Calcium Involvement in Aminophospholipid Exposure and Microparticle Formation during Platelet Activation: A Study Using Ca^{2+}-ATPase Inhibitors†

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ABSTRACT: The development of procoagulant activity and microparticle formation during platelet activation is known to depend on an increase in cytosolic Ca^{2+} levels. We have studied the mechanisms leading to these events using FITC-labeled recombinant annexin V, a protein which binds with a high affinity to aminophospholipids, in flow cytometry. In particular, we show that the Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid are as potent inducers of aminophospholipid exposure and microparticle formation as the ionophore A23187. In contrast, 2,5-di-*tert*-butyl-1,4-benzohydroquinone induced negligible microparticle formation, although platelets abundantly bound annexin V−FITC. That platelet activation had occurred was confirmed by binding studies with VH10, a monoclonal antibody specific for the α-granule membrane glycoprotein GMP-140, and by prothrombinase activity measurements. These results demonstrate that microvesiculation is not an automatic response to aminophospholipid exposure. The Ca^{2+}-ATPase inhibitors induced different intracellular Ca^{2+} levels as measured using fluo-3 as a calcium dye. These were 10 ± 4 μM (n = 11) for thapsigargin (3 μM), 19.6 ± 2.2 μM (n = 8) for cyclopiazonic acid (100 μM), and 0.619 ± 0.137 μM (n = 8) for 2,5-di-*tert*-butyl-1,4-benzohydroquinone (100 μM). Calpain activity, as assessed in platelets by analyzing the degradation of cytoskeletal proteins, was only observed with agents that stimulated microparticle formation. Phospholipid transbilayer movement was studied by measuring annexin V binding during platelet activation. Results showed that aminophospholipid exposure induced by ionophore A23187 ($t_{1/2}$ = 133 ± 14 s) was more rapid than that induced by TG ($t_{1/2}$ = 280 ± 30 s), although the rate-limiting step in the assay was the binding of annexin V to activated platelets ($t_{1/2}$ = 70−80 s). Interestingly, the presence of annexin V itself during the activation inhibited microparticle formation, although degradation of platelet proteins by calpain continued to occur. Our results clearly show (i) that aminophospholipid exposure and platelet microvesiculation are independent but closely regulated events and (ii) that while both processes are associated with an increase in intracellular Ca^{2+}, microvesiculation additionally requires Ca^{2+}-induced calpain activation and a fusion process inhibited by annexin V.

Plasma membranes have an asymmetric distribution of phospholipids that is lost during cell activation, incorporation of amphipathic agents, aging, and fusion (Devaux, 1991; Bevers et al., 1991). Such processes may be accompanied by microparticle formation (Bevers et al., 1991). The mechanism involved in the loss of phospholipid asymmetry and in microparticle formation is still debated, although it has been proposed to result from an inhibition of the putative aminophospholipid translocase (Devaux, 1991) or from activation of the cytosolic thiol-protease calpain (Sims et al., 1989; Fox et al., 1991; Bassé et al., 1993). Other hypotheses for aminophospholipid exposure include aminophospholipid scrambling at the point where the plasma membrane fuses to form the budding vesicle and the existence of a specific protein that scrambles the lipids (Zwaal et al., 1993). More recently, the phospholipid redistribution has been shown to require the presence of phosphatidylinositol 4,5-bisphosphate on the external leaflet of the plasma membrane, inferring a role for this phosphoinositide in the process (Sulpice et al., 1994). There is evidence to suggest that loss of phospholipid asymmetry and the exposure of phosphatidylserine at the cell surface may serve as a signal for cell−cell recognition (Schroit et al., 1985) and promote blood coagulation (Bevers et al., 1983). In this respect, blood platelets are a suitable model for study, since these phenomena occur readily in these cells and lead to the appearance of procoagulant activity. Such an activity can be measured using the prothrombinase activity test (Bevers et al., 1983) or by flow cytometry using annexin V as a probe (Thiagarajan & Tait, 1991; Dachary-Prigent et al., 1993), taking advantage of the high affinity of this protein for aminophospholipids (Thiagarajan & Tait, 1990).

Considerable evidence has accumulated to show that increases in cytosolic free Ca^{2+} concentration are involved in all of the major functional responses of platelets to stimulation, including shape change, aggregation, and secretion, as well as expression of procoagulant activity and microparticle formation. In this respect, the Ca^{2+} ionophore A23187 is a potent activator of platelets and is very active in inducing the last two processes (Sims et al., 1989; Dachary-Prigent et al., 1993). In view of the physiologic

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role of Ca^{2+}-ATPases in controlling Ca^{2+} levels in cells (Haynes, 1993), we have investigated the relationship between the increase in cytosolic Ca^{2+} brought about by inhibition of the Ca^{2+}-ATPases in platelets and the induction of procoagulant activity and microparticle production. Two Ca^{2+}-ATPases of molecular mass 100 and 97 kDa have been characterized in intracellular membranes of human platelets (Papp et al., 1991) and have been shown to be involved in the sequestration of this ion into the dense tubular system of the platelet. They were recently identified as respectively the SERCA2-b (Enouf et al., 1992) and the SERCA3 isoform (Wuytack et al., 1994). These microsomal ATPases are sensitive to inhibitors. Depending on the dose, thapsigargin (TG)[1] predominantly acts on the 100-kDa species (Papp et al., 1991); 2,5-di-*tert*-butyl-1,4-benzohydroquinone (tbuBHQ) inhibits primarily the 97-kDa species (Papp et al., 1992), and cyclopiazonic acid (CPA) inhibits both ATPases with equal potency (Papp et al., 1993). These inhibitors have been used previously to study platelet activation in relation to Ca^{2+} (Thastrup et al., 1987; Brüne & Ullrich, 1991a,b; Tao & Haynes, 1992; Malcom & Fitzpatrick, 1992). Results have shown that the Ca^{2+} pool released by ATPase inhibitors overlaps that released by inositol 1,4,5-trisphosphate (Authi et al., 1993; Kass et al., 1989). At the same time, phosphorylation of two major proteins, P20 and P47, which is generally characteristic of platelet activation by physiologic agonists, also occurs when platelets are incubated with TG (Thastrup et al., 1987; Hashimoto et al., 1993). So the mechanisms induced by these inhibitors may share some pathways in common with those induced by physiologic agonists. However, one advantage of these agents for our studies is that they act independently of membrane receptors, thus bypassing the early steps of signal transduction. As in our previous work (Dachary-Prigent et al., 1993), we have detected procoagulant platelets and microparticles by fluorescence-gated flow cytometry using annexin V-FITC as a probe. Prothrombinase activity measurements were performed in parallel. FITC-labeled VH10, a monoclonal antibody to GMP-140 (P-selectin), an α-granule membrane glycoprotein that is expressed on the platelet surface following secretion, was used as an independent marker of platelet activation. Results were correlated with the elevation of intracellular Ca^{2+} levels as probed with the calcium dye fluo-3 (Vanderberghe & Ceuppens, 1990; Merrit et al., 1990). Finally, the induced activity of the cytosolic Ca^{2+}-activated protease, calpain, was assessed by analyzing the degradation of platelet proteins by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) followed by protein staining (Dachary-Prigent et al., 1993). Results were compared with those obtained for platelets activated with the Ca^{2+} ionophore A23187. Our studies clearly demonstrate that aminophospholipid exposure and microparticle formation are independent events since it was possible to induce the former without the later. Furthermore, they show that the appearance of aminophospholipids on the platelet surface precedes microvesiculation, which requires calpain activation induced by a large extracellular Ca^{2+} influx. Finally, we show that annexin V itself is a powerful inhibitor of a late step in microparticle formation.

[1] Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; TG, thapsigargin; CPA, cyclopiazonic acid; tbuBHQ, 2,5-di-*tert*-butyl-1,4-benzohydroquinone; DTT, dithiothreitol; FSC, forward light scatter; FL1, fluorescence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NBD-phosphatidylserine, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylserine.

EXPERIMENTAL PROCEDURES

Materials. Prostaglandin E1 (PGE1), bovine serum albumin (BSA, fatty acid free), dimethyl sulfoxide (DMSO), apyrase (grade I), cyclopiazonic acid, Triton X-100, dithiothreitol, and probenecid were purchased from Sigma Chemical Co. (St. Louis, MO). Collagen (equine tendon) was from Hormon-Chemie (Munich, Germany), and ionophore A23187, thapsigargin, and 2,5-di-*tert*-butyl-1,4-benzohydroquinone were from Calbiochem (La Jolla, CA). 1-Acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphatidylserine (NBD-phosphatidylserine) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Human α-thrombin (specific activity, 3000 units/mg), factor II, and factor X were purified as already described (Freyssinet et al., 1989). Factor V and Russell viper venom (RVV) were purchased from Diagnostica Stago (Asnières, France), and the chromogenic substrate S2238 used to measure prothrombinase activity was from Kabi Diagnostica (Stockholm, Sweden). Fluo-3 was from Molecular Probes (Eugene, OR), and dithionite and FITC were from Aldrich (Strasbourg, France). Recombinant annexin V (Maurer-Fogy et al., 1988) was a generous gift from Bender+Co (Vienna, Austria). The monoclonal antibody VH10, specific for the α-granule glycoprotein GMP-140, was prepared in the Centre Régional de Transfusion Sanguine, Bordeaux, France (Dachary-Prigent et al., 1993).

Fluorescence Labeling of Proteins. Recombinant annexin V was reconstituted in distilled water and labeled with FITC as described by us for native human annexin V (Dachary-Prigent et al., 1993). After the incubation with FITC, the sample was subjected to a concentration and dialysis step in a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, OR) against 50 mM Tris-HCl and 100 mM NaCl buffer, pH 7.5, and then gel-filtered on a PD10 column (Pharmacia-France, St. Quentin-en-Yveline, France) equilibrated with the same buffer. The molar concentration of each annexin V−FITC preparation was calculated by determining the amino acid composition in a measured volume of solution as described (Dachary-Prigent et al., 1993). The FITC/protein molar ratios ranged from 4 to 6 and were calculated using the following extinction coefficient for FITC: $\epsilon_{495} = 78\,000\ M^{-1}\ cm^{-1}$. The monoclonal antibody VH10 was labeled with FITC as previously described by us (Dachary-Prigent et al., 1993). The concentration of VH10 was calculated using the extinction coefficient $\epsilon_{280}{}^{1\%} = 14$ for the antibody. The FITC/antibody molar ratio was 2.4.

Preparation of Gel-Filtered Platelets and Their Activation. Gel-filtered platelets were prepared from the blood of adult volunteer donors as previously described (Sims et al., 1989; Dachary-Prigent et al., 1993), except that the platelet-rich plasma, containing apyrase (25 μg/mL) and PGE_1 (0.1 μg/mL), was directly gel-filtered on Sepharose CL-2B equilibrated in 137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.1% (w/v) glucose, 0.1% (w/v) BSA, and 10 mM HEPES, pH 7.5 (assay buffer). Platelets eluting in the void volume were adjusted to 5×10^7/mL in assay buffer which now also contained 2 mM $CaCl_2$. Samples (500 μL) were incubated for 10 min at 37 °C in the presence of the following agents: ionophore A23187 (3

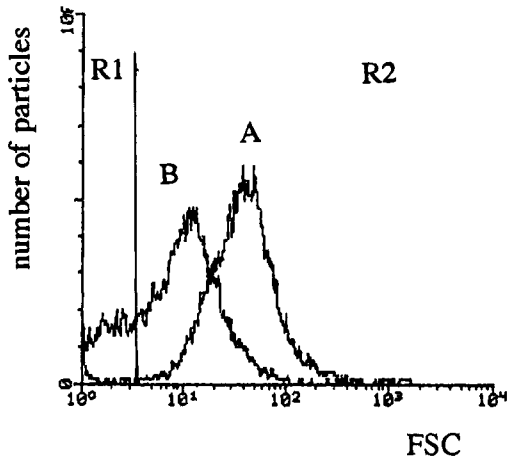

FIGURE 1: Detection of platelets and microparticles in flow cytometry. Shown are the forward light scatter (FSC) histograms of unstimulated platelets (A) and those activated with 3 μM ionophore A23187 (B). Note that the pattern is shifted to the left after incubation with the ionophore, and a shoulder is clearly observed. A gate is drawn (vertical line) delimiting the region containing the microparticles (R1) from that of the remnant platelets (R2).

μM), TG (0.3 or 3 μM), CPA (50 or 100 μM), and tbuBHQ (100 μM). These agents were dissolved in DMSO; the final DMSO concentration did not exceed 1% in the platelet suspension. Samples were not stirred after an initial mixing. Aliquots (100 μL) of stimulated or control platelets (5×10^6 cells) were incubated with saturating amounts of annexin V−FITC or VH10−FITC (respectively 150 nM and 1.65 μg/mL) for 10 min at room temperature. Samples were diluted 5-fold prior to direct analysis by flow cytometry.

Detection of Platelets and Platelet-Derived Microparticles by Flow Cytometry. Samples were analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Le Pont-de-Claix, France) as already described (Dachary-Prigent et al., 1993). Excitation was from an argon laser at 488 nm. Fluorescence emission was monitored using a 530/30 band-pass filter. The light scatter and the fluorescence signal were set at logarithmic gain. Ten thousand events were analyzed. The lower limit of the platelet gate (R2) was defined on the forward light scatter (FSC) histogram of ionophore-activated platelets (Figure 1), which is shifted to the left compared to that of unstimulated platelets, as already described (Dachary-Prigent et al., 1993). Every particle to the left of this gate is considered as a microparticle (R1). As a control, a platelet suspension activated by the Ca^{2+} ionophore was centrifuged for 10 min at 2000*g* over a 20% sucrose layer. The supernatant was seen to contain >95% low-density microparticles labeled with annexin V−FITC and situated in the R1 region of the FSC/FL1 representation, the platelets having sedimented to the bottom of the tube (results not shown).

Preloading of Platelets with Fluo-3. Gel-filtered platelets (2.5×10^8/mL) were incubated at 37 °C for 30 min with 5 μM fluo-3 (from a 1 mM stock solution in DMSO) in the assay buffer without Ca^{2+} as previously described (Merrit et al., 1990). EDTA (2 mM) was then added, and platelets were pelleted by centrifugation at 1000*g*. After one wash in the assay buffer containing 2 mM EDTA and 2.5 mM probenecid, fluo-3-loaded platelets were resuspended in the assay buffer containing 2.5 mM probenecid and kept at 37 °C. Probenecid is an organic anion-transport inhibitor which prevents fluo-3 leakage from platelets (Merrit et al., 1990). Under these conditions, the fluorescence baseline for unstimulated fluo-3-loaded platelets was stable for several hours.

Measurement of Intracellular Ca^{2+} Concentrations by Spectrofluorimetry. Measurements of cytosolic Ca^{2+} concentrations were performed in a Fluoromax spectrofluorimeter (Spex Industries, Inc., Edison, NJ). All procedures were taken at 37 °C under stirring. The excitation and emission wavelengths were 495 and 530 nm, respectively. Fluo-3-loaded platelets (1.5 mL at 2.5×10^7 cells/mL) in assay buffer were incubated for 15 min at 37 °C with 2 mM $CaCl_2$ before their stimulation. After the determination of the fluorescence baseline for 20 s, the inhibitor was added, and the fluorescence was recorded with time for about 10 min, as in the flow cytometry experiments. $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$ where K_d is the dissociation constant of fluo-3 for Ca^{2+} and is 860 nM at 37 °C (Merrit et al., 1990), and F is the fluorescence of the experimental sample. The calibration procedure consists first of obtaining F_{max} by lysing platelets with 0.05% Triton X-100 and then F_{min} by adding 4 mM EGTA in Tris−saline buffer, pH 8.4. This procedure was performed for each sample under study.

Kinetic Studies of Aminophospholipid Exposure on Activated Platelets. Samples (500 μL containing 5×10^6 platelets) in assay buffer containing 2 mM $CaCl_2$ were preincubated with 150 nM annexin V-FITC for 10 min at 37 °C. After the determination of the fluorescence baseline on the FACScan, the agonist was added at time zero with a quick stir. Then, data were collected for 3 s every 45 s on the FACScan using the Chronys software. The mean fluorescence intensities of the data acquired at each time point were converted into a linear mode and plotted versus time.

Prothrombinase Activity Measurements. Prothrombinase activity was measured by following the hydrolysis of S2238 substrate by newly formed thrombin on microtiter enzyme-linked immunosorbent assay plates. In brief, unstimulated or activated platelets were added to microtiter wells (10^6 platelets/well) that had been previously coated with BSA (5 g/L). Samples were incubated with added factor Xa (1 nM, RVV-activated) and factor Va (1 nM, thrombin-activated) for 2 min at 37 °C to assemble the prothrombinase complex. The prothrombin substrate (3 μM) was then added. After 4 min at 37 °C, the reaction was quenched by adding 50 μL of the suspension to 150 μL of 5 mM EDTA. Volumes (12 μL) of the quenched mixture were then added to wells containing the chromogenic substrate S2238 (150 μM), and the liberation of the chromophore was monitored continuously at 405 nm at room temperature in a microplate reader (SLT Labinstruments, Grodig/Salzburg, Austria) using kinetic software from SLT Labinstruments. The slope of the curve was determined and expressed as absorbance/min; it is directly proportional to the amount of thrombin formed.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE) of Platelet Proteins as a Measure of Calpain Activity. SDS−PAGE of platelet proteins was performed as previously described by us (Dachary-Prigent et al., 1993). In brief, platelets were activated as for the flow cytometry experiments and pelleted in the presence of 2 mM EGTA, and the proteins were solubilized by incubating for 1 h at 37 °C in the presence of 2% (w/v) SDS and 5 mM dithiothreitol (to reduce disulfides). Electrophoresis was performed on 7% polyacrylamide slab gels, and proteins were revealed by Coo-

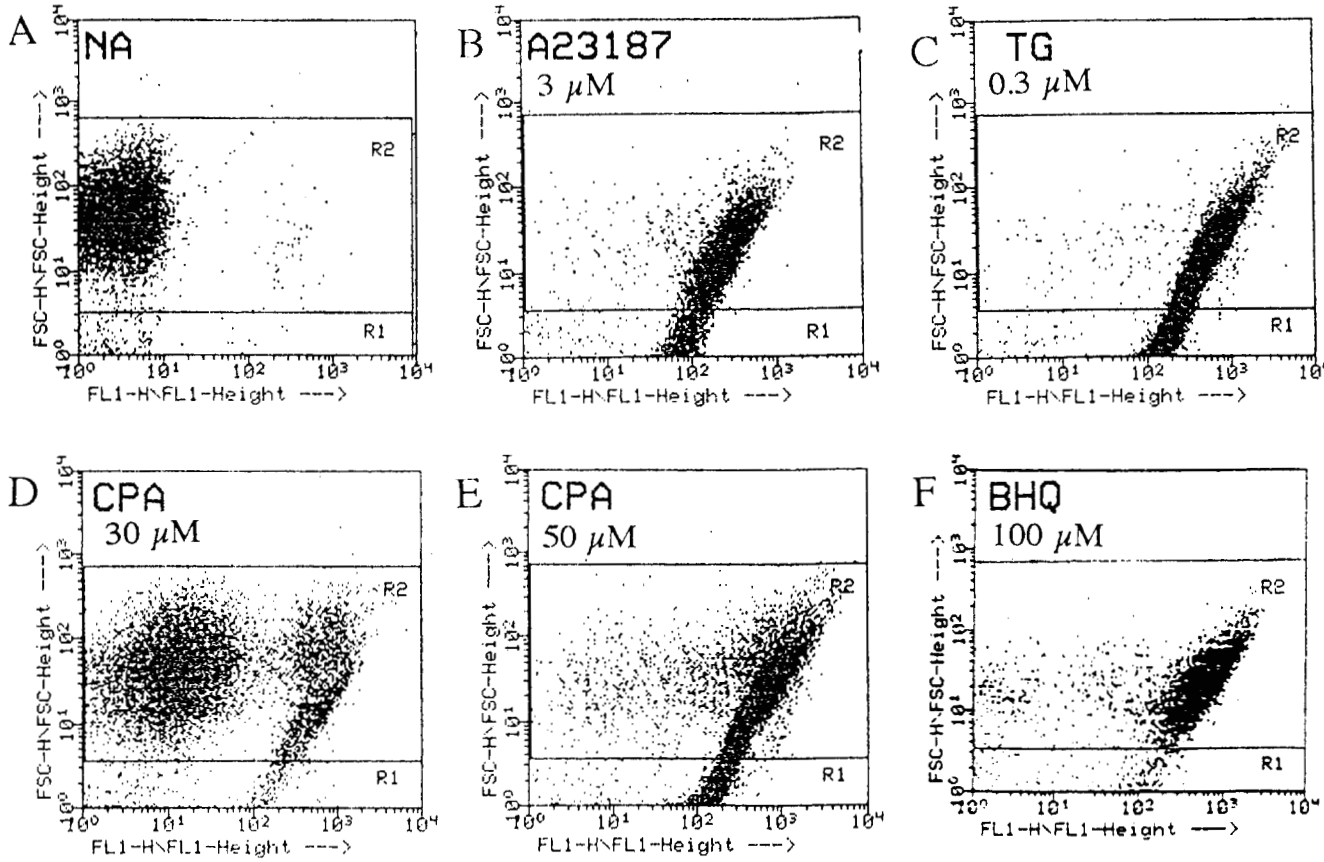

FIGURE 2: Aminophospholipid exposure and microparticle formation as detected with annexin V-FITC. The dot plots of fluorescence (FL1) versus forward light scatter (FSC) correspond respectively to nonactivated (NA) platelets (A), and to platelets activated by 3 μM calcium ionophore A23187 (B), 0.3 μM TG (C), 30 μM CPA (D), 50 μM CPA (E), and 100 μM tbuBHQ (F). The areas R1 and R2 correspond respectively to microparticles and to remnant platelets as defined in Figure 1. A representative experiment is illustrated.

massie Blue R staining. Protein migration was compared to that of high and low molecular weight protein standards (Bio-Rad, Melville, NY) electrophoresed in parallel.

Statistics. Results are presented as mean values ± SD for the number n of experiments. Statistical significance was determined by paired t testing, with a p value of 0.1 or less considered significant.

RESULTS

Addition of Ca^{2+}-ATPase Inhibitors to Platelets Results in Aminophospholipid Exposure and Microparticle Formation. Figure 2 compares the FSC versus FL1 representations for platelets challenged with the ionophore A23187, TG, CPA, and tbuBHQ. In confirmation of our earlier results (Dachary-Prigent et al., 1993), 3 μM ionophore A23187 induced both (i) extensive aminophospholipid exposure on platelets, as shown by the marked shift to the right of the fluorescence intensity, and (ii) extensive microparticle formation, as seen by the appearance of a large number of events in the R1 region (Figure 2B). Incubation with 0.3 μM TG had a similar effect with both the exposure of aminophospholipids on the platelets and the formation of annexin V-positive microparticles (Figure 2C). In contrast, 30 μM CPA induced incomplete activation, with only a subpopulation of platelets (approximately 20% of the total population) being fully labeled with annexin V and with limited microparticle shedding (Figure 2D). Nevertheless, many of the platelets showed an increase in annexin V binding compared to nonactivated platelets (Figure 2A). This partial response is a function of the dose of CPA used, for with platelets from the same donor, 50 μM CPA was a potent inducer of both phenomena (Figure 2E). Strikingly, with 100 μM tbuBHQ, whereas the bulk of the platelets were strongly labeled with annexin V, few microparticles were formed (Figure 2F). The above results are representative of those obtained during the course of our study. Notwithstanding, some interdonor variability in the response was noted. Platelets of some individuals did not respond fully to 0.3 μM TG and to 50 μM CPA under our standard experimental conditions (i.e., 10 min of incubation at 37 °C), but all responded maximally to 3 and 100 μM, respectively, of TG and CPA. For 100 μM tbuBHQ, the range of responses between individuals was larger and occasionally included no or partial platelet binding of annexin V. However, for each of four donors giving a maximal platelet response (as in Figure 2), microparticle formation was always negligible in the presence of tbuBHQ.

A qualitative analysis showed that the ionophore A23187 and the higher doses of TG and CPA were equally effective in inducing microparticle formation (data not given). The mean fluorescence intensities for annexin V-FITC bound to microparticles and to remnant platelets are compared in Table 1. The microparticles formed by the different agents bound approximately the same amount of annexin V, and the fluorescence values were about one-third of those for remnant platelets. Notwithstanding, when the respective sizes of microparticles (about 0.2 μm in diameter, Bevers et al., 1991) and platelets (from 2 to 4 μM in diameter) are taken into

Table 1: Mean Fluorescence Intensities of Annexin V-FITC Bound to Microparticles and to Platelets[a]

agent	microparticles	platelets
none	ND	3
A23187, 3 μM	154	407
TG, 0.3 μM	170	437
TG, 3 μM	143	392
CPA, 50 μM	164	422
CPA, 100 μM	162	441
tbuBHQ, 100 μM	ND	474

[a] Platelets were incubated for 10 min at 37 °C, and samples were analyzed by flow cytometry as detailed in the Experimental Procedures. Microparticles and remnant platelets were discriminated by their associated light scatter signal, and aminophospholipid exposure was assessed by annexin V−FITC binding. Mean fluorescence intensities were calculated on 10 000 events acquired in R1 and R2 as defined in Experimental Procedures. ND, not detected. Data were from a single experiment but were representative of four similar experiments where platelets responded well with tbuBHQ.

Table 2: Prothrombinase Activity in Suspensions of Activated Platelets[a]

agent	prothrombinase activity (A/min)	p
control	0.063 ± 0.023 (n = 5)	nd
A23187, 3 μM	0.709 ± 0.125 (n = 6)	
TG, 0.3 μM	0.523 ± 0.137 (n = 5)	<0.05
TG, 3 μM	0.776 ± 0.121 (n = 6)	<0.4
CPA, 50 μM	0.740 ± 0.077 (n = 4)	<0.7
CPA, 100 μM	0.837 ± 0.103 (n = 4)	<0.1
tbuBHQ, 100 μM	0.642 ± 0.165 (n = 4)	<0.5

[a] Platelets were incubated with each agent as described in the flow cytometry experiments. Aliquots of the platelet suspension were taken to measure the enhancement of thrombin formation in the prothrombinase assay performed with the chromogenic substrate S2238. The amount of thrombin formed is proportional to the rate of liberation of the chromophore, expressed as absorbance/min (A/min). Only results from donors where the total platelet population expressed aminophospholipids in response to tbuBHQ are taken into account here. n = number of donors tested; nd, not determined. Values of p were calculated for each pair constituted of one inhibitor and the calcium ionophore A 23187.

account, such values suggest a higher density of aminophospholipids on the microparticle surface, as already discussed (Comfurius et al., 1990; Dachary-Prigent et al., 1993). For donors responding well with tbuBHQ, the annexin V-positive platelets always exhibited the highest mean fluorescence intensity, as microparticles are not released.

Exposure of Procoagulant Lipids As Measured by the Prothrombinase Activity Test. A means to confirm that platelets are procoagulant as a result of aminophospholipid exposure and microparticle shedding was to perform prothrombinase activity measurements on aliquots of the activated platelet suspensions. Results showed that the basal prothrombinase activity was increased 8−13-fold by these agents (Table 2). This functional test measured the combined procoagulant activity of both platelets and microparticles. It should be noted that tbuBHQ produced a 10-fold increase in prothrombinase activity, confirming its ability to induce aminophospholipid translocation. A significant difference was observed with 100 μM CPA compared to ionophore A23187 (p < 0.1) despite the fact that equivalent numbers of microparticles were produced. This was, however, in agreement with the higher mean fluorescence intensity seen with annexin V for remnant platelets with CPA (Table 1), suggesting a higher density of aminophospholipids on the surface membrane.

Detection of Platelets and Microparticles with VH10-FITC, a Monoclonal Antibody to GMP-140. Figure 3 shows that both platelets and shed microparticles were labeled with VH10-FITC. This confirmed that platelet activation had occurred and was accompanied by secretion. The absence of shedding of microparticles for platelets incubated with tbuBHQ was also confirmed (see the relative absence of particles in region R1 in Figure 3F). Notwithstanding, tbuBHQ induced as much binding of VH10, as was seen with the other ATPase inhibitors or ionophore A23187. Results for platelets activated by thrombin under conditions giving maximal secretion are shown for comparison (Figure 3B); these platelets exhibited extensive labeling with VH10 and yet also only gave rise to limited microparticle formation.

Intracytosolic Free Ca^{2+} Concentration Measurements. Quantitative measurements were performed first with platelets incubated with the ATPase inhibitors in the presence of 2 mM external Ca^{2+} (Table 3). Addition of 3 μM TG or 100 μM CPA to fluo-3-loaded platelets caused a large increase in cytosolic Ca^{2+} levels (10 ± 4 μM, mean ± SD, n = 11; 19.6 ± 2.2 μM, mean ± SD, n = 8, respectively), while with 100 μM tbuBHQ, the Ca^{2+} increase was much lower (0.619 ± 0.137 μM, mean ± SD, n = 8). In addition, the times taken to reach the stationary state were within 1 min for the first two inhibitors, whereas it took several minutes for tbuBHQ (results not shown).

In order to confirm that inhibition of Ca^{2+}-ATPases from the platelet dense tubular system had occurred, measurements were also performed in the absence of extracellular Ca^{2+}, a situation where only release of the internal Ca^{2+} pool would contribute to the response. Results showed that the basal cytoplasmic Ca^{2+} concentration increased when platelets were incubated with the inhibitors (Table 4). The less efficient inhibitor was tbuBHQ (0.243 ± 0.055 μM, mean ± SD, n = 8) while the Ca^{2+} levels attained with TG and CPA were higher and not statistically different one from the other (about 0.4 μM, p < 0.6). This confirmed that tbuBHQ was acting differently on the platelet ATPases (see introduction). Overall, our results show that increases in cytosolic Ca^{2+} concentration resulted from both depletion of sensitive internal pools and influx from the extracellular fluids.

Time Course for Aminophospholipid Exposure As Detected by Annexin V-FITC. The platelets were preincubated for 10 min with annexin V-FITC, the fluorescence baseline was determined, and then ionophore A23187 or TG was added (arrow on Figure 4). The fluorescence was recorded at 45-s time intervals. After a short lag phase, the mean fluorescence intensity progressively increased, reaching a maximum after 8−10 min. Under our experimental conditions, half-times for maximal annexin V binding were 133 ± 14 s (mean ± SD, n = 5) and 280 ± 30 s (mean ± SD, n = 4) for 3 μM ionophore A 23187 and 3 μM TG, respectively. Control incubations in the absence of agonist showed no spontaneous aminophospholipid transfer. Unexpectedly, analysis of FSC/FL1 dot-plots at several time points during these experiments showed that little or no microparticle formation was occurring. Results are shown for the ionophore A23187 in Figure 5 but were identical for TG. A consequence of the lack of microparticle shedding was that the mean fluorescence intensity reached a higher value than when platelet activation was performed prior to the addition of annexin V (compare Figure 5 with Figure 2). During the initial stages of platelet activation, a platelet subpopulation having an intermediate

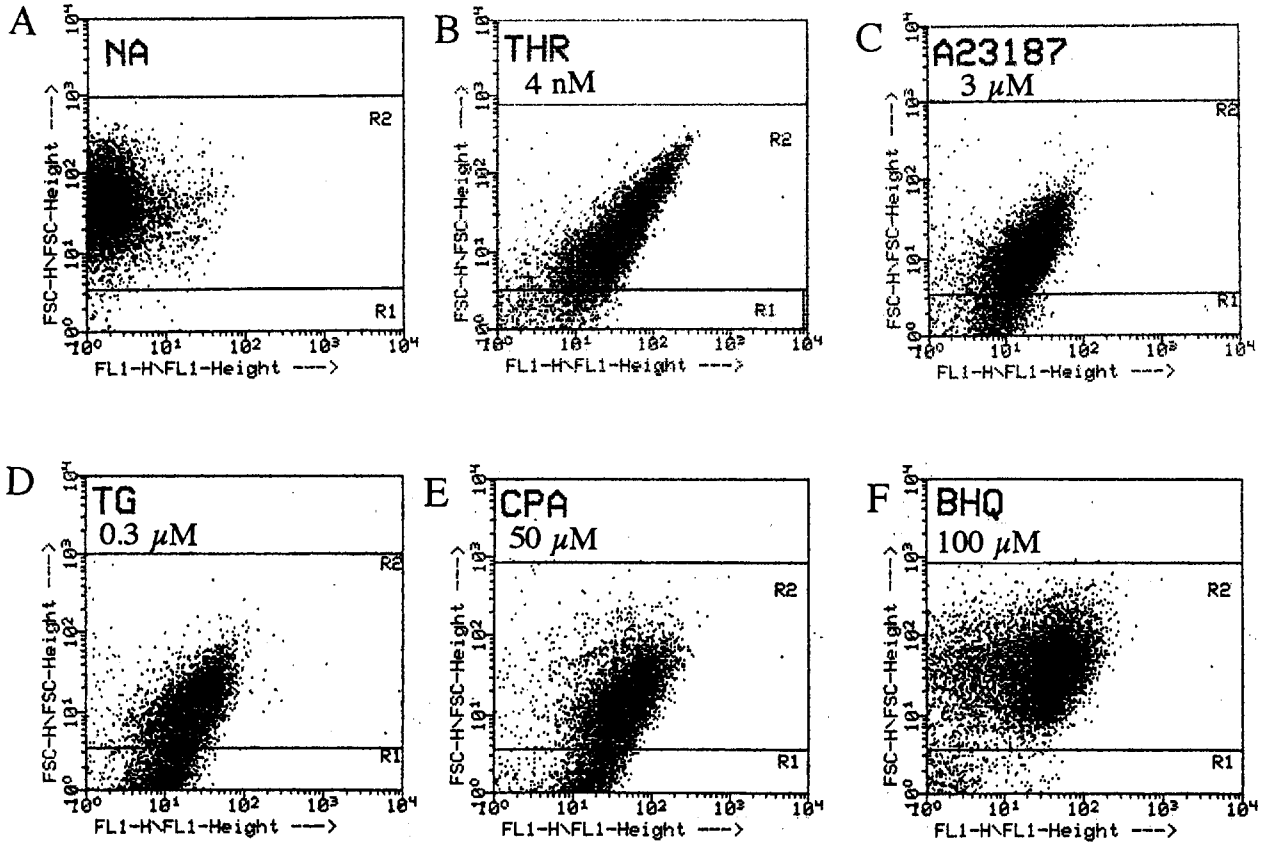

FIGURE 3: GMP-140 expression as detected by the binding of VH10-FITC. The dot plots of fluorescence (FL1) versus forward light scatter (FSC) correspond respectively to nonactivated (NA) platelets (A) and to platelets activated by 4 nM thrombin (THR) (B), 3 μM A23187 (C), 3 μM TG (D), 50 μM CPA (E), and 100 μM tbuBHQ (F). The areas R1 and R2 correspond to microparticles and to remnant platelets, respectively. A representative experiment is illustrated.

Table 3: Cytosolic Ca^{2+} Concentrations Induced by the Inhibitors in the Presence of 2 mM External Ca^{2+} [a]

inhibitor	$[Ca^{2+}]_i$, μM basal	$[Ca^{2+}]_i$, μM induced	*n*
A 23187, 3 μM	0.164 ± 0.045	>20	6
TG, 3 μM	0.222 ± 0.059	10 ± 4	11
CPA, 100 μM	0.209 ± 0.043	19.6 ± 2.2	8
tbuBHO, 100 μM	0.183 ± 0.039	0.619 ± 0.137	8

[a] Fluo-3-loaded platelets (1.5 mL at 2.5 × 10^7 cells/mL) were incubated for 15 min at 37 °C with 2 mM Ca^{2+} before their stimulation. After the determination of the fluorescence baseline, the inhibitor was added and the fluorescence was recorded with time for about 10 min. $[Ca^{2+}]_i$ was calculated before (basal) and after a 10-min incubation (induced) with the inhibitor as described in Experimental Procedures. Results are means ± SD; *n* is the number of individual experiments.

Table 4: Cytosolic Ca^{2+} Concentrations Induced by the Inhibitors in the Absence of External Ca^{2+}

inhibitors	$[Ca^{2+}]_i$, μM basal	$[Ca^{2+}]_i$, μM induced	*n*
TG, 3 μM	0.151 ± 0.034	0.385 ± 0.094	5
CPA, 100 μM	0.100 ± 0.037	0.433 ± 0.140	6
tbuBHQ, 100 μM	0.146 ± 0.033	0.243 ± 0.055	8

[a] Conditions are as in Table 3 except that measurements were performed in the absence of Ca^{2+} in the external medium.

fluorescence was detected (Figure 5A). This could result from incomplete annexin V fixation to the exposed aminophospholipids on this subpopulation (see Discussion) or from the fact that different subpopulations of platelets showed different sensitivity to activation by ionophore A23187.

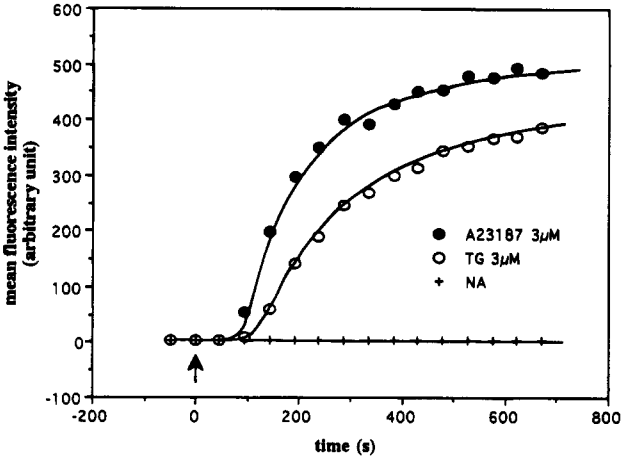

FIGURE 4: Kinetics of aminophospholipid exposure as assessed by annexin V-FITC binding. Platelets (10^7/mL) were preincubated at 37 °C for 10 min with annexin V-FITC (150 nM) and then run in the FACScan using the Chronys software. After the determination of the fluorescence baseline, 3 μM A23187 or 3 μM TG was added (time 0), and data were acquired for a 3-s passage of cells every 45 s during a total period of about 12 min at room temperature. Results were expressed as the mean fluorescence intensity of the platelet population at each time point. A representative experiment is illustrated.

Calpain Activity As Assessed by the Degradation of Platelet Proteins. The role of calpain in microparticle formation and in the surface exposure of aminophospholipids was assessed by SDS−PAGE analysis of platelet protein degradation (Figure 6). Extensive hydrolysis of the cytoskeletal proteins filamin, talin, and myosin heavy chain was observed when

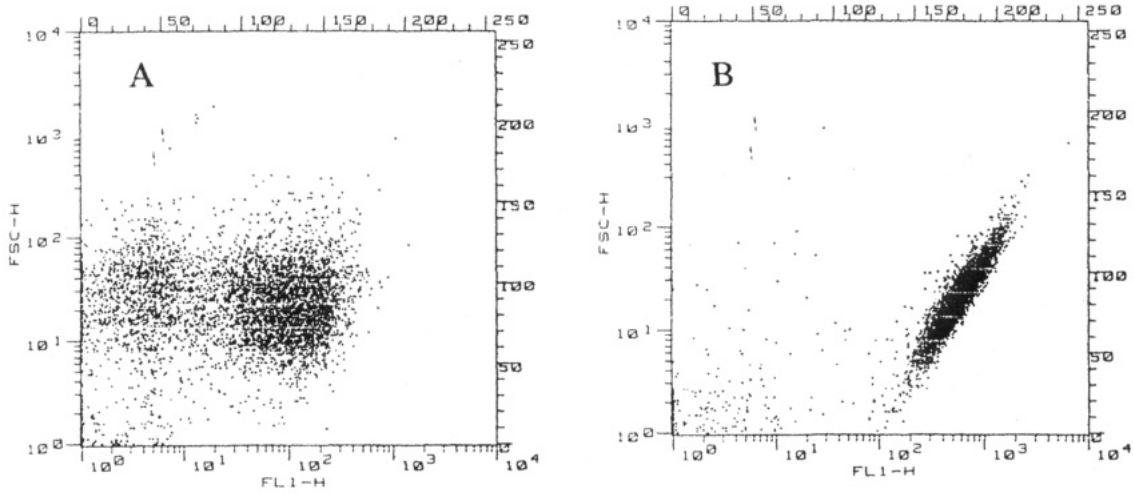

FIGURE 5: Dot plot of fluorescence (FL1) versus forward light scatter (FSC) of platelets activated by 3 μM A23187 in the presence of annexin V at two time points during the time course as illustrated in Figure 4. These were (A) 140 s after the addition of the agonist and (B) at the end of the incubation. No gate was set. The virtual absence of microparticles in the R1 region is clearly demonstrated (compare with Figure 2). Note that a platelet population showing an intermediate labeling is observed in (A).

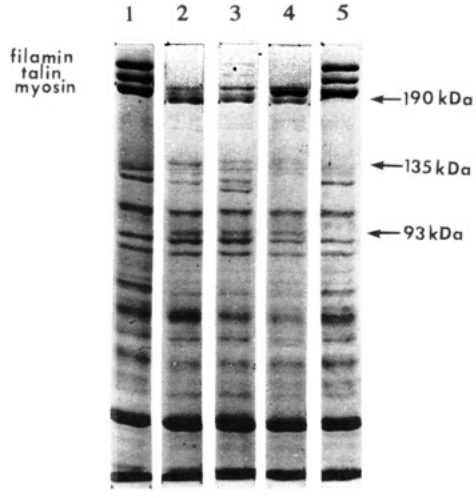

FIGURE 6: Calpain-induced degradation of high molecular weight platelet proteins as assessed by Coomassie Blue R staining after SDS−PAGE. Shown are the protein pattern for unstimulated platelets (lane 1) and those for platelets activated by 3 μM A23187 (lane 2), 3 μM TG (lane 3), 100 μM CPA (lane 4), and 100 μM tbuBHQ (lane 5). Arrows show major degradation products resulting from the calpain-induced degradation of filamin, talin, and myosin: 190 kDa (filamin and talin), 135 kDa (myosin), and 93 kDa (filamin).

platelets were incubated with 3 μM ionophore A23187 (lane 2), 3 μM TG (lane 3), and 100 μM CPA (lane 4). In contrast, no hydrolysis was observed with 100 μM tbuBHQ (lane 5) where the pattern resembled that of unstimulated platelets (lane 1). No calpain activity was detected when platelets were incubated with the above agonists in assay buffer without added Ca^{2+} or in the presence of 2 mM EGTA (results not shown). The same protein degradation patterns as in Figure 6 were observed when platelet activition was performed in the presence of annexin V (result not shown). This means that the binding of annexin V to platelets did not interfere with calpain activation.

DISCUSSION

Our studies were performed in order to more precisely define the relationship between intracellular Ca^{2+} increase, aminophospholipid exposure, and microparticle formation during platelet activation. A specific feature of our work was the use of Ca^{2+}-ATPase inhibitors as a means of inducing changes in intracellular Ca^{2+} levels. The results showed that two of these agents, TG and CPA, were able to induce extensive aminophospholipid exposure and microparticle formation. In contrast, tbuBHQ induced aminophospholipid exposure but little microparticle formation (Figure 2). The relationship between the platelet responses and intracellular Ca^{2+} levels was studied using fluo-3 as the Ca^{2+} dye. The relatively low affinity of this probe for Ca^{2+} (K_d = 860 nM at 37 °C) (Merrit et al., 1990) permits measurements of intracellular free Ca^{2+} concentrations up to 10−20 μM (Minta et al., 1989). Results in the absence of extracellular Ca^{2+} (Table 4) imply a release of internal Ca^{2+} pools, thus confirming that, in our experimental conditions, the Ca^{2+}-ATPase inhibitors have reached their intracellular targets. However, under these conditions, platelets neither shed microparticles nor expressed aminophospholipids at their surface. The inability of annexin V to bind to aminophospholipids in the absence of Ca^{2+} (Thiagarajan & Tait, 1990) and the requirement of Ca^{2+} to assemble the prothrombinase complex in the prothrombinase assay necessitated the use of other procedures to assess these phenomena. In fact, flow cytometry with VH10-FITC as a Ca^{2+}-independent marker showed that secretion occurred without microparticle release. Furthermore, by means of the fluorescence assay for phospholipid asymmetry using dithionite to selectively reduce NBD-labeled phospholipids in the outer leaflet of the membrane bilayer (McIntyre & Sleight, 1991; Müller et al., 1994), we have shown in flow cytometry that, when incorporated into the inner leaflet of the plasma membrane of resting platelets, NBD-phosphatidylserine was not exposed when platelets were activated with TG or CPA in the absence of added Ca^{2+} (unpublished results).

The high levels in intracellular Ca^{2+} measured in the presence of extracellular Ca^{2+} indicate that Ca^{2+} influx has occurred (Table 3). Partial release of the intracellular Ca^{2+} pool has previously been shown to induce the activation of Ca^{2+} entry into cells (Putney, 1990). TG is known to induce both the release of Ca^{2+} from intracellular stores and Ca^{2+} influx across the membrane (Tao & Haynes, 1992). Although less information is available concerning CPA, the

level of intracellular free Ca^{2+} clearly showed that considerable Ca^{2+} influx must also be occurring. The much lower rise in Ca^{2+} levels in the presence of tbuBHQ implies that this agent does not induce a massive influx. This can result from the fact that tbuBHQ is specific for the SERCA3 isoform of the Ca^{2+}-ATPases (Papp et al., 1992). Another reason could be that tbuBHQ is known to inhibit cyclooxygenase in contrast to TG (Brüne & Ullrich, 1991a,b; Brüne et al., 1993). In the presence of inhibitors of cyclooxygenase, a lower rise in cytosolic Ca^{2+} concentration has been reported in platelets activated by TG (Authi et al., 1993). Overall, these results could mean that an amplification mechanism involving thromboxane formation is necessary for microvesiculation. However, it cannot be excluded that tbuBHQ may have an unknown additional inhibitory action (also discussed below). Comparable levels of intracellular Ca^{2+} were obtained with 4 nM thrombin (Smeets et al., 1993), which is also a poor inducer of microparticle formation. Interestingly, low doses of TG have been reported to synergistically increase the platelet procoagulant response to thrombin with respect to both an increased shedding of microparticles and the outward scrambling of NBD-phospholipid analogs (Smeets et al., 1993, 1994).

The use of 30 μM CPA in the dose-dependence study (Figure 2E) resulted in a subpopulation that was maximally labeled with annexin V and thus had fully exposed aminophospholipids. This subpopulation had also shed microparticles, as confirmed by the lower mean FSC of the platelets (results not shown). Thus it is possible to conclude that here aminophospholipid exposure and microparticle formation occurred in concert, and that the cytosolic Ca^{2+} concentrations required for both processes were exceeded. However, the remaining platelets bound less annexin V (Figure 2E) with no change in FSC (data not shown), a result which can be interpreted as indicating a lower degree of aminophospholipid exposure without microparticle formation. Such a heterogeneity in the platelet response has also been observed in flow cytometry during a kinetic study of the activation of fluo-3-loaded platelets with CPA and TG where two platelet subpopulations were observed, one exhibiting maximal fluorescence and increasing numerically with time, and in which cytosolic Ca^{2+} was maximal, and the other with intermediate fluorescence and where the size of the subpopulation decreased as the Ca^{2+} level rose (results not shown). Significantly, microparticles were most abundant when the bulk of the platelets exhibited maximal fluorescence. When all of the results are taken together, it appears that microvesiculation occurs when platelets have maximally exposed aminophospholipids and high intracellular Ca^{2+} levels. This hypothesis is supported by the fact that microvesicle release from erythrocytes treated by ionophore A23187 has been shown to require Ca^{2+} concentrations higher than those needed to obtain flip-flop of phospholipids (Henseleit et al., 1990). Further studies are planned to investigate the temporal relationship between Ca^{2+} rises and aminophospholipid exposure.

In the presence of Ca^{2+}, prothrombinase activity measurements showed that activated platelet suspensions, containing both microparticles and platelets, were highly procoagulant (Table 3). The fact that platelets treated with tbuBHQ expressed so much prothrombinase activity confirms that microparticle release is not required for the expression of this activity, although microparticle release increases the total prothrombinase activity present. Prothrombinase activity for thrombin-activated platelets is lower, a finding related to partial aminophospholipid exposure and microvesiculation (Sims et al., 1989; Dachary-Prigent et al., 1993). As thrombin induces a cytosolic Ca^{2+} increase in platelets similar to that induced by tbuBHQ (see above), this result suggests that there is not a direct relationship between Ca^{2+} levels and aminophospholipid exposure. However, it is possible that the Ca^{2+} response to thrombin may be heterogeneous within the total platelet population and that the overall prothrombinase activity comes from the responding platelets.

Procoagulant platelets and microparticles were both labeled with VH10-FITC (Figure 3). This labeling, while showing that each of the Ca^{2+}-ATPase inhibitors induced α-granule secretion, confirmed that microparticles were formed during exocytosis or after α-granule secretion has occurred. The kinetics of aminophospholipid exposure were studied by the continuous labeling of platelets with annexin V-FITC at a saturating concentration during activation (Figure 4). Here, the rate-limiting step was the binding of annexin V to activated platelets for which the $t_{1/2}$ for maximal binding was about 70−80 s (data not shown). As a result, it was not possible to establish a direct time correlation between the rates for aminophospholipid exposure and the cytosolic Ca^{2+} increase. Nevertheless, the rate for aminophospholipid exposure induced by the ionophore A23187 found in this study ($t_{1/2} = 133 \pm 14$ s at room temperature) compares well with the results from a study performed using the back-exchange technique and a paramagnetic phosphatidylserine analog: 1 min at 37 °C and a few minutes at 20 °C (Bassé et al., 1993). Thus, annexin V-FITC binding is a useful alternative method to study the transbilayer aminophospholipid movement which appears to be an essential part of microparticle production.

More evidence for this conclusion was provided by the finding that when the annexin V-FITC was added prior to ionophore A23187 or TG, it prevented microparticle formation without affecting the surface expression of aminophospholipids (Figure 5). Confirmation was performed using platelets labeled with NBD-phosphatidylserine (McIntyre & Sleight, 1991; Muller et al., 1994). Results showed that when platelets were activated by ionophore A23187 in the presence of annexin V, the percentage of microparticles in region R1 was similar to the background level obtained for control unstimulated platelets. As annexin V has been detected in a wide variety of tissues and in different cell types, this is an important observation that may be related to the physiologic functions of the annexin family which are still unknown (Raynal et al., 1994). It has been reported that while annexin I mediates the *in vitro* fusion of human neutrophil plasma membranes with phospholipid vesicles, annexin V inhibits such a fusion process (Osrhy et al., 1991). Extrapolation of these results to our studies suggests that the binding of annexin V to charged phospholipids in the presence of Ca^{2+} could prevent the fusion process, implying that both charged lipids and Ca^{2+} are required for budding and microparticle formation. Our results confirm that recombinant annexin V, or a structurally related protein, may have a potential therapeutic use in the inhibition of microparticle production in prethrombotic states. Anticoagulant and anti-inflammatory properties of annexin V have already been shown in preliminary *in vivo* studies in animals (Römisch et al., 1991; Chollet et al., 1992).

The relationship between calpain-mediated proteolysis of cytoskeletal proteins and vesicle formation is controversial (Sims et al., 1989; Fox et al., 1991; Bassé et al., 1993). The present study showed that calpain activity was extensive in platelets activated with ionophore A23187, TG, or CPA in the presence of external Ca^{2+}, conditions where microparticles were formed (Figure 6). In contrast, no calpain activity was seen when divalent cations were absent from the incubating buffer. These results strongly imply that calpain activation is associated with Ca^{2+} influx across the membrane. Strikingly, calpain activity was not observed for platelets activated by tbuBHQ, which did not lead to microparticle release. Addition of the Ca^{2+} ionophore A23187 to platelets preincubated with tbuBHQ in the presence of extracellular Ca^{2+} resulted in microvesiculation and calpain activation (results not shown), ruling out direct inhibition of calpain by tbuBHQ. The lack of calpain activation in platelets activated with tbuBHQ is thus probably related to the insufficient cytosolic Ca^{2+} levels induced by this agent (Kuboki et al., 1987). As annexin V prevented microparticle formation while calpain was activated, microvesiculation would appear to be at least a two-step process, one factor being calpain activity, probably by increasing membrane flexibility following proteolysis of cytoskeletal proteins (Bassé et al., 1994). Our results for calpain extend and reinforce previous studies which have shown that microparticle production is inhibited when platelets are preincubated with membrane-soluble inhibitors of the enzyme such as E-64d or calpeptin prior to the addition of ionophore A23187 (Fox et al., 1990, 1991; Bassé et al., 1993; Dachary-Prigent et al., 1993).

The molecular mechanism responsible for aminophospholipid translocation has not been defined. It has previously been speculated that overloading of the outer leaflet of the plasma membrane in aminophospholipids at the expense of the inner leaflet results in an imbalance that leads to membrane spiculation and budding according to the bilayer couple model (Sheetz & Singer, 1974). Since no microparticle formation was observed with tbuBHQ, this appears to mean that transmembrane movement of aminophospholipids alone is insufficient to lead to microparticle formation in platelets. Evidence that phospholipid movement precedes microparticle formation was confirmed by kinetic studies performed with ionophore A23187 and TG where a parallel decrease in fluorescence intensity of the annexin V-positive platelets occurred concomitant with microvesiculation (results not illustrated). Furthermore, aminophospholipid exposure can occur even when microparticle formation is inhibited or reduced, either with annexin V in the incubation medium (present work) or when platelets are activated by ionophore A23187 in the presence of thiol-reactive agents (Fox et al., 1990, 1991; Bassé et al., 1993; Dachary-Prigent et al., 1993), and also in the presence of high-potassium buffer (Williamson et al., 1992). Thus aminophospholipid exposure appears to be a necessary step that leads to microvesiculation. This step and the associated mechanism (Zwaal et al., 1993) may be deficient in the Scott syndrome, an inherited disorder where platelets and red cells have a normal phospholipid asymmetry, yet both aminophospholipid exposure and microparticle formation are deficient despite normal calpain activation following stimulation (Sims et al., 1989; Bevers et al., 1992).

In conclusion, our studies confirm that microparticle formation in platelets depends on a rise in cytoplasmic Ca^{2+} levels probably associated with a large Ca^{2+} influx. They further show that an increase in intracellular Ca^{2+} activates the mechanisms responsible for transbilayer movement of aminophospholipids, calpain activation followed by the degradation of cytoskeletal proteins, and a membrane fusion process. They demonstrate that annexin V can inhibit microvesiculation, confirming the potential usefulness of annexin V, or a structurally related protein, as an inhibitor of microparticle release and of the development of procoagulant activity. Such studies using Ca^{2+}-ATPase inhibitors may help our understanding of the way in which physiologic agonists can induce these platelet responses.

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