of the scramblase by calpain, the major Ca^{2+}-activated protease in platelets, is unlikely, because preincubation of platelets with calpeptin, a membrane-permeable inhibitor of this protease, was without effect (unpublished observations). Further experiments will be required to dissect out the contribution of these and other such complicating mechanisms to the kinetics that is actually observed.

The nature of the mechanism by which the lipids are scrambled has been a puzzle for several years. Several mechanisms have been proposed, including release of aminophospholipids from cytoskeletal interactions (Williamson et al., 1982; Comfurius et al., 1985, 1989), accelerated transbilayer movement through nonbilayer structures created at the site of membrane fusion events (Wiedmer et al., 1990), and formation of $Ca^{2+}-PIP_2$ complexes that promote or facilitate transbilayer movement of phospholipids (Sulpice et al., 1994). Evidence against all of these mechanisms has been presented (Williamson et al., 1992; Comfurius et al., 1990; Bevers et al., submitted). While it may be argued that lipid degradation products resulting from phospholipase activation could enhance transbilayer lipid movement, this is unlikely because lipid scrambling is not observed in thrombin-activated platelets.

The obvious possibility that lipid scrambling is mediated by the formation of a protein-based flip site has not been refuted, but has been difficult to support, in part because the lipid headgroup nonspecificity of the mechanism makes it similar to transbilayer movements produced by membrane perturbants such as ethanol (Schwichtenhovel et al., 1992). The evidence presented here for the sensitivity of the platelet scramblase to the sulfhydryl oxidizer PDA is the first to positively implicate a protein in this process. The large difference in the concentrations of oxidizer (PDA) required for inhibition (0.5 mM) and of reducer (DTT) required to partially restore activity (20 mM) suggests that the SH-reactive site of the scramblase is located in the core of the membrane, where it would be accessible to the more hydrophobic PDA. This inhibitory effect of PDA on lipid scrambling has previously been overlooked, perhaps in part because the oxidizing agent induces lipid movement at higher concentrations or after more prolonged incubations (Comfurius et al., 1990), thereby masking its ability to block induction of the scramblase. The demonstration of sensitivity to sulfhydryl oxidation may be helpful in the development of procedures for reconstituting the activity, which are likely to be essential in identifying the protein or proteins that support it.

REFERENCES

Balch, C., Morris, R., Brooks E., & Sleight, R. G. (1994) *Chem. Phys. Lipids 70*, 205−212.

Bassé, F., Gaffet, P., Rendu, F., & Bienvenüe, A. (1993) *Biochemistry 32*, 2337−2344.

Bevers, E. M., Comfurius, P., Rijn van, J. L. M. L., Hemker, H. C., & Zwaal, R. F. A. (1982) *Eur. J. Biochem. 122*, 429−436.

Bevers, E. M., Comfurius, P., & Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta 736*, 57−66.

Bevers, E. M., Tilly, R. H., Senden, J. M. G., Comfurius, P., & Zwaal, R. F. A. (1989) *Biochemistry 28*, 2382−2387.

Bevers, E. M., Wiedmer, T., Comfurius, P., Shattil, S. J., Weiss, H. J., Zwaal, R. F. A., & Sims, P. J. (1992) *Blood 79*, 380−388.

Bitbol, M., Fellmann, P., Zachowski, A., & Devaux, P. F. (1987) *Biochim. Biophys. Acta 904*, 268−282.

Bratton, D. L. (1994) *J. Biol. Chem. 269*, 22517−22523.

Chandra, R., Joshi, P. C., Bajpai, V. K., & Gupta, C. M. (1987) *Biochim. Biophys. Acta 902*, 253−262.

Colleau, M., Hervé, P., Fellmann, P., & Devaux, P. F. (1991) *Chem. Phys. Lipids 57*, 29−37.

Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1985) *Biochim. Biophys. Acta 815*, 143−148.

Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1989) *Biochim. Biophys. Acta 983*, 212−216.

Comfurius, P., Senden, J. M. G., Tilly, R. H. J., Schroit, A. J., Bevers, E. M., & Zwaal, R. F. A. (1990) *Biochim. Biophys. Acta 1026*, 153−160.

Connor, J., & Schroit, A. J. (1988) *Biochemistry 27*, 848−851.

Connor, J., & Schroit, A. J. (1990) *Biochemistry 29*, 37−43.

Connor, J., Pak, C. H., Zwaal, R. F. A., & Schroit, A. J. (1992) *J. Biol. Chem. 267*, 19412−19417.

Dachary-Prigent, J., Freyssinet, J.-M., Pasquet, J.-M., Carron, J.-C., & Nurden, A. T. (1993) *Blood 81*, 2554−2565.

Demel, R. A., Jansen, J. W. C. M., van Dijck, P. W. M., & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 1−10.

Devaux, P. F., & Zachowski, A. (1994) *Chem. Phys. Lipids 73*, 107−120.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., & Henson, P. M. (1992) *J. Immunol. 148*, 2207−2216.

Gruber, H. J., & Schindler, H. (1994) *Biochim. Biophys. Acta 1189*, 212−224.

Haest, C. W. M. (1982) *Biochim. Biophys. Acta 694*, 331−352.

Henseleit, U., Plasa, G., & Haest, C. W. M. (1990) *Biochim. Biophys. Acta 1029*, 127−135.

Holmsen, H., & Weiss, H. J. (1979) *Annu. Rev. Med. 30*, 119−134.

Martin, T. W., Joist, J. H., Bauman, J. E., & Lagunoff (1985) *J. Biol. Chem. 260*, 2852−2856.

McIntyre, J. C., & Sleight, R. G. (1991) *Biochemistry 30*, 11819−11827.

Moriyama, Y., & Nelson, N. (1988) *J. Biol. Chem. 263*, 8521−8527.

Morrot, G., Zachowski, A., & Devaux, P. F. (1990) *FEBS Lett. 266*, 29−32.

Pomorski, T., Herrmann, A., Zachowski, A., Devaux, P. F., & Müller, P. (1994) *Mol. Membr. Biol. 11*, 39−44.

Reed, C. F., Swisher, S. N., Marinetti, G. V., & Eden, E. G. (1960) *J. Lab. Clin. Med. 56*, 281−285.

Rosing, J., Bevers, E. M., Comfurius, P., Hemker, H. C., Dieijen van, G., Weiss, H. J., & Zwaal, R. F. A. (1985a) *Blood 65*, 1557−1561.

Rosing, J., Rijn van, J. L. M. L., Bevers, E. M., Dieijen van, G., Comfurius, P., & Zwaal, R. F. A. (1985b) *Blood 65*, 319−332.

Schlegel, R. A., Stevens, M., Lumley-Sapanski, K., & Williamson, P. (1993) *Immunol. Lett. 36*, 283−288.

Schroit, A. J., & Zwaal, R. F. A. (1991) *Biochim. Biophys. Acta 1071*, 313−329.

Schwartz, R. S., Chiu, D. T.-Y., & Lubin, B. (1985) *Curr. Top. Hematol. 4*, 63−112.

Schwichtenhovel, C., Deuticke, B., & Haest, C. W. M. (1992) *Biochim. Biophys. Acta 1111*, 35−44.

Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751−3755.

Sims, P. J., Wiedmer, T., Esmon, C. T., Weiss, H. J., & Shattil, S. J. (1989) *J. Biol. Chem. 264*, 17049−17057.

Smeets, E. F., Heemskerk, J. W. M., Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1993) *Thromb. Haemostas. 70*, 1024−1029.

Smeets, E. F., Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1994) *Biochim. Biophys. Acta 1195*, 281−286.

Sulpice, J. C., Zachowski, A., Devaux, P. F., & Giraud, F. (1994) *J. Biol. Chem. 269*, 6347−6354.

Thiagarajan, P., & Tait, J. F. (1991) *J. Biol. Chem. 266*, 24302−24307.

Verhallen, P. F. J., Bevers, E. M., Comfurius, P., & Zwaal, R. F. A. (1987) *Biochim. Biophys. Acta 903*, 206−217.

Verhoven, B. V., Schlegel, R. A., & Williamson, P. (1992) *Biochim. Biophys. Acta 1104*, 15−22.

White, J. G. (1983) *Blood Cells 9*, 237−261.

Wiedmer, T., Shattil, S. J., Cunningham, M., & Sims, P. J. (1990) *Biochemistry 29*, 623−632.

Williamson, P., & Schlegel, R. A. (1994) *Mol. Membr. Biol. 11*, 199−216.

Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.-R., & Schlegel, R. A. (1982) *Cell 30*, 725−733.

Williamson, P., Algarin, I., Bateman, J., Choe, H.-R., & Schlegel, R. A. (1985) *J. Cell Physiol. 123*, 209−214.

Williamson, P., Kulick, A., Zachowski, A., Schlegel, R. A., & Devaux, P. F. (1992) *Biochemistry 31*, 6355−6360.

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