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Function of intracellular $[Ca^{2+}]_i$ in exocytosis and transbilayer movement in human platelets surface-labeled with the fluorescent probe 1-(4-(trimethylammonio)phenyl)-6-phenyl-1,3,5-hexatriene

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Ellipsometry indicated that 1-(4-(trimethylammonio)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) bound to platelets in a reversible and saturable way. Accordingly, the fluorescence intensity (F) of a suspension of TMA-DPH-labeled platelets was described as a quantity, determined by the amount of TMA-DPH bound to the platelet surface. Most platelet activators elevated F to a degree that correlated well with the secretion of serotonin evoked by these activators. The increase in F levels reflected the increase in outer membrane surface area following exocytosis. However, activators that evoked prolonged (> 2.5 min) and strong (> 600 nM) elevations of cytosolic $[Ca^{2+}]_i$ increased F to levels that were much higher than expected from the maximal increase in surface area due to exocytosis. This high increase in F was caused by inward transbilayer movement of TMA-DPH over the plasma membrane and the subsequent labeling of cytosolic membrane sides. The kinetics of exocytosis and changes in cytosolic $[Ca^{2+}]_i$ were studied by stopped-flow mixing of platelets with agonist. Thrombin-induced exocytosis had a delay of only 3 s, which was shortened when external $CaCl_2$ or ADP was present. This correlated well with a faster rise in $[Ca^{2+}]_i$ in the presence of $CaCl_2$ or ADP, indicating that exocytosis was linked in time to elevation of $[Ca^{2+}]_i$. By itself, ADP was unable to evoke exocytosis and it elicited a $[Ca^{2+}]_i$ transient of much shorter duration than thrombin, but with similar maximum. We concluded that both exocytosis and transbilayer movement were associated with elevation of $[Ca^{2+}]_i$: exocytosis required a moderate, relatively prolonged rise and transbilayer movement was accompanied by a stronger rise of even longer duration. Influx of external Ca^{2+} was essential for transbilayer movement, but not for exocytosis.

Introduction

1-(4-(Trimethylammonio)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) was developed as a fluorescent probe for the phospholipid-water interface [1]. When added to a cell suspension, it partitions from the aqueous phase over the available outer membrane [2,3]. Being virtually non-fluorescent in aqueous solution, TMA-DPH becomes very bright once incorporated in membranes [1,2]. Thus, the steady-state fluorescence intensity (F) of platelets in TMA-DPH-containing medium was found to increase upon exocytosis [4,5], due to the increase in outer membrane surface area by fusion of the plasma membrane with endomembranes from secretory vesicles [6]. However, the quantification of exocytosis from such increased F levels was rather unsatisfactory so far. For instance, the Ca^{2+}-ionophore ionomycin has been found to evoke a much higher F increase than thrombin did, in spite of the fact that the ionomycin-induced secretion of serotonin was slightly lower than the secretion with thrombin [5]. This inconsistency is caused partially by the poor insight into the factors determining the incorporation of TMA-DPH

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Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; dimethyl-BAPTA/AM, 5,5'-dimethyl-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; DOPC, dioleoyl phosphatidylcholine; DOPS, dioleoyl phosphatidylserine; F, fluorescence intensity; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TMA-DPH, 1-(4-(trimethylammonio)phenyl)-6-phenylhexa-1,3,5-triene.

into the plasma membrane, and also by the ability of some, but not all, agonists to activate the transbilayer movement of TMA-DPH across the membrane, which results in additional increases in F levels [7].

It is well known that the stimulus-exocytosis coupling in platelets is linked to elevation of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$ [8,9]. This has been demonstrated most directly in experiments with permeabilized platelets [10,11] and with platelets treated with Ca^{2+}-ionophore [12], where an artificial rise in $[Ca^{2+}]_i$ resulted in secretion, a process sensitized by the activation of protein kinase C. In intact platelets, most agonists elevate $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from internal stores and by influx of Ca^{2+} from extracellular origin [13]. Inhibition of these Ca^{2+} fluxes – e.g., by loading the platelets with an intracellular Ca^{2+} scavenger – appeared to lower agonist-induced secretion [14], suggesting a function of Ca^{2+} also in the non-permeabilized cells. Nevertheless, it is not completely clear, what levels of $[Ca^{2+}]_i$ are necessary for exocytosis in intact platelets, nor why some Ca^{2+}-mobilizing agonists like thrombin are secretory whereas others like ADP are not.

In this paper, we firstly studied the interaction of TMA-DPH with platelets and propose that F of TMA-DPH-labeled platelets is largely determined by equilibrium binding of the probe to the platelet surface, which justified the use of a binding model to quantify changes in outer membrane surface area following exocytosis. By kinetic measurements of exocytosis with TMA-DPH-labeled platelets and of changes in $[Ca^{2+}]_i$ with fura-2 label, using a stopped-flow procedure [15,16], we found that the occurrence of exocytosis was associated with a fairly prolonged rise in $[Ca^{2+}]_i$, and that there was a timely link between the $[Ca^{2+}]_i$ rise and the start of exocytosis. Detailed comparance of the agonist-induced changes in $[Ca^{2+}]_i$ and changes in TMA-DPH F suggested that activation of transbilayer movement was associated with a more vigorous elevation of $[Ca^{2+}]_i$ – in terms of level and duration of the transient – than activation of the exocytotic response. The latter observation may be of physiological importance, since transbilayer movement has been shown to occur along with loss of the phospholipid asymmetry over the plasma membrane and with increased thrombin-generating, procoagulant activity of the platelet surface [7,17].

Materials and Methods

Chemicals. Dimethyl-BAPTA/AM, fura-2/AM and TMA-DPH *p*-toluenesulfonate were from Molecular Probes (Eugene OR, USA), ionomycin from Serva (Heidelberg, Germany), DOPC and thapsigargin from Sigma (St. Louis, MO, USA), DOPS from Avanti Polar Lipids (Pelham, AL, USA), and thromboxane A_2 analogue U46619 was a gift from Upjohn (Kalamazoo, MI, USA). Bovine thrombin was purified and quantified according to Rosing et al. [18] or came from Sigma.

Ellipsometry. Films of a planar phospholipid bilayer were stacked on 0.6 cm^2 silicon slides [19]. Adsorption of TMA-DPH to such bilayers was measured with an automated ellipsometer according to Cuypers et al. [20]. The binding experiments were performed in hydrophilic quartz cuvettes containing 5 ml Hepes buffer (composition as below) at 20°C. The binding constant (K_d) of TMA-DPH to the phospholipid bilayer was determined from fitting the Langmuir isotherm, Eqn. 1

$$\Gamma = \Gamma_{max}\, C_{free}/(C_{free} + K_d) \quad (1)$$

where Γ and Γ_{max} are the adsorbed and the maximally adsorbed mass (measured in $\mu g/cm^2$), respectively. C_{free} is the concentration of unbound TMA-DPH and was approximated by the total TMA-DPH concentration, since the surface area of phospholipids was relatively small. Small unilamellar phospholipid vesicles were prepared by ultrasonication [21].

Platelet isolation. Human platelets were obtained from blood, freshly drawn from healthy volunteers. Except for the stopped-flow experiments, platelet isolation was according to Ref. 22. Platelets were washed twice in the presence of apyrase (0.1 U ADPase/ml) and were suspended in Hepes buffer (pH 7.4), containing 137 mM NaCl, 10 mM Hepes, 5 mM glucose, 2.7 mM KCl and 2 mM $MgCl_2$. Platelet concentration was calculated from the optical density of a diluted suspension at 405 nm.

Stopped-flow experiments were performed with platelets purified according to Sage and Rink [15]. Briefly, platelet-rich plasma was incubated with aspirin (100 μM) and apyrase (0.1 U ADPase/ml) for 45 min at 37°C. Fura-2/AM (4 μM) was added, when desired. The cells were washed and suspended in modified Hepes buffer (pH 7.4), containing 145 mM NaCl, 10 mM Hepes, 10 mM glucose, 5 mM KCl, 5 mM $MgCl_2$ and apyrase (0.1 U ADPase/ml). This procedure allowed straightforward comparison with earlier stopped-flow measurements [15].

TMA-DPH fluorescence measurements. Steady-state F was measured at excitation and emission wavelengths of 360 and 430 nm, respectively. TMA-DPH from a 10 mM stock solution in dimethyl sulfoxide was diluted into (modified) Hepes buffer, and the diluted solution was continuously stirred at room temperature. This solution was added to an equal volume of suspended platelets 2 min before the start of each experiment. Usually, fluorescent light was measured with an automated spectrofluorometer [23]. Corrections were made, where necessary, for fluorescence from scattered exciting light and background scattering. Control experiments showed that only a minor fraction of the externally applied TMA-DPH was internalized into the

platelets: less than 3% of the fluorescence was recovered after centrifugation of the platelets and resuspension of the pellet in TMA-DPH-free buffer.

Kinetic experiments were performed by stopped-flow fluorometry [15], where fluorescence data were sampled at a high rate of 50 s^{-1} for 10–20 s; nine repetitive injections were carried out at 15 to 30-s intervals and the nine resulting scans were averaged.

Platelet activation. Activation of TMA-DPH-labeled platelets was measured in quartz cuvettes containing $2.5–5 \cdot 10^7$ platelets/ml, 1 μM TMA-DPH and the required agonists at a low stirring rate of 200 rpm (37°C). Before use, the cuvettes were cleaned with ethanol/2 M HCl (50:50, v/v) and rinsed with water. Serotonin secretion was measured under the same conditions, but in the absence of TMA-DPH and with platelets that were preloaded [6] with [*side chain* 2-^{14}C]serotonin. Loading of platelets with dimethyl-BAPTA was according to Davies et al. [14]. Loading with fura-2 and measurements of $[Ca^{2+}]_i$ in quartz cuvettes were as described [15], and calibration of fura-2 fluorescence ratios to nM levels of $[Ca^{2+}]_i$ was according to Grynkiewicz et al. [24]. Platelet aggregation was not influenced by the presence of TMA-DPH and all agonists were checked for possible interference with TMA-DPH fluorescence. Activation experiments with ADP were carried out within 45 min of final preparation of the cells, because of rapid desensitization of the ADP response.

Stopped-flow kinetics of platelet activation. Aspirin-treated platelets ($1 \cdot 10^8$/ml) in modified Hepes buffer containing 5 μM TMA-DPH and 1 mM $CaCl_2$ or 1 mM EGTA, as desired, were mixed by stopped-flow injection with an equal volume of buffer containing TMA-DPH, $CaCl_2$ or EGTA, and the required agonist at a temperature of 37°C. This resulted in rapid occupation (< 30 ms) of platelet receptors and in synchronous activation of the cells. Averaged fluorescence scans (see above) were corrected for changes in F due to re-equilibration, by subtracting agonist-free control records from the stimulated records. Kinetics of agonist-induced rises in $[Ca^{2+}]_i$ were determined using fura-2-preloaded platelets at 37°C as described [15], except that records obtained at excitation wavelenghts of 340 and 380 nm were divided and expressed as a ratio.

Binding model for TMA-DPH. A binding model was applied, in which the fluorescence intensity (F) of a platelet suspension was assumed to be proportional to the fraction of TMA-DPH bound to the platelets (Γ) according to $\Gamma = a\ F$, where a is the proportionality constant. Binding was considered to be restricted to one type of site, defined by an apparent K_d and Γ_{max} value. Note that the values of F and, thus, a were dependent on the instrumental settings.

Accordingly, F can be described as a function of the free TMA-DPH concentration (C_{free}), using the classical binding formula

$$F = F_{max} C_{free} / (C_{free} + K_d) \quad (2)$$

The maximal binding, Γ_{max}, and the maximal fluorescence, F_{max}, at a given platelet concentration are related by $\Gamma_{max} = a\ F_{max}$. The non-fluorescent C_{free} is a fraction of the total TMA-DPH concentration (C_T), according to Eqn. 3

$$C_{free} = C_T - a\ F \quad (3)$$

As a first approximation, F_{max} at a given platelet concentration was estimated from the measured F levels by a double inverse plot of F and C_T. The obtained F_{max} value appeared to be a linear function of the platelet concentration, as required. Using this estimation and using Eqns. 2 and 3, the matrix of measured F values at various TMA-DPH and platelet concentrations was analyzed by derivative-free nonlin-

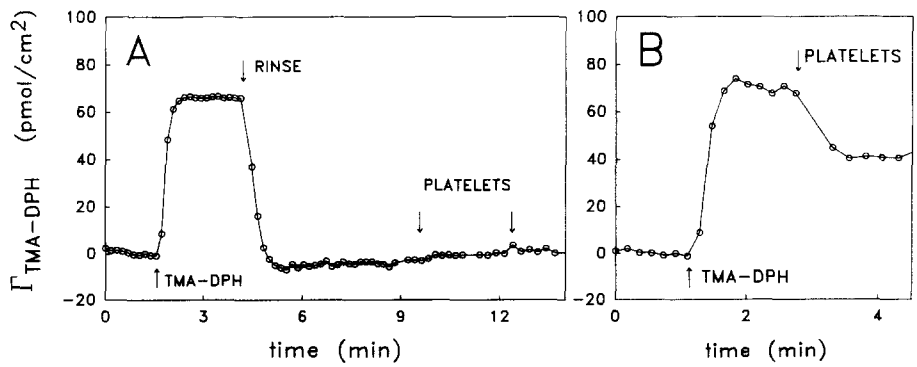

Fig. 1. Binding of TMA-DPH to phospholipids and platelets. A planar bilayer of DOPC/DOPS (80:20, mol/mol) was stacked on a hydrophilic silicon slide, and changes in mass (Γ) of the slide were measured by ellipsometry at 20°C. The mass of the bilayer was 420 ng/cm^2 (i.e., 500 pmol/cm^2). (A) The addition of TMA-DPH (1 μM) resulted, after equilibrium, in an increase of the bilayer mass by 30 ng/cm^2, equivalent to a binding of 66 pmol TMA-DPH/cm^2). Rinsing with TMA-DPH-free buffer resulted in complete desorption. The planar bilayer mass was not changed by the addition of two aliquots of platelets (each $1 \cdot 10^7$/ml). (B) The addition of platelets ($2 \cdot 10^7$/ml) following TMA-DPH (1 μM) resulted in desorption as a consequence of the binding of the probe to the platelets. Data given are representative for at least seven experiments.

ear regression with BMDP-AR statistical software (University of California, Los Angeles). Three-parametric analysis gave best fitting values for a, K_d and F_{max}. Adequacy of the fit was judged from calculation of pseudo $R^2 = 1.0 - (\text{sum of squared residuals}/(n - 1) \cdot \text{variance})$; this parameter amounted to 0.964 ± 0.014 (S.E., $n = 8$). The value of F_{max} fitted in this way closely approached the previously estimated one.

Results

Binding of TMA-DPH to phospholipid bilayers and platelets

Ellipsometry has been used to detect the binding of nanogram amounts of protein to a planar bilayer of phospholipids, stacked on a silicon slide [20,25]. This technique was applied to study the interaction of TMA-DPH with phospholipids. When TMA-DPH was added to a stacked bilayer of DOPC/DOPS (80:20, mol/mol), the bilayer mass increased significantly, indicating binding to the phospholipid surface (Fig. 1A). Subsequent rinsing with TMA-DPH-free buffer resulted in complete desorption to the level of the original mass of the bilayer, suggesting that the binding was reversible and the bilayer structure remained intact. The amount of adsorbed TMA-DPH was concentration-dependent and saturable: binding curves gave a K_d of 5.7 μM and a maximal binding, Γ_{max}, of 410 pmol/cm^2. Similar binding characteristics were measured with a stacked bilayer of DOPC, i.e., a K_d of 6.9

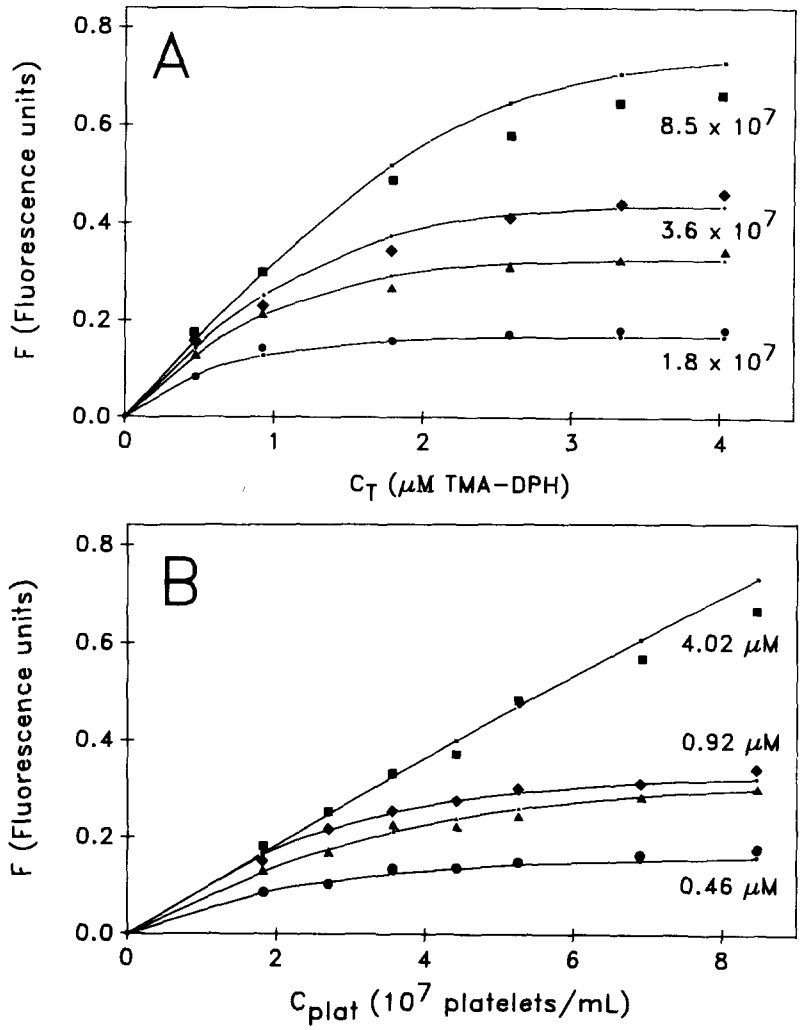

Fig. 2. Quantification of fluorescence of TMA-DPH-labeled platelets. Platelets were unstimulated or were activated with 2 nM thrombin, as indicated. Prostaglandin E_1 (3 μM) was added to inhibit changes in activation during the measurements. Solid symbols represent the measured fluorescence intensities (F) with errors < 0.003 (S.E., $n = 8$) of suspensions containing various concentrations of TMA-DPH (C_T) and platelets (C_{plat}). (A) Plots of F and C_T at 1.8 (●), 3.6 (▲) and 8.5 (■) $\cdot 10^7$ unstimulated platelets/ml; also a plot of thrombin-activated platelets at $3.6 \cdot 10^7$ platelets/ml (◆). (B) Plots of F and C_{plat} at 0.46 (●), 0.92 (▲) and 4.02 (■) μM TMA-DPH; plot of thrombin-activated platelets at 0.92 μM TMA-DPH (◆). Using Eqns. 2 and 3, best values were estimated for a, K_d and F_{max} by nonlinear regression analysis (see Materials and Methods). These were $a = 2.30$ μM, $K_d = 0.44$ μM and $F_{max} = 0.114$ ml/10^7 platelets. The corresponding values estimated for thrombin-activated platelets differed only in $F_{max} = 0.154$ ml/10^7 platelets. Drawn lines represent recalculated values of F, using these parameters and Eqns. 2 and 3.

μM and Γ_{max} of 350 pmol/cm^2. In contrast, TMA-DPH did not bind to uncovered silicon slides (data not shown).

Once bound, TMA-DPH desorbed from the DOPC/DOPS bilayer when either sonicated vesicles of DOPC/DOPS (not shown) or blood platelets (Fig. 1B) were added. Since neither the vesicles (not shown) nor the platelets (Fig. 1A) influenced the bilayer mass, this points to a shift in equilibrium distribution of the TMA-DPH, apparently due to its binding to the added vesicles or platelets. The binding amounted to 291 ± 24 (S.E., $n = 10$) pmol/10^7 platelets at $2 \cdot 10^7$ platelets/ml and 1 μM TMA-DPH, and it increased with the probe concentration. Activation of the platelets with thrombin resulted in a significant increase of desorption of TMA-DPH from the bilayer: the extra binding to platelets caused by 2 nM thrombin was 74 ± 14 (S.E., $n = 7$) pmol/10^7 platelets.

Quantification of changes in platelet surface area from TMA-DPH fluorescence intensities

Knowing that TMA-DPH reversibly binds to platelets, it was tempting to quantify TMA-DPH F in terms of equilibrium binding. As would be expected for a binding-related quantity, F was saturated at high TMA-DPH and high platelet concentrations (Fig. 2A,B). We tested the validity of a simple binding model, in which F was assumed to reflect the amount of membrane-bound TMA-DPH (see Materials and Methods). Binding constants were estimated from the measured fluorescence data by using Eqns. 2 and 3 and an automated fitting procedure and, subsequently, theoretical F levels were calculated with these constants (Fig. 2A,B). These fitted levels closely resembled the actually measured ones, except at platelet concentrations above $7 \cdot 10^7$/ml where the values deviated, possibly due to the high turbidity of such suspensions. Binding parameters determined were an apparent K_d of 0.60 ± 0.18 μM and Γ_{max} of 248 ± 18 pmol/10^7 platelets (S.E., $n = 4$).

According to the binding model, Γ_{max} and the maximally reached F level (F_{max}) are proportional to the platelet membrane surface area and, thus, these parameters are expected to increase upon exocytosis. In agreement with earlier observations, platelet activation with secretory thrombin (2 nM) resulted in increased F levels (Fig. 2). When the fluorescence data of thrombin-activated platelets were fitted into the model, we found a K_d of 0.51 ± 0.12 μM and Γ_{max} of 354 ± 28 pmol/10^7 platelets (S.E., $n = 3$), suggesting that the apparent binding affinity was virtually unchanged by the activation, whereas the binding capacity was increased by about 43%. The latter value is close to the estimated thrombin-evoked increase in outer membrane surface area of 55%, determined from the increased phospholipid hydrolysis by exogenously added phospholipase A_2 and sphingomyelinase under nonlytic conditions [26].

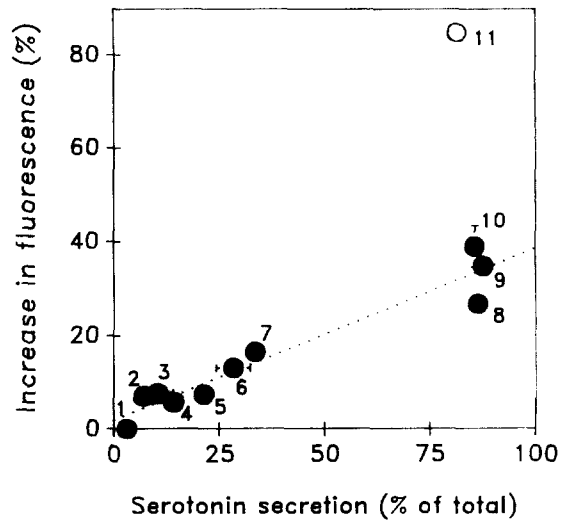

Fig. 3. Correlation between the effects of platelet activators on serotonin secretion and TMA-DPH fluorescence. Platelets ($2.5 \cdot 10^7$/ml) incubated with TMA-DPH (1 μM) were activated as indicated, and the increase in F was examined after 5 min of activation. In parallel experiments, platelets prelabeled with [^{14}C]serotonin were activated under the same conditions (but without TMA-DPH) and secretion of serotonin was measured. Data are mean values ± S.E. ($n = 4$–10); note that error bars are often smaller than the symbol sizes. Numbers refer to the activation conditions used: 1, ADP (40 μM); 2, U46619 (1 μM); 3, NaF (10 mM); 4, thapsigargin (0.5 μM) + EGTA (1 mM); 5, collagen (20 μg/ml); 6, ionomycin (0.5 μM) + EGTA (1 mM); 7 and 8, thrombin (0.2 and 2 nM); 9, trypsin (0.5 μg/ml); 10, thapsigargin (0.5 μM) + $CaCl_2$ (1 mM). Curve-fitting gave a straight line with $r = 0.96$ ($n = 10$). Additionally, 11, ionomycin (0.5 μM) + $CaCl_2$ (1 mM).

Quantification of exocytosis from changes in TMA-DPH fluorescence

The effects of a number of platelet activators on TMA-DPH fluorescence were compared with those on serotonin secretion. We found a good correlation between both processes using the following activators (all applied for 5 min): ADP, collagen, thrombin, trypsin, ionomycin/EGTA, thromboxane A_2 analogue U46619, GTPase activator fluoride, the endomembrane Ca^{2+}-ATPase inhibitor [27] thapsigargin (Fig. 3). Dose-response curves gave comparable results for each type of measurements (not shown). Typically, ionomycin in the presence of $CaCl_2$ evoked an increase in F of about 85% (Fig. 3), i.e., much larger than expected from the serotonin release and in agreement with the earlier data from Kubina et al. [5]. As has been demonstrated [7], this extra increase is caused by transbilayer movement of TMA-DPH over the plasma membrane followed by a re-labeling of the outer membrane surface with aqueous probe molecules [7], which process, consequently, is likely to interfere with exocytosis when ionomycin/$CaCl_2$ is used as activator (see also below).

TABLE I

Estimation of agonist-induced increase in platelet outer membrane surface from TMA-DPH fluorescence intensities

Platelets labeled with TMA-DPH were activated with the indicated agonists, as described for Fig. 3. Percentages increase of F are given with platelets from different donors (mean ± S.E., $n = 6$–10), measured after 5 min of activation. From these F values increases in outer membrane surface area (Γ_{max}) were estimated using eqns. 2 and 3 and $\Gamma = a\ F$, and assuming that only F_{max} changes upon activation. Data for ionomycin/$CaCl_2$ and thapsigargin/$CaCl_2$ are not included, because of the (possible) interference of transbilayer movement of TMA-DPH with these agonists. Mean values for a (2.30 μM), K_d (0.60 μM) and F_{max} (0.108 ml/10^7 platelets) for unstimulated platelets were taken from four independent experiments (see Materials and Methods).

Agonist	Increase in	
	F (%)	Γ_{max} (%)
Control	0	0
ADP (40 μM)	0.1 ± 0.06	0.1
U46619 (1 μM)	7.1 ± 1.4	8.9
NaF (10 mM)	7.6 ± 0.9	9.6
Ionomycin (0.5 μM) + EGTA (1 mM)	13.1 ± 1.7	16.7
Trypsin (0.5 μg/ml)	26.7 ± 1.3	35.6
Thrombin (0.2 nM)	16.5 ± 1.1	21.3
Thrombin (2 nM)	34.0 ± 1.5	46.6

However, in some (2 out of 8) experiments, prolonged (10 min) incubation of the platelets with thapsigargin/$CaCl_2$ resulted in somewhat higher F levels than expected, suggesting that transbilayer movement of TMA-DPH may also occur with thapsigargin as activator. In agreement with this, as indicated below, thapsigargin in the presence of both $CaCl_2$ and a second agonist was found to evoke similarly high increases in F as those reached with ionomycin/$CaCl_2$.

The binding model predicts that, under normal incubation conditions, considerable fractions of TMA-DPH are depleted from the aqueous phase due to the binding to platelets (see Fig. 2). For example, from Eqn. 1 and the previously estimated values for K_d of 0.60 μM and Γ_{max} of 248 pmol/10^7 platelets it can be calculated that at 1 μM TMA-DPH and $2 \cdot 10^7$ platelets/ml a fraction of about 28% of the probe is platelet-bound. Consequently, the increase in F induced by secretory agonists is likely to underestimate the actual increase in membrane surface area. Estimation of the activation-induced changes in F_{max} or Γ_{max} corrects for such a depletion effect. Thus, Table I, presenting changes in Γ_{max} of agonists that are unlikely to evoke transbilayer movement, indicates that thrombin induced the highest increase in outer membrane surface area by 46.6%. Other activators were less potent in evoking exocytosis, in agreement with data from Holmsen and Weiss [28]. Indeed, transmission electron micrographs showed that thrombin was able to release most of the platelet granules under these conditions (data not given).

Effect of platelet activators on exocytosis and rise in $[Ca^{2+}]_i$

The early kinetics of $[Ca^{2+}]_i$ rises have been studied by stopped-flow mixing of fura-2-loaded platelets with high doses of agonists, a procedure resulting in synchronous activation of the cells [15,16]. After ratiometric analysis of the 340 and 380 nm fura-2 fluorescence tracings, we found that such stopped-flow mixing with ADP caused an immediate, but rather transient, $[Ca^{2+}]_i$ rise (Fig. 4A), whereas thrombin evoked a somewhat delayed and more prolonged rise (Fig. 4B). Typically, the two agonists hardly differed in the maximal levels of $[Ca^{2+}]_i$ reached, but were much different in the duration of the Ca^{2+} signal.

The same stopped-flow technique was used to study exocytosis (Fig. 5). After correction for probe equilibration (Fig. 5B), stopped-flow mixing of TMA-DPH-labeled platelets with 40 μM ADP resulted in constant F levels for at least 10 s (Fig. 5C), whereas mixing with 11 nM thrombin gave a rapid increase of F (Fig. 5D), indicative of exocytosis with an early onset (2.5 s) and high velocity (F increased by 23%, and Γ_{max} by 25% in 5 s).

As shown in Table II the delay in onset of thrombin-induced $[Ca^{2+}]_i$ rise and thrombin-induced increase in TMA-DPH F were significantly shortened by the presence of external $CaCl_2$ or ADP. This suggested that exocytosis was accelerated by $CaCl_2$- and/or ADP-mediated events, but also that a faster Ca^{2+} response gave rise to an earlier onset of exocytosis. Apparently, the rapid and short-lived $[Ca^{2+}]_i$ signal evoked by ADP did not promote exocytosis by itself, but it synergized with the thrombin-induced Ca^{2+} response (Fig. 4C) to evoke more rapid secretion (Fig. 5D). By similar reasoning, the thrombin-induced Ca^{2+} influx component of elevated $[Ca^{2+}]_i$, being more rapid than the internal mobilization of Ca^{2+} [15], seemed to accelerate the exocytosis response.

Effect of platelet activators on transbilayer movement of TMA-DPH and rise in $[Ca^{2+}]_i$

Ionomycin, in the presence of $CaCl_2$ but not with EGTA, could still elevate TMA-DPH F levels of platelets that were preactivated with thrombin (Fig. 6A) and had secreted most of their granular contents (data not shown, compare Fig. 3). Apparently, the exocytosis- and transbilayer movement-dependent increases in F required different conditions of activation. Loading the platelets with the intracellular Ca^{2+} scavenger dimethyl-BAPTA prior to activation, resulted in inhibition of both the thrombin- and ionomycin-induced increases in F (Fig. 6B), suggesting that exocytosis as well as transbilayer movement required elevated $[Ca^{2+}]_i$. This raised the possibility that exocytosis and transbilayer movement may have different thresholds in terms of $[Ca^{2+}]_i$ elevation.

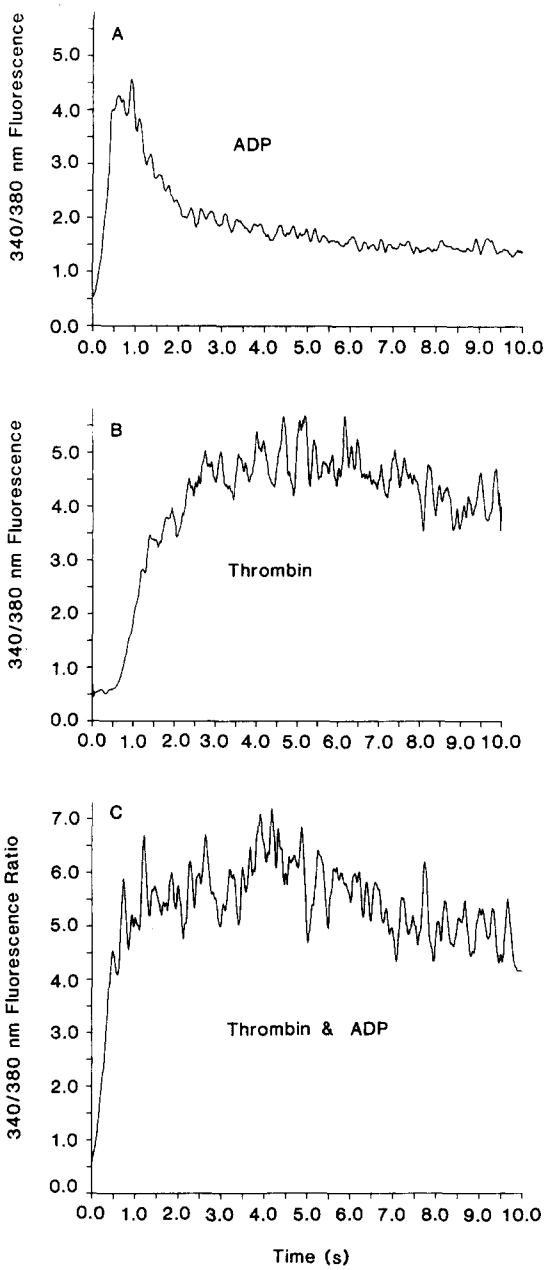

Fig. 4. Stopped-flow kinetics of rise in $[Ca^{2+}]_i$. Fura-2-loaded, aspirinated platelets ($1 \cdot 10^8$/ml) were activated by stopped-flow with an equal volume of agonist-containing buffer. Traces show the ratio of fura-2 fluorescence at 340 to 380 nm excitation wavelength. (A) Activation with 40 μM ADP (final concentration); (B) activation with 11 nM thrombin; (C) activation with 11 nM thrombin plus 40 μM ADP. Note the 10-s time scale. Data are given of experiments representative for three experiments.

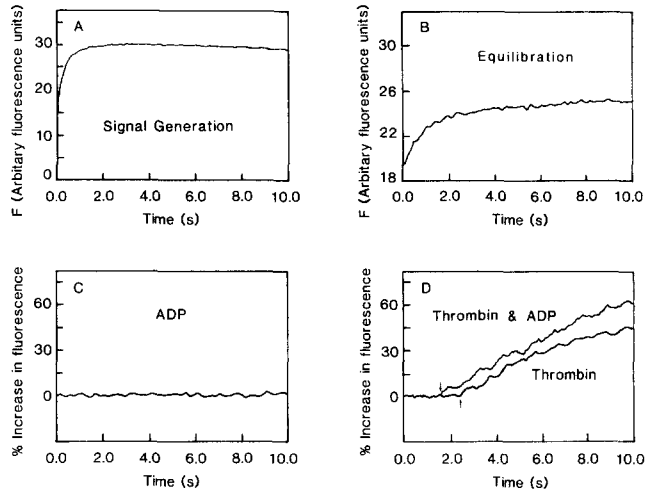

Fig. 5. Stopped-flow kinetic analysis of exocytosis. (A) Sub-second generation of TMA-DPH fluorescence. Aspirinated platelets ($1 \cdot 10^8$/ml) in modified Hepes buffer were mixed by stopped-flow with an equal volume of buffer containing TMA-DPH (5 μM). Injection time was at 0 s. Fluorescence F reached 50% of its final value after 10 ms. (B) Re-equilibration of TMA-DPH. Platelets ($1 \cdot 10^8$/ml) in buffer with TMA-DPH (5 μM) and $CaCl_2$ (1 mM) were mixed by stopped-flow with an equal volume of TMA-DPH/$CaCl_2$-containing buffer. The increase in F is probably due to a change in equilibrium, i.e., to the increased binding of free TMA-DPH to the cell surface due to a more favourable probe/platelet ratio. (C) Tracing of activation with 40 μM ADP (final concentration) after correction for equilibration, by substracting the tracing of agonist-free control. Conditions of mixing as in (B). (D) Tracings of activation with 11 nM thrombin $\pm$ 40 μM ADP after correction for equilibration. Mixing conditions as in (B). Increase in fluorescence after a delay (arrow) indicates start of exocytosis. Note the 10-s time scale. Data are given of experiments representative of 3–10 experiments.

In platelets, the Ca^{2+}-ATPase inhibitor thapsigargin is a potent Ca^{2+}-mobilizing agent, evoking the release of intracellular Ca^{2+} [29] and the influx of extracellular Ca^{2+} [30]. Fig. 7 shows that thapsigargin in combination with thrombin and $CaCl_2$ was able to induce a similarly high, transbilayer movement-dependent, increase in F as ionomycin/$CaCl_2$. The nearly complete secretion of serotonin induced by thrombin/$CaCl_2$ un-

TABLE II

Delay in onset of $[Ca^{2+}]_i$ rise and exocytosis in stopped-flow activated platelets

Aspirin-treated, fura-2-preloaded platelets ($1 \cdot 10^8$/ml) suspended in modified Hepes buffer were rapidly activated by stopped-flow mixing with the indicated agonists, and onset of the rise in $[Ca^{2+}]_i$ was determined from the fura-2 fluorescence recordings. In parallel experiments, platelets (not loaded with fura-2) in the presence of 5 μM TMA-DPH were activated by stopped-flow mixing with buffer containing agonist(s) and TMA-DPH, and the delay in onset of exocytosis was determined. Buffers contained either $CaCl_2$ or EGTA (both 1 mM), as indicated, and final concentrations of thrombin and ADP were 11 nM and 40 μM, respectively. Mean values $\pm$ S.E. (n) of delay times are given.

Activation condition	Delay in	
	rise of $[Ca^{2+}]_i$ (ms)	exocytosis (ms)
$CaCl_2$ + thrombin	430 ± 20 (4)	2540 ± 120 (10)
$CaCl_2$ + thrombin + ADP	10 ± 10* (7)	1950 ± 150* (9)
EGTA + thrombin	500 ± 10 (4)	2940 ± 70 (10)
EGTA + thrombin + ADP	190 ± 20* (7)	2240 ± 190* (9)

* Significantly different from the parallel experiments in the absence of ADP with $P < 0.01$ (Student's t-test).

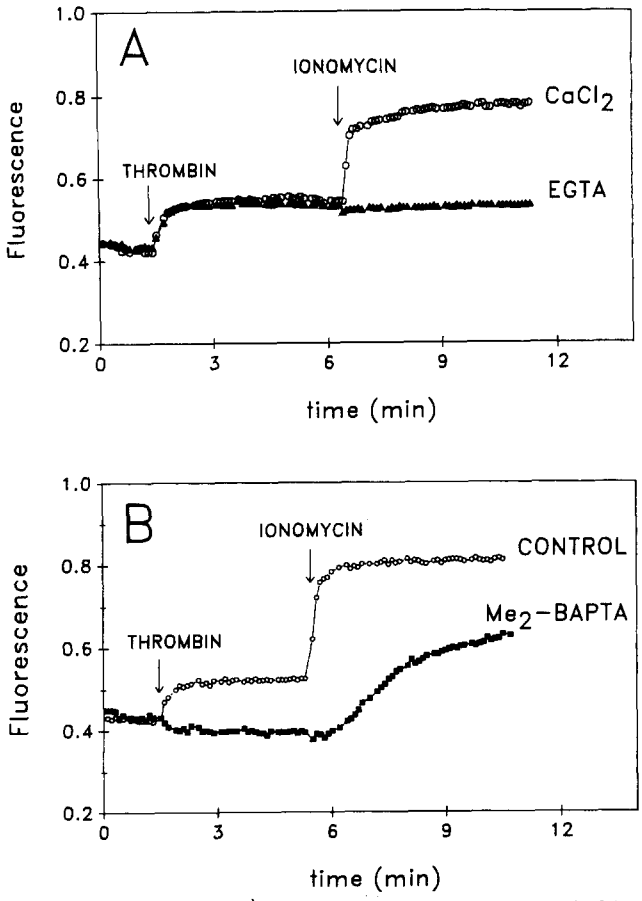

Fig. 6. Effects of thrombin and ionomycin on fluorescence of TMA-DPH-labeled platelets. Platelets in TMA-DPH buffer were activated with 2 nM thrombin and 0.5 μM ionomycin, as indicated. Conditions were as in Fig. 3. (A) Fluorescence recordings of activations in the presence of 1 mM $CaCl_2$ (○) or 1 mM EGTA (△). (B) Fluorescence recordings of control (○) or dimethyl-BAPTA-loaded (■) platelets activated in the presence of 1 mM $CaCl_2$. Error of measured F values was < 0.003 (S.E., $n = 4$).

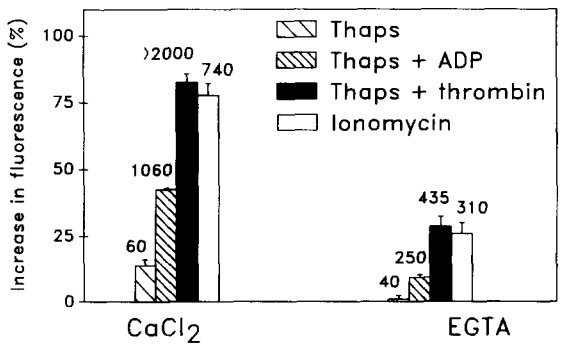

Fig. 7. Effects of thapsigargin and ionomycin on fluorescence of TMA-DPH-labeled platelets. Platelets ($2.5 \cdot 10^7$/ml) were labeled with TMA-DPH (2 μM) in the presence of $CaCl_2$ (1 mM) or EGTA (1 mM). Activation was with thapsigargin (thaps, 50 nM), ADP (40 μM), thrombin (2 nM) or ionomycin (1 μM), as indicated, and percentage increase of F was measured after 5 min of activation (mean ± S.E., $n = 3$). Numbers represent $[Ca^{2+}]_i$ (in nM), measured in fura-2-loaded platelets in parallel experiments after 0.25 min of activation.

der these conditions was not further increased by the addition of thapsigargin (data not shown). Thrombin could be replaced by ADP, although in the latter case the elevation of TMA-DPH F was somewhat less (Fig. 7). In the absence of external $CaCl_2$ or second agonist, the thapsigargin-provoked increase in F was reduced to 28% or lower, i.e., to the level reached by exocytosis alone (compare Fig. 3).

By measuring the Ca^{2+} response of the platelets under these activation conditions, we found that thapsigargin/$CaCl_2$ in combination with thrombin or ADP, like ionomycin/$CaCl_2$, raised $[Ca^{2+}]_i$ to very high, (almost) micromolar concentrations (Fig. 7). Further comparison of the thapsigargin/agonist-provoked effects on TMA-DPH fluorescence and on $[Ca^{2+}]_i$ changes showed that replacement of $CaCl_2$ by EGTA or inhibition of the Ca^{2+} influx by Ni^{2+} resulted in complete abolishment of the strong increase in F (i.e., the component exceeding the exocytosis-related increase of about 28%) and in appreciable reduction of the prolonged rise in $[Ca^{2+}]_i$ (Table III). Preincubation of the platelets with prostaglandin E_1 had similar inhibitory effects, although this treatment was only partially active for the combination thapsigargin/thrombin. Careful examination of all data showed that transbilayer movement was associated with an increase in $[Ca^{2+}]_i$ of > 600 nM that persisted for at least 2.5 min and was dependent on the presence of external $CaCl_2$ (Table III).

TABLE III

Synergistic effect of agonists on thapsigargin-evoked increase in TMA-DPH fluorescence and rise in $[Ca^{2+}]_i$[a]

Left column: platelets were labeled with TMA-DPH in the presence of 1 mM $CaCl_2$ and activated with thapsigargin (thaps) and/or other agonists, as indicated. Further conditions were as in Fig. 7. Percentages increase of F are given, measured after 5 min of activation. Middle and right columns: fura-2-preloaded platelets were activated in the presence of $CaCl_2$ (1 mM), and $[Ca^{2+}]_i$ was determined after 0.25 and 2.5 min. Where indicated, the platelets were preincubated with prostaglandin E_1 or $NiCl_2$ for 2 min, or EGTA was present instead of $CaCl_2$. All data are mean values (± S.E.) of three triplicate experiments.

Activation condition	Increase in F (%)	$[Ca^{2+}]_i$ (nM)	
		0.25 min	2.5 min
Vehicle	0.0	105	95
Thrombin (2 nM)	23.0 (0.8)	900	445
Thaps (50 nM) + thrombin (2 nM)	81.6 (1.6)	> 2000	> 2000
+ prostaglandin E_1 (6 μM)	69.1 (4.9)	835	> 2000
+ Ni^{2+} (5 mM)	25.4 (3.7)	400	305
+ EGTA (1 mM)	30.6 (2.4)	435	135
Thaps (50 nM) + ADP (40 μM)	47.8 (2.4)	1060	660
+ prostaglandin E_1 (6 μM)	19.9 (6.0)	125	270
+ Ni^{2+} (5 mM)	23.6 (3.2)	510	370
+ EGTA (1 mM)	13.8 (4.6)	250	145

Discussion

Quantification of TMA-DPH fluorescence in terms of binding

Based on the results of binding studies with ellipsometry, we used a simple binding formula (Eqn. 2), in which F of TMA-DPH-labeled platelets was considered to be determined by the binding of TMA-DPH to the platelet surface. Application of this model to thrombin-activated platelets showed that the number of apparent binding sites (Γ_{max}) was increased compared to non-activated platelets, whereas the apparent K_d was virtually unchanged. This binding approach thus could be used to predict changes in the platelet outer membrane surface caused by exocytosis.

Calculation learns that considerable fractions of TMA-DPH may interact with the platelet surface. At $2 \cdot 10^7$ platelets/ml and 1 μM TMA-DPH, ellipsometry and fluorescence studies predicted a binding of 290 and 140 pmol/10^7 platelets, respectively. Assuming a total amount of phospholipids of 4 nmol/10^7 platelets, of which 20–25% is located in the outer leaflet of the plasma membrane [26,31], the surface phospholipids comprise 800–1000 pmol/10^7 platelets. Consequently, the number of surface-bound TMA-DPH molecules is of the same order of magnitude as the number of surface-exposed phospholipid molecules. Since we have no evidence that the probe disrupts the lipid bilayer structure (see Fig. 1), it is likely that only minor fractions of the membrane-bound TMA-DPH penetrate into the bilayer and become fluorescent. It is difficult to quantify the number of actually incorporated molecules, but time-resolved fluorescence decay studies of TMA-DPH-labeled platelet suspensions have predicted fractions of incorporated TMA-DPH that are at least one magnitude lower than the fractions of membrane-bound TMA-DPH calculated in this paper [2,7]. Thus, the results suggest that the amount of membrane-incorporated TMA-DPH is much smaller than, but nevertheless is determined by, the amount of membrane-bound probe.

The question arises as to the physical basis for the relationship between TMA-DPH binding and fluorescence. Fluorescence decay studies have indicated that TMA-DPH is fluorescent only after its incorporation into the membrane bilayer [1,32]. Thus, F ultimately must be a function of the number of membrane-incorporated probe molecules. The amphipathic structure of TMA-DPH suggests it forms into aggregates or micelles in aqueous environment. It is plausible that the positively charged TMA-DPH aggregates first bind to the polar heads of membrane phospholipids, after which only some TMA-DPH molecules incorporate into the bilayer and develop fluorescence. Conclusively, the steady-state F level may be determined not primarily by the number of incorporated TMA-DPH molecules, but by the equilibrium between aqueous and membrane-bound aggregates. Typically, ellipsometry gave binding values for TMA-DPH that were somewhat higher than those obtained with fluorescence studies (see above). Putatively, part of the platelet-bound TMA-DPH aggregates/micelles, as detected by ellipsometry, is not in direct contact with the membrane surface and, thus, cannot equilibrate with the membrane-incorporated, fluorescent probe molecules.

Quantification of exocytosis

For most platelet activators, we found a good correlation between serotonin secretion and increase in TMA-DPH F levels (Fig. 3), meaning that exocytosis can be quantified adequately by measuring the changes in TMA-DPH fluorescence. However, ionomycin and thapsigargin, when applied in such a way that $[Ca^{2+}]_i$ was strongly elevated, increased F to much higher levels than expected from the exocytosis-induced increase in platelet surface area (Figs. 3 and 7). Apparently, transbilayer movement-dependent, high increases in F interfere with exocytosis only under these activating conditions.

It has been found that platelet agonists, particularly the Ca^{2+} ionophore A23187, may give rise to the shedding of microparticles from the platelet surface [33]. Such platelet-derived microparticles will show TMA-DPH fluorescence. However, with the secretory agonists, microparticle formation could not account for more than 2% of the fluorescence in our experiments. On the other hand, it can't be excluded that microparticle formation interferes to some extent with the F levels measured with transbilayer-evoking agonists.

Function of $[Ca^{2+}]_i$ in exocytosis

Our experiments with intact platelets confirm earlier ones with permeabilized platelets that elevated cytosolic Ca^{2+} is involved in exocytosis [10,11]. However, it is clear that in platelets, like in many cells, other processes of the stimulus-secretion coupling synergize with the Ca^{2+} signalling pathway, e.g., G-protein- and protein kinase C-mediated processes [34–37]. The present stopped-flow experiments give more precise insight into the regulatory function of $[Ca^{2+}]_i$ in the secretion process. The increase in $[Ca^{2+}]_i$ seems to be an early, initiating factor in exocytosis, as suggested by the immediate $[Ca^{2+}]_i$ rise with ADP and the accelerating effect of ADP on thrombin-evoked exocytosis (Table II). Thus, such an early rise in $[Ca^{2+}]_i$ may generate a primary activation stage, after which other processes downstream in the signal transduction cascade are sensitized and accelerated. Apart from this, stopped-flow studies with TMA-DPH label also demonstrated that thrombin-induced exocytosis was quite rapid: the membrane surface area increased by 25% within 5 s of activation. Similar results have been

obtained by Wüthrich et al. [38], who measured the release of trapped acriflavine and found that thrombin-induced secretion started after a delay time of only 1.5 s.

Involvement of $[Ca^{2+}]_i$ in exocytosis and transbilayer movement

The present TMA-DPH studies and fura-2 measurements suggest that elevated $[Ca^{2+}]_i$ is involved in exocytosis as well as in transbilayer movement of TMA-DPH. The results suggest that both level and duration of the $[Ca^{2+}]_i$ rise are relevant: a moderate, relatively prolonged rise may result in exocytosis, whereas a stronger, more prolonged rise result in transbilayer movement. With regard to exocytosis, this may explain why ADP is not secretory although it raised $[Ca^{2+}]_i$ to similarly high levels as thrombin (see Fig. 4) and, as we have shown recently [39], it can activate phospholipase C and, thus, protein kinase C, transiently. The present stopped-flow data (Fig. 4) may suggest that the duration of the ADP-induced $[Ca^{2+}]_i$ rise is too short to evoke secretion. Previously, we have observed that the ADP-induced Ca^{2+} signal in single platelets is composed of multiple, separate spikes of elevated $[Ca^{2+}]_i$, in contrast to the thrombin signal which consisted of a continuously elevated plateau level [40]. From this observation it seems that in platelets a prolonged, and not an oscillatory, elevation of $[Ca^{2+}]_i$ is needed for exocytosis.

As shown in Table III, the transbilayer movement of TMA-DPH, recognized by the exceptionally high increases in F levels, was only observed with activator combinations that led to substantial (> 600 nM) and prolonged (2.5 min) elevations in $[Ca^{2+}]_i$. The presence of external $CaCl_2$ was necessary to reach these high levels (Table III). It has been demonstrated that the transbilayer movement of TMA-DPH occurs along with loss of the phospholipid asymmetry over the plasma membrane, the appearance of phosphatidylserine at the plasma membrane surface, and with the development of procoagulant (i.e., thrombin-generating) activity on the surface [7,17]. Thus, the present results with ionomycin and thapsigargin suggest that development of procoagulant activity in platelets is associated with a prolonged, high rise in $[Ca^{2+}]_i$. Bevers et al. [7] have shown that Ca^{2+} ionophores are strong activators of procoagulant activity if external $CaCl_2$ is present. Preliminary experiments indicated that also thapsigargin in combination with thrombin and $CaCl_2$ (i.e., conditions where also micromolar levels of $[Ca^{2+}]_i$ can be reached) also stimulates this activity [2].

[2] E.F. Smeets, P. Comfurius, J.W.M. Heemskerk and E.M. Bevers (unpublished observations).

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