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Bidirectional Transbilayer Lipid Movement in Human Platelets As Visualized by the Fluorescent Membrane Probe 1-[4-(Trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene[†]

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ABSTRACT: Transbilayer movement of the fluorescent membrane probe TMA-DPH [1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene] in the plasma membrane of human platelets was investigated by measuring fluorescence intensity and fluorescence decay. Labeling of unstimulated platelets by TMA-DPH results in a rapid increase in fluorescence intensity, leveling off within 1 min. Dilution of platelets into buffer without TMA-DPH leads to an almost complete rapid efflux of TMA-DPH, indicating that TMA-DPH labels only the outer leaflet of the plasma membrane. Transbilayer movement of the fluorescent probe in unstimulated platelets could be observed upon prolonged incubation and occurs with a $t_{1/2}$ of 60-90 min. Stimulation of platelets with thrombin directly after the initial rapid uptake of TMA-DPH results in a fast increase in membrane-bound TMA-DPH, fully explained by the increase in plasma membrane caused by secretion of intracellular storage organelles. No indications for increased transbilayer movement of the probe were found, since dilution of thrombin-stimulated TMA-DPH-labeled platelets into buffer without TMA-DPH indicated no uptake of TMA-DPH by intracellular membranes. In contrast to thrombin, stimulation of TMA-DPH-labeled platelets with the Ca^{2+} -ionophore ionomycin results in a much larger increase in fluorescence intensity. This process is accompanied by labeling of intracellular membranes as indicated by incomplete efflux of TMA-DPH after dilution of the stimulated platelets. Thus, stimulation of platelets by ionomycin gives rise to rapid and massive inward movement of TMA-DPH ($t_{1/2} \sim 10-12$ s). Prolonged incubation of platelets in the absence of any stimulus allows labeling of the total lipid pool, including intracellular membranes. Dilution experiments with these platelets showed that rapid outward movement of TMA-DPH only occurs upon stimulation with ionomycin. The high rate of transbilayer movement of TMA-DPH—both inward and outward—during stimulation by ionomycin corresponds to the randomization of phospholipids that has been described previously for platelets stimulated by Ca^{2+} -ionophore A23187 [Bevers et al. (1983) *Biochim. Biophys. Acta* 736, 57-66]. These results suggest that stimulation by ionophore causes the formation of local defects (flip sites) in the plasma membrane along which both endogenous phospholipid and exogenous added lipidlike compounds can cross the bilayer in both directions. Further examination of the "flip sites" during platelet stimulation by ionomycin showed that they were only transient, disappearing within 1 min following addition of the stimulus.

The plasma membrane of blood platelets is characterized by an asymmetrical distribution of the different phospholipid classes over both leaflets: the inner leaflet is enriched in phosphatidylethanolamine and contains virtually all the phosphatidylserine (PS)¹ of the plasma membrane, whereas

the outer monolayer is composed of a major part of the phosphatidylcholine and contains almost all the sphingomyelin (Schick et al., 1976; Chap et al., 1977; Perret et al., 1979; Bevers et al., 1983). This phospholipid distribution closely resembles that of the erythrocyte membrane (Verkley et al., 1973; Gordesky et al., 1975). In erythrocytes two different mechanisms have been considered to account for transbilayer asymmetry: translocation mediated by an ATP-dependent

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¹ Abbreviations: PS, phosphatidylserine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; *F*, fluorescence intensity; $\langle \tau \rangle$, average fluorescence lifetime.

transport protein (translocase) (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Zachowski et al., 1986; Tilley et al., 1986; Connor & Schroit, 1987) and selective interaction of lipids with cytoskeletal components (Mommers et al., 1979; Haest, 1982; Mohandas et al., 1985; Williamson et al., 1987). Both mechanisms have also been recognized in platelets (Sune et al., 1987; Comfurius et al., 1989; Bevers et al., 1989).

In contrast to the erythrocyte, transbilayer asymmetry in platelets can be dramatically changed upon cell activation (Bevers et al., 1983, 1987). Depending on the stimulus, phospholipid transbilayer asymmetry of the plasma membrane is more or less lost during platelet activation. As a consequence of this randomization of phospholipids, PS becomes exposed at the outer surface of the plasma membrane. The latter event is of great physiological importance since it leads to a significant acceleration of blood clotting (Rosing et al., 1985). Activation-dependent loss of phospholipid asymmetry is a rapid phenomenon: when platelets are activated by calcium ionophore, phospholipid asymmetry is virtually lost within 1 min (Verhallen et al., 1987). Such a rapid process which involves all membrane phospholipids (Bevers et al., 1983) suggests the formation of nonspecific flip sites along which both inward and outward movement of lipids is enhanced.

The occurrence of nonspecific flip sites in the plasma membrane during platelet activation was investigated employing the fluorescent membrane probe TMA-DPH. This probe has been reported to incorporate instantaneously in the outer monolayer of the plasma membrane (Kuhry et al., 1983; Kubina et al., 1987). Due to the partition equilibrium (Kuhry et al., 1983), most of the TMA-DPH will remain in solution where it is hardly fluorescent (Prendergast et al., 1981). Transbilayer movement of TMA-DPH (Cranney et al., 1983) will result in labeling of intracellular compartments including the plasma membrane inner leaflet, accompanied by more uptake of probe from the solution and a corresponding increase in fluorescence intensity (Kuhry et al., 1983, 1985). Thus, the rationale for the present study was that loss of transbilayer asymmetry in platelets, which involves both inward and outward movement of phospholipids (Bevers et al., 1983), would be accompanied by an increase in fluorescence intensity due to incorporation of TMA-DPH in intracellular membrane compartments.

EXPERIMENTAL PROCEDURES

Fatty acid free human serum albumin was obtained from Sigma Chemical Co. Ionomycin was from Calbiochem, U.S.A. Thrombin was purified according to Rosing et al. (1980). TMA-DPH was purchased from Molecular Probes Inc. (Eugene, OR) and was dissolved in *N,N*-dimethylformamide and stored at -20°C . Other (bio)chemicals used were of the highest grade commercially available.

Platelet Isolation. Human platelets were isolated as outlined previously (Bevers et al., 1982) by differential centrifugation of blood, freshly drawn from healthy volunteers who received no medication. Blood was anticoagulated with acid-citrate-dextrose. Platelets were washed twice in an isotonic buffer at pH 6.6, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , and 5 mM glucose (Hepes buffer) in the presence of 0.05% fatty acid free human serum albumin. After washing, platelets were resuspended in Hepes buffer, pH 7.4, without albumin (albumin interferes with TMA-DPH measurements) and stored at a concentration of 2×10^8 platelets per mL. Platelet lysis was determined by assaying liberation of lactate dehydrogenase (EC 1.1.1.27) in a 12000g supernatant according to Wroblewski and La Due (1955).

Fluorescence Measurements. All fluorescence measurements were performed in quartz cuvettes. TMA-DPH was added at a final concentration of $1 \mu\text{M}$ to suspensions of 2×10^7 platelets/mL unless otherwise stated. The final concentration of the solvent *N,N*-dimethylformamide was kept below 0.02 vol %. Thrombin or ionomycin was added at final concentrations of 2 nM and $0.5 \mu\text{M}$, respectively. All agents used during fluorescence measurements were tested for their effect on the fluorescence properties of TMA-DPH and were found to be without any effect. Fluorescence intensities (F) were recorded at 37°C under moderate stirring (250 rpm) with a SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port and a stirring device (Hellma). Excitation and emission wavelengths were set at 365 and 450 nm, respectively. F from light-scatter or background fluorescence varied between 1 and 2% and was neglected.

Fluorescence decay was determined with a laser-pulsed single photon counting fluorimeter, described in detail elsewhere (Visser et al., 1985; Van Hoek et al., 1987). TMA-DPH was excited at 310 nm and emission was monitored at 450 nm. All measurements were performed at 37°C without stirring, while activation and labeling of the platelets were carried out under stirring.

Time-resolved data were analyzed as described in detail by Vos et al. (1987). A biexponential function generally used by others was employed to describe the fluorescence decay of TMA-DPH from which the average fluorescence lifetime, $\langle\tau\rangle$, was calculated (Stubbs et al., 1984; Van Langen et al., 1987; Straume & Litman, 1987). In addition, a tetraexponential function, fitted from lower channel numbers, was used to include the fluorescence lifetime of TMA-DPH in solution [fixed at 38 ps according to Verhallen (1988)]. This procedure allows estimation of the fraction of TMA-DPH in solution and thus of the fraction membrane-bound TMA-DPH.

RESULTS

Addition of TMA-DPH to unstimulated platelets results in an immediate rise in fluorescence intensity (F), which becomes stable in 1 min (Figure 1A). The increase in F is due to incorporation of TMA-DPH in the platelet membrane, where it has a much higher average fluorescence lifetime than TMA-DPH in aqueous solution (Prendergast et al., 1981; Verhallen, 1988). On the basis of its physicochemical properties, TMA-DPH is expected to incorporate exclusively in the outer leaflet of the plasma membrane. This is supported by the observation that F increases more than 2-fold upon sonication of unstimulated platelets in the presence of TMA-DPH (Figure 1A). At first sight, however, a 4- to 5-fold increase in F has to be expected since the amount of lipid present in the outer leaflet of the platelet plasma membrane comprises approximately 20–25% of the total phospholipids (Chap et al., 1977; Perret et al., 1979; Bevers et al., 1983). Time-resolved fluorescence spectroscopy allows calculation of the fraction of membrane-bound TMA-DPH, which is summarized in Table I. Immediately after addition of $1 \mu\text{M}$ TMA-DPH to 2×10^7 platelets/mL 4.5% TMA-DPH is incorporated in the membrane. Upon sonication the membrane-bound fraction of TMA-DPH is 5-fold increased to 20.5%. It is emphasized that F cannot be directly interpreted as the number of membrane-bound molecules since F is also dependent on $\langle\tau\rangle$, which decreases upon platelet sonication (Table I), indicating that the increase in F is an underestimation of the increase in membrane-bound TMA-DPH.

When platelets that are labeled with $1 \mu\text{M}$ TMA-DPH for 3 min are activated by 2 nM thrombin, F increases about 1.5-fold (Figure 1A). This has already been observed by

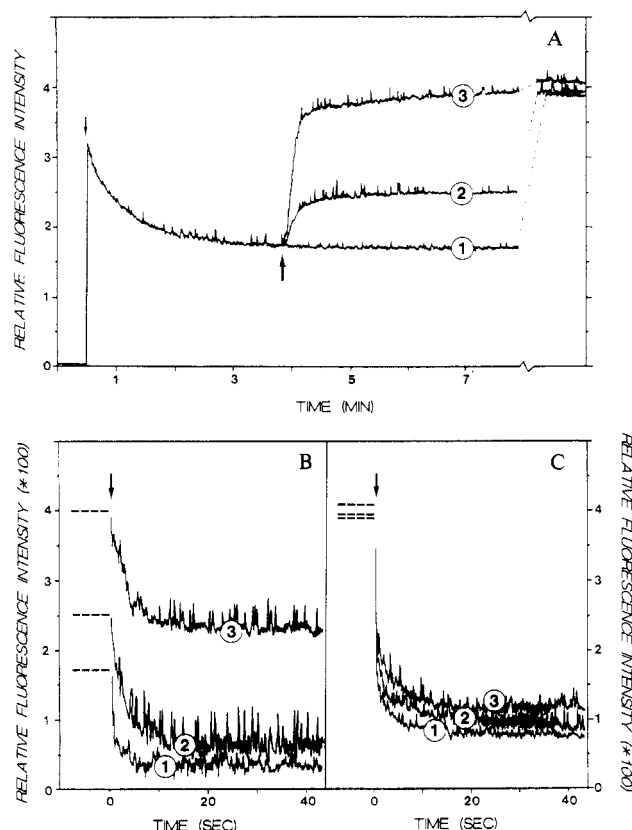


FIGURE 1: Changes in membrane-bound pools of TMA-DPH during platelet activation. (A) Washed human platelets (2×10^7 platelets/mL) were labeled with TMA-DPH ($1 \mu\text{M}$) in HEPES buffer in the presence of 3 mM Ca^{2+} (thin arrow). After stabilization of the fluorescence signal platelets were left unstimulated (1) or stimulated by 2 nM thrombin (2) or by $0.5 \mu\text{M}$ ionomycin (3), as indicated by the bold arrow. Stimulation was carried out under moderate stirring (250 rpm). At the end of the measurement each platelet preparation was sonicated (dashed line), and F was measured again by using the same instrumental settings. (B, C) Subsamples from the very same platelet preparations used in A were 100 times diluted (arrow) in HEPES buffer without TMA-DPH, before (B) and after (C) sonication. The instrumental signal magnification was 100-fold increased with respect to the settings used for A. The dashed horizontal lines indicate F of each platelet preparation before dilution and represent 1% of F in panel A, just before (B) or after (C) sonication. Trace numbers are the same as in panel A. Recordings shown are representative for six experiments. All manipulations were carried out at 37°C under moderate stirring. For further details see the Experimental Procedures.

Table I: Fluorescence Lifetime and Fraction of Membrane-Bound TMA-DPH in Human Platelets^a

| | $\langle\tau\rangle$ (ns) ($\pm\text{SEM}$) | bound fraction (%) ($\pm\text{SEM}$) |
|---|--|---|
| unstimulated platelets (1 min after labeling) | 6.82 ± 0.02 | 4.5 ± 0.3 (22) |
| stimulated with thrombin (2 nM) | 6.72 ± 0.04 | 7.3 ± 0.4 (36) |
| stimulated with ionomycin ($0.5 \mu\text{M}$) | 5.09 ± 0.01 | 15.3 ± 0.3 (75) |
| platelets sonicated for 3 min | 4.89 ± 0.28 | 20.5 ± 2.0 (100) |
| unstimulated platelets (140 min after labeling) | 4.65 ± 0.02 | 16.7 ± 1.0 (81) |

^a 2×10^7 platelets/mL were incubated with $1 \mu\text{M}$ TMA-DPH. Data shown are mean values from three different experiments. The bound fraction is expressed as a percentage of total TMA-DPH added. In parentheses the bound fractions are expressed as a percentage of the maximum labeling observed in platelet sonicate.

Kubina et al. (1987), who correlated this increase in F with serotonin release, suggesting that the increased uptake of TMA-DPH is due to the increase in plasma membrane after exocytosis. Platelet lysis was 1.5%, which is too small to account for the increase in F . Fluorescence decay analysis after thrombin stimulation showed that the bound fraction of

TMA-DPH increases from 4.5 to 7.3% (Table I), while the average lifetime does not change significantly. In comparison with the total amount of membrane-bound TMA-DPH in a platelet sonicate, these values correspond to 22 and 36%, respectively. Assuming that TMA-DPH is only incorporated in the outer monolayer of the plasma membrane (at short labeling times), these values are representative of the amount of phospholipid present in the outer monolayer. Moreover, these values are in perfect agreement with the percentage of total phospholipid hydrolysis observed by exogenously added phospholipase A_2 and sphingomyelinase under nonlytic conditions (Beyers et al., 1983). The increase in F after sonication of thrombin-stimulated platelets indicates that a significant fraction of the total platelet lipid pool remains unlabeled by TMA-DPH.

The supposition that TMA-DPH in unstimulated and thrombin-stimulated platelets is exclusively located in the outer monolayer of the plasma membrane was tested by dilution experiments, which are based on the rapid equilibrium distribution of TMA-DPH between membranes and aqueous solution. It was found that within 20 s after dilution of unstimulated platelets, F rapidly and almost completely disappeared (Figure 1B) due to rapid efflux of TMA-DPH from the plasma membrane into solution where it is hardly fluorescent. Thrombin-activated platelets showed a similar decrease of F after dilution, suggesting that TMA-DPH has not crossed the plasma membrane during stimulation by thrombin.

A completely different picture was obtained when these experiments were carried out with ionomycin-stimulated platelets. Activation of platelets by $0.5 \mu\text{M}$ ionomycin resulted in a more than 2-fold increase in F of TMA-DPH (Figure 1A). This increase in F is much higher than with thrombin, in spite of the same extent of platelet secretion induced by both activators (data not shown). Sonication of the ionomycin-stimulated platelets hardly increased F any further (Figure 1A), which indicates that virtually all platelet membranes become labeled by TMA-DPH during stimulation by Ca^{2+} ionophore. Platelet lysis did not exceed 2.6%. Calculation of the membrane-bound fraction of TMA-DPH showed a value of 15.3% compared to 20.5% for a platelet sonicate (Table I). Moreover, judged from the decrease in average lifetime of TMA-DPH in ionomycin-stimulated platelets, the increase in F indeed underestimates the increase in membrane-bound TMA-DPH. These findings strongly suggest that TMA-DPH labels virtually all membranes within platelets during stimulation by ionomycin. Dilution of ionomycin-activated platelets reveals the presence of a much larger nondilutable pool of TMA-DPH than observed with unstimulated or thrombin-activated platelets (Figure 1B). This nondilutable pool most likely results from penetration of TMA-DPH to the inner leaflet of the plasma membrane and other intracellular membranes of the platelet during stimulation by ionomycin. Alternatively, the nondilutable pool may be due to irreversible binding of TMA-DPH in the ionomycin-containing platelet plasma membrane. However, this possibility can be excluded, since platelets sonicated after stimulation by ionomycin show a similar steady-state level of F after dilution as do unstimulated or thrombin-stimulated platelets (Figure 1C). Thus, it is concluded that rapid transbilayer movement of TMA-DPH over the plasma membrane is the cause for the high levels of F observed after stimulation by ionomycin.

Figure 1A shows that upon stimulation with ionophore, TMA-DPH becomes translocated into the platelet almost to the maximal level within less than 1 min, suggesting that the permeability barrier for this probe has disappeared under those

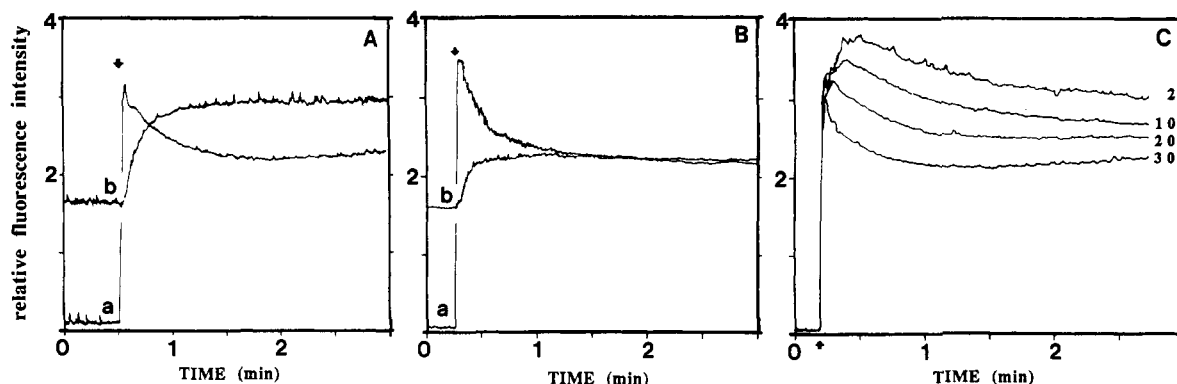


FIGURE 2: Temporary character of flip sites in activated platelets. (A) Labeling of ionomycin-activated platelets with TMA-DPH, 1 min after (a) or before (b) activation. Arrow indicates addition of TMA-DPH (a) or ionomycin (b). (B) Labeling of thrombin-activated platelets with TMA-DPH, 1 min after (a) or before (b) activation. Arrow indicates addition of TMA-DPH (a) or thrombin (b). (C) Labeling of ionomycin-activated platelets with TMA-DPH (arrow) at different times after activation, indicated in seconds by the numbers at the end of the recordings. Details are described in the Experimental Procedures. Data shown are representative for four experiments.

conditions. This, however, seems to be incompatible with the results of the dilution experiment in Figure 1B, where a significant part of the probe is retained in the internal membranes over a period of more than 3 min. Thus, it seems that rapid transbilayer movement of TMA-DPH during activation by ionomycin is enabled only transiently. This possibility was examined by comparing the fluorescence intensity of TMA-DPH when added before or after stimulation of the platelets. Table I demonstrates that upon stimulation with ionomycin (τ) decreases to a value that is not significantly different from the value obtained with sonicated platelets. Hence, the environmental difference that determines the difference in (τ) between the inner and outer monolayer disappears upon stimulation with ionomycin. With thrombin as stimulating agent (τ) did not change significantly (Table I). Nevertheless, (τ) appears to be independent of whether TMA-DPH was added to the platelets before or after activation by thrombin. Therefore, quantitative comparison of F when TMA-DPH is added before or after the stimulus is allowed both for ionomycin- and thrombin-activated platelets. It was found that when TMA-DPH was added 1 min after ionomycin, F increased to 1.5-fold the basal level observed for unstimulated platelets whereas a 2-fold increase in F is observed when TMA-DPH was added prior to ionomycin (Figure 2A). In contrast, no difference in increase of F was found between addition of the probe before or after stimulation with thrombin (Figure 2B). The transient character of rapid transbilayer movement of TMA-DPH during ionomycin activation was further detailed by adding the probe at various time intervals after ionomycin. It can be seen from Figure 2C that when the time interval between addition of the stimulus and addition of the probe is increased, the level of increase in F is gradually lowered. Addition of TMA-DPH more than 30 s after ionomycin did not lead to a further diminution of the increase in fluorescence signal. Thus, it is suggested that during stimulation of platelets by ionomycin rapid transbilayer movement, as sensed by TMA-DPH, takes place for a time period of about 30 s.

The results described above demonstrate an inward-directed translocation of TMA-DPH over the platelet plasma membrane. However, the probe can also be used to monitor outward transport, i.e., from the inner leaflet to the outer leaflet of the plasma membrane. Prolonged incubation of platelets with TMA-DPH in the absence of any stimulus allows the probe to become gradually incorporated in the total lipid pool with an estimated $t_{1/2}$ of 60–90 min (Verhallen, 1988). As shown in Table I, after a 140-min incubation the fraction of membrane-bound TMA-DPH is increased to 16.7%, close to

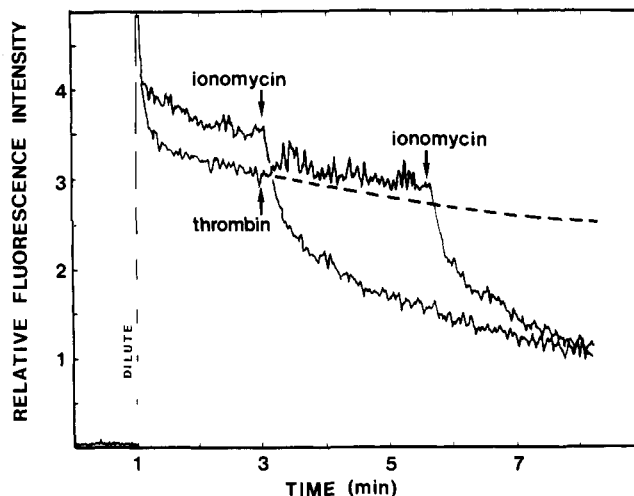


FIGURE 3: Outward transport of TMA-DPH during platelet activation. Washed human platelets (2×10^8 platelets/mL) were equilibrated at 37 °C with TMA-DPH ($5 \mu\text{M}$) for 140 min in HEPES buffer in the absence of Ca^{2+} . F was registered after 100-fold dilution of the platelet suspension in HEPES buffer containing 3 mM CaCl_2 . Ionomycin ($0.5 \mu\text{M}$) or thrombin (2 nM) was added 2 min after dilution. Stimulation by thrombin was followed (2.5 min later) by addition of $0.5 \mu\text{M}$ ionomycin. Dashed line represents the time course of F in unstimulated platelets upon dilution.

the values observed with ionomycin-stimulated platelets or platelet sonicate. The average lifetime after a 140-min incubation is decreased to 4.65 ns, indicating that the probe has gained access to a different environment. Subsequent dilution of these platelets causes a rapid efflux of TMA-DPH from the outer monolayer of the plasma membrane according to the principle of mass action. The remaining fluorescence intensity is caused by residual probe present in the inner leaflet of the plasma membrane and the total pool of internal membranes. Efflux of TMA-DPH from the latter two pools is dependent on the rate of outward transport through the plasma membrane. As shown in Figure 3, this is a rather slow process, which can be greatly accelerated upon stimulation with ionomycin. This increase in rate of efflux is not observed when platelets are stimulated by thrombin, in spite of the same extent of secretion induced by both stimuli. The minor increase in F that results from thrombin stimulation is most likely caused by additional plasma membrane that becomes available as part of the secretory event, taking up diluted probe from the extracellular compartment. Thus, the outward transport of TMA-DPH is not accelerated upon stimulation with thrombin. Moreover, subsequent addition of ionomycin to thrombin-stimulated platelets causes rapid efflux of internalized TMA-

DPH. In conclusion, this experiment clearly demonstrates that during stimulation by ionomycin also the rate of outward transport of TMA-DPH is greatly accelerated.

DISCUSSION

In the present investigations, platelet stimulation by thrombin and by ionomycin was compared, because of the different potencies of these activators to alter transmembrane lipid asymmetry (Bever et al., 1983, 1987; Comfurius et al., 1985). A change in membrane lipid asymmetry in platelets was shown to be most clearly reflected by the generation of a procoagulant surface, i.e., the exposure of PS in the outer leaflet of the plasma membrane. This can be sensitively measured as an increase in the rate of prothrombin activation by the coagulation factor Xa-Va complex, the formation of which is critically dependent on a PS-containing lipid surface. Using this method, it has been shown that platelet stimulation by thrombin hardly affects the distribution of PS, while Ca^{2+} ionophore leads to a considerable exposure of PS in the outer leaflet (Bever et al. 1983). This change in lipid asymmetry induced by ionophore is by no means restricted to PS. Nonlytic degradation of phospholipids by means of phospholipases has revealed that loss of PS asymmetry is accompanied by randomization of the other phospholipids as well (Bever et al., 1983). Moreover, it has been demonstrated that the ionophore-induced randomization process—at least for PS—is completed in less than 1 min (Verhallen et al., 1987). A rapid and abundant flip-flop of lipids most likely occurs along local disturbances of the bilayer structure (flip sites) as suggested earlier (Bergmann et al., 1984), though direct or indirect involvement of membrane proteins cannot be excluded (van Deenen, 1981). The present results clearly demonstrate that the amphiphilic fluorescent probe TMA-DPH is able to detect the occurrence of these flip sites during platelet stimulation.

Stimulation of washed human platelets by thrombin increased the relative fluorescence intensity of TMA-DPH, which can be directly interpreted as an increase in membrane-bound TMA-DPH, considering the absence of any change in average lifetime of the probe. The increase in membrane-bound TMA-DPH has been clearly correlated with the release reaction (Kuhry et al., 1983), which involves fusion of granule membranes with the plasma membrane. Stimulation of platelets by thrombin was not accompanied by an increase in transbilayer movement of TMA-DPH. This seems a rather surprising result, since it could be expected that during fusion of granule membranes with the plasma membrane flip-flop of a relatively small and aspecific lipid probe as TMA-DPH would be enhanced due to disturbances of the bilayer structure, which inevitably accompany the secretory process. It is conceivable that actual fusion of membranes is too rapid and involves too low a percentage of total membrane lipids to allow a detectable amount of TMA-DPH to gain access to these potential flip sites.

During stimulation of platelets by ionomycin, TMA-DPH becomes subject to massive transbilayer movement. Inward transport of TMA-DPH is inferred from the combined increase in F and decrease in lifetime, as well as the increase in the membrane-bound fraction of TMA-DPH as measured by laser-pulsed fluorescence spectroscopy. It is well-known that stimulation of platelets, in particular with Ca^{2+} ionophore, is accompanied by shedding of microvesicles from the plasma membrane (Sims et al., 1988, 1989). From differential centrifugation it was estimated that approximately 20% of the TMA-DPH signal can be ascribed to membrane microvesicles shed upon stimulation with ionomycin (data not shown). This value is in good agreement with the distribution of procoag-

ulant activity between shed vesicles and remnant cells as originates during platelet stimulation (Bever et al., 1982; Rosing et al., 1985).

The increase in membrane-bound TMA-DPH upon ionomycin stimulation cannot only be explained by additional plasma membrane becoming available as a result of the release reaction, since this increase was much higher than that observed with thrombin-stimulated platelets in spite of the decrease in average lifetime upon stimulation with ionomycin. Moreover, the appearance of a nondilutable pool of TMA-DPH after stimulation strongly suggests incorporation of probe in internal membranes. The time scale of rapid flip-flop of TMA-DPH is well in accordance with that of rapid flip-flop of PS during generation of platelet procoagulant activity by the Ca^{2+} -ionophore A23187 (Verhallen et al., 1987). Ionomycin is equally potent as A23187 in inducing randomization of PS over the platelet plasma membrane as judged from the dose-response relationships (unpublished results). The results presented in this study demonstrate that stimulation by ionomycin not only accelerates the inward-directed transport of TMA-DPH but also causes a rapid translocation of the probe from the inner to the outer leaflet of the plasma membrane. Thus, it is suggested that during stimulation of platelets by ionomycin flip sites are generated that provide a means for bidirectional transport, explaining the rapid loss of phospholipid transbilayer asymmetry and the rapid flip-flop of TMA-DPH. This supports the aspecific character of the flip sites, which favors the concept that disturbances of the bilayer structure are responsible for increased flip-flop. The results show that these sites, induced through the action of ionomycin, exist for less than 1 min.

The nature of the flip sites as well as the mechanism by which these sites are formed remains unclear. Involvement of a translocase in the activation-dependent loss of transbilayer asymmetry of the platelet plasma membrane seems, a priori, unlikely for the following reasons: (1) the translocase specifically transports amino phospholipids, while rapid flip-flop in activated platelets also involves sphingomyelin and phosphatidylcholine (Bever et al., 1983); (2) stimulation of rapid flip-flop in platelets can be observed only at increased cytosolic Ca^{2+} levels (Verhallen et al., 1987), at which translocase activity is inhibited (Zachowski et al., 1986); (3) the outward flip rate in activated platelets [$t_{1/2}$ less than 1 min (Verhallen et al., 1987)] is more rapid than inward transport rates by the translocase [$t_{1/2}$ for PS approximately 7 min at 37 °C (Sune et al., 1987)]; (4) translocase is normally identified as a unidirectional transporter of amino phospholipids toward the cell interior, although the possibility of outward transport by this protein has been suggested (Williamson et al., 1987).

Loss of PS asymmetry has been correlated with degradation of cytoskeletal proteins by the endogenous Ca^{2+} -dependent platelet protease calpain (Verhallen et al., 1987, 1988). It was proposed that degradation or modification of the cytoskeletal structure results in a loss of interaction with PS, allowing this lipid to participate in transbilayer movement. However, it is not immediately obvious how cytoskeletal degradation as such would lead to disturbance of the bilayer structure and subsequent randomization of endogenous lipids as well as an exogenous added lipidlike compound such as TMA-DPH.

We have previously proposed the involvement of diglycerides (Comfurius et al., 1985), known to form hexagonal phases in the normal bilayer structure (Cullis & de Kruijff, 1979) that are responsible for enhanced transbilayer movement of lipids. Although this mechanism could partially explain flip-flop in platelets stimulated by collagen plus thrombin, it seems less

likely to occur during activation by ionophore, since this stimulus is known to cause only minor diglyceride formation in platelets (Rittenhouse-Simmons, 1981). Alternatively, disturbances of the bilayer structure could occur during fusion of the plasma membrane that takes place upon shedding of membrane microvesicles as was recently suggested by Sims et al. (1989).

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