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Effects of exogenous phospholipids on platelet activation

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Intercalation of amphipaths into the plasma membrane of platelets has a marked effect on their morphology. Incubation of platelets with phosphatidylcholines (PC) results in rounding of the platelet body and spiculation, while incubation with aminophospholipids such as dilauroylphosphatidylserine (DLPS) results in a biphasic shape change consistent with the bilayer couple model (Sheetz, M.P. and Singer, S.J. (1982) Proc. Natl. Acad. Sci. USA 71, 4457–4461) and with the activity of an aminophospholipid translocator facilitating transverse bilayer diffusion (Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416). The present study extends this work to investigate the effects of PC and PS on platelet responses to a natural agonist, thrombin. PC incorporation produces a concentration-dependent progression of shape changes, beginning with surface ruffling and development of fine spicules, followed by sphering of the cell body, and ending with the apparent loss of spicules. PC reduces platelet responses to thrombin only under conditions that promote membrane vesiculation, seen morphologically as a loss of spicules and biochemically as a loss of ^{14}C-PC labeled membrane. PS homologues of varying acyl chain composition induce concentration- and time-dependent platelet sphering. Incorporation of PS inhibits thrombin-induced platelet shape change, granule secretion, and protein phosphorylation. Inhibition of these responses requires transit of the exogenous PS to the cytofacial leaflet of the membrane bilayer.

Introduction

Upon binding of agonists, platelets undergo a complex transformation. A natural agonist such as thrombin induces a striking alteration in the cellular morphology; flat disc-like platelets change into rounded forms with many spike-like projections. This change is followed by an array of biochemical events, including secretion of two kinds of cytosolic granules, protein phosphorylation, activation of phosphoinositide-specific phospholipase C, a rise in cytosolic calcium, reorganization of cytoskeletal elements, and aggregation and clot formation. The mechanistic integration of these and the numerous other events involved in the hemostatic response is still incompletely understood (for review, see Ref. 1).

Platelet morphology also can be altered by exogenous amphipaths, which may intercalate selectively into the inner or outer membrane monolayer and alter relative occupancy of the two leaflets of the bilayer [2–4]. Phosphatidylcholine (PC), for example, accumulates in the outer leaflet of the bilayer and induces formation of fine filopodia, similar to those resulting from exposure to natural agonists such as platelet-activating factor [2]. This PC-induced filopod formation occurs in the absence of certain other platelet activation sequelae (e.g., protein phosphorylation). In contrast, phosphatidylserine (PS) accumulates in the inner leaflet of the bilayer, sequestered by action of the aminophospholipid translocase [5–7]. Treatment with PS also modifies platelet shape, transforming discoid platelets into spheroid and indented forms.

In this work, we investigate the extent to which normal activation responses are either mimicked or inhibited by exogenous PC and PS homologues, alone or in combination with thrombin. Spiculating amphipaths such as dilauroylphosphatidylcholine (DLPC) inhibit thrombin activation, primarily due to membrane

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Abbreviations: CPZ, chlorpromazine; DLPC, dilauroylphosphatidylcholine; DLPS, dilauroylphosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; lyso-PC, lysopalmitoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleolyphosphatidylserine; PC, phosphatidylcholine; PS, phosphatidylserine; PRP, platelet-rich plasma.

vesiculation and attendant loss of membrane components. In contrast, the sphering agent DLPS suppresses activation responses, but only under conditions that permit its translocation to the inner monolayer of the membrane.

Materials and Methods

Materials

L-3-Phosphatidylcholine, 1,2-di[1-^{14}C]myristoyl (specific activity 108 mCi/mmol, 20 μCi/ml) and 5-hydroxy[*side-chain*-2-^{14}C]tryptamine creatinine sulfate ([^{14}C]serotonin, specific activity 57 Ci/mmol, 50 μCi/ml) were obtained from Amersham (Arlington Heights, IL). Carrier free [^{32}P]P_i (spec. act. 5 mCi/ml) was obtained from New England Nuclear (Wilmington, DE). Dimyristoylphosphatidylserine (DMPS) was obtained from Calbiochem (San Diego, CA); dioleoylphosphatidylserine (DOPS) and 1-palmitoyl-2-oleoylphosphatidylserine (POPS) were purchased from Avanti Polar Lipids (Birmingham, AL). All other biochemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Platelet isolation

Isolation procedures were carried out at room temperature (22°C) to prevent premature platelet activation. Blood was drawn by venipuncture from healthy adult volunteers who had denied taking any medications for at least 2 weeks prior to donation. Blood was collected in 0.1 vol. of 11.4% sodium citrate (pH 6.4), and platelet-rich plasma (PRP) was separated from erythrocytes and leukocytes by centrifugation at $150 \times g$ for 20 min. PRP was then centrifuged at $150 \times g$ for 15 min to remove residual erythrocytes and leukocytes. The pH of the PRP was adjusted to 6.5 with 150 mM citric acid and platelets were separated from plasma by centrifugation for 10 min at $700 \times g$. The cells were next washed twice in citrate washing buffer (137 mM NaCl, 6 mM sodium citrate, 5 mM citric acid, 5.5 mM glucose, 0.25% essentially fatty acid-free BSA, 2 U/ml creatine phosphokinase, and 0.7 mM creatine phosphate (pH 6.5)) as described [8]. The final platelet pellet was resuspended in calcium-free Tyrodes buffer (137 mM NaCl, 5 mM Hepes, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5.6 mM glucose, 8 U/ml creatine phosphokinase, 0.7 mM creatine phosphate, 0.1% BSA (pH 7.4)). Platelets were allowed to rest 15–60 min at 37°C prior to use. The final cell concentration in all experiments was adjusted to $2 \cdot 10^8$ per ml unless otherwise specified. Typical morphological indices for platelets prepared by low pH centrifugation were +0.2 to +0.6, calculated using the scale described below. Subsequent operations were performed at 37°C unless otherwise indicated.

Quantitation of cell morphology

Platelets were examined by phase contrast microscopy after fixation in buffered glutaraldehyde (0.5% glutaraldehyde, 5 mM EDTA, 50 mM sodium phosphate (pH 7.4)). Cell morphology was classified as densely colored smooth spheres (−1), discs without spicules (0), discs with smooth edges and 1–3 spicules (+1), discs or spheres with a ruffled edges and more than 2 spicules (+2), and ruffled spheres without well-defined spicules (+3). The average score for a field of 100 cells was taken as the morphological index. Errors associated with counting replicate samples were typically less than 0.15 MI unit. Scanning electron microscopy was performed on samples of glutaraldehyde-fixed cells. Cells were prepared as described previously (Ref. 9; Fig. 1), or were first sequentially stained with 1% osmium tetroxide, 1% tannic acid, and 1% uranyl acetate followed by dehydration in a graded ethanol series and resuspension in hexamethyldisilazane for 30 min (Fig. 2). The two methods gave similar results. Fixed platelet samples were dried on aluminum stubs and sputter-coated with 12 nm gold prior to examination.

Preparation of amphipaths

Lipid stock solutions were prepared in chloroform and stored at −20°C under an inert atmosphere. Lipid vesicles were prepared by placing an aliquot of the lipid in a glass test tube, removing the chloroform under a stream of argon, adding phosphate buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate (pH 7.4)) and subsequently sonicating the preparation to clarity under argon in a bath sonicator. Chlorpromazine (CPZ) was dissolved in isotonic saline (150 mM NaCl). At the concentrations employed in this study, amphipaths did not induce lysis, as shown by the absence of release of lactate dehydrogenase.

Determination of serotonin secretion

PRP was incubated at 37°C with [^{14}C]serotonin (0.2 μCi per ml of PRP) for 1 h. The cells were then washed and isolated as described above. Platelets prelabeled with [^{14}C]serotonin were incubated with 2.5 μM imipramine (to inhibit uptake of released serotonin) and amphipath or PBS for several min, as indicated in the figure legends. In some cases, as indicated, sodium orthovanadate (final concentration, 500 μM, in PBS) was added 30 min prior to the addition of the amphipath or thrombin. Thrombin (0.5–0.7 U/ml, final concentration) was then added and the incubation continued. Aliquots were removed both before and after the addition of thrombin, as noted in the figure legends, and fixed in 10 vols. of ice-cold buffered glutaraldehyde. A portion of the fixed cells was reserved for assessment of morphology. The remaining cells were pelleted at $8800 \times g$ for 90 s. Radioactivity

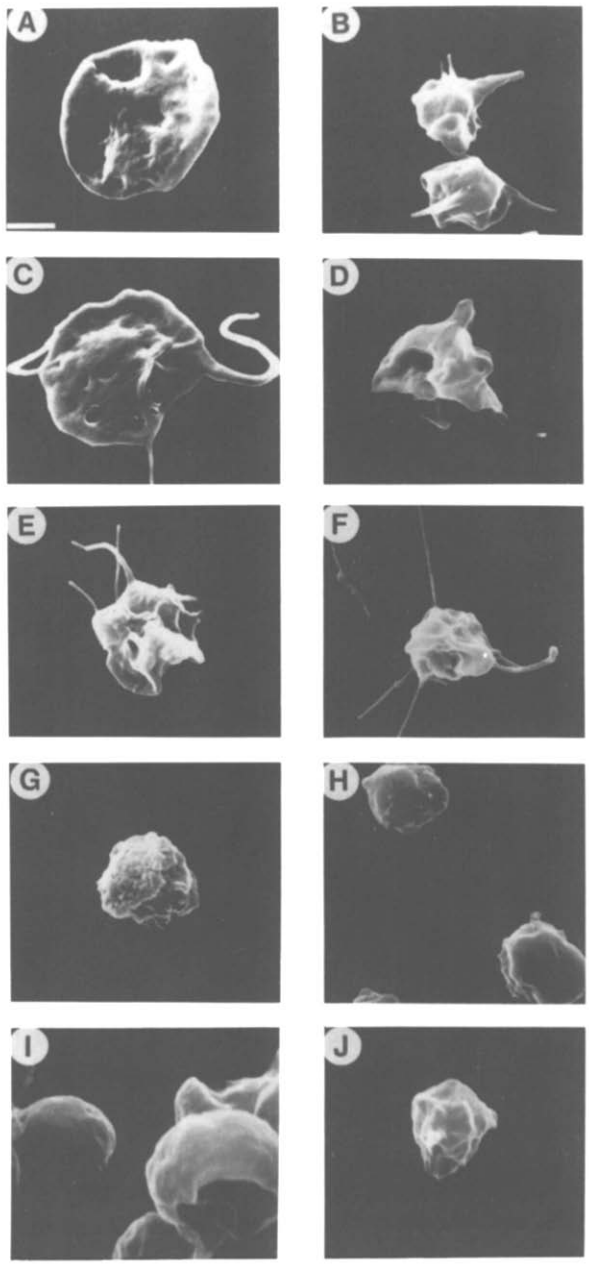

Fig. 1. Effect of DLPC and CPZ on platelet morphology. Scanning electron micrographs of platelets resting (A), after pretreatment with amphipath (C, E, G, I), and after subsequent activation with thrombin (B, D, F, H, J). The cells were pretreated with DLPC at concentrations of 0 μM (A and B), 5 μM (C and D), 15 μM (E and F), 30 μM (G and H) or CPZ at a concentration of 30 μM (I and J). Bar, 1 μm.

in the supernatant was measured by liquid scintillation spectrometry. The extent of serotonin secretion was expressed as a proportion of the total secreted in the presence of thrombin alone (this value was typically 85–90% of the total radioactivity incorporated).

Determination of release of lipid from plasma membranes

Isolated platelets (approximately $4 \cdot 10^9$ cells) were suspended in 1.4 ml of Tyrodes buffer. Radiolabeled dimyristoylphosphatidylcholine (DMPC) vesicles were prepared by sonicating 0.2 μCi of [^{14}C]DMPC to clarity, under argon in 0.10 ml of 150 mM NaCl. The platelet suspension was incubated with the [^{14}C]DMPC vesicles for 90 min, then diluted with 14 ml of citrate wash buffer. The pH was then adjusted to 6.5 with 150 mM citric acid, and the platelets pelleted by centrifugation to remove unincorporated radiolabel. Platelets were then resuspended in Tyrodes buffer at a final concentration of $2 \cdot 10^8$ cells/ml. Labeled platelets were incubated with various amphipaths, at the concentrations indicated in the figure legends, and subsequently pelleted at $8800 \times g$ for 90 s. Release of plasma membrane was determined by quantifying radioactivity in the supernatant.

Quantitation of protein phosphorylation

Isolated platelets ($1.6 \cdot 10^9$ cells) were allowed to equilibrate with 1 mCi [^{32}P]P_i for 90 min at 37°C. Radiolabeled platelets were treated with amphipath, alone or in combination with thrombin. In some cases, platelets were pretreated with 500 μM sodium orthovanadate prior to the addition of amphipath or thrombin, as described in the figure legends. Phosphorylated proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, according to the method of Laemmli

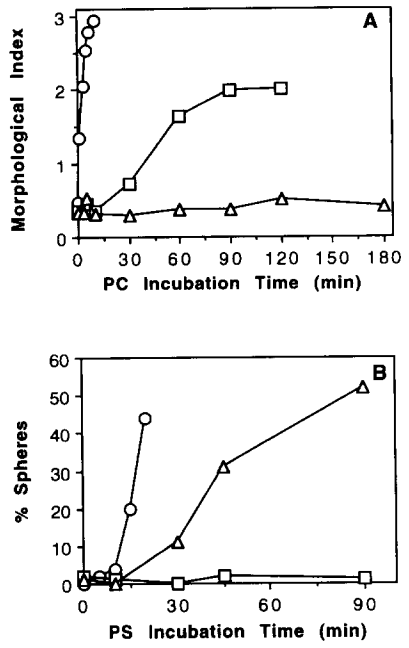

Fig. 2. Kinetics of PC- and PS- induced platelet shape change. Cells were incubated with phospholipids. At the indicated intervals samples of cells were fixed in buffered glutaraldehyde and cell morphologies assessed by phase microscopy. (A) Morphology of cells treated with 35 μM DLPC (circles), DMPC (squares), or DOPC (triangles). (B) Morphology of cells treated with 15 μM DLPS (circles), 25 μM DMPS (squares), or 250 μM DMPS (triangles). Data in B are expressed as the percentage of sphered or indented cells in the sample cell population.

[10]. The resulting gels were subjected to autoradiography using Kodak X-OMAT film preflashed to ensure linearity of response [11]. Incorporation of ^{32}P into proteins was determined either by densitometry of the resulting autoradiograms or by slicing the gels into 0.5 cm slices and measuring the ^{32}P present by Cerenkov counting; the two techniques gave similar results.

Results

Morphological alterations in the presence of spiculating amphipaths

As previously reported [2,5], treatment of isolated platelets with exogenous amphipaths resulted in a significant modification of their resting morphology. Platelets incubated with zwitterionic amphipaths such as DLPC or DMPC developed spicules, generally longer and finer than the filopodia produced during thrombin activation. Treatment of discoidal platelets (stage 0, Fig. 1A) with DLPC (5 μM) resulted in formation of stage +1 platelets, discoidal cells possessing one or several spicules (Fig. 1C). At greater concentrations, DLPC (15 μM) produced stage +2 platelets with spheroid cell bodies and more numerous spicules (Fig. 1E). At still higher DLPC concentrations (30 μM) platelets lost their spicules and adopted a spherical, highly ruffled shape (stage +3 platelets, Fig. 1G).

The kinetics of PC-induced platelet spiculation was dependent on the acyl chain composition of the PC (Fig. 2A). DLPC (35 μM) induced platelet spiculation and loss of spicules within a few minutes of addition, while DMPC (35 μM), with longer acyl chains, required more prolonged incubation (1–2 h) to effect shape changes. Incubation with PC of greater chain length, such as dioleolyphosphatidylcholine (DOPC; 35 μM), failed to affect platelet morphology even after 3 h of incubation. Much greater concentrations of DOPC (e.g., > 1000 μM) were required to induce spiculation (data not shown).

Morphological alterations in the presence of sphering amphipaths

Treatment of discoid platelets with cationic amphipaths such as CPZ (Fig. 1I) or with aminophospholipids such as PS (Fig. 3) converted the discoid platelets to spheroid and indented forms (stage −1). The kinetics of platelet response to exogenous PS varied, depending on the PS acyl chain composition. DLPS (di

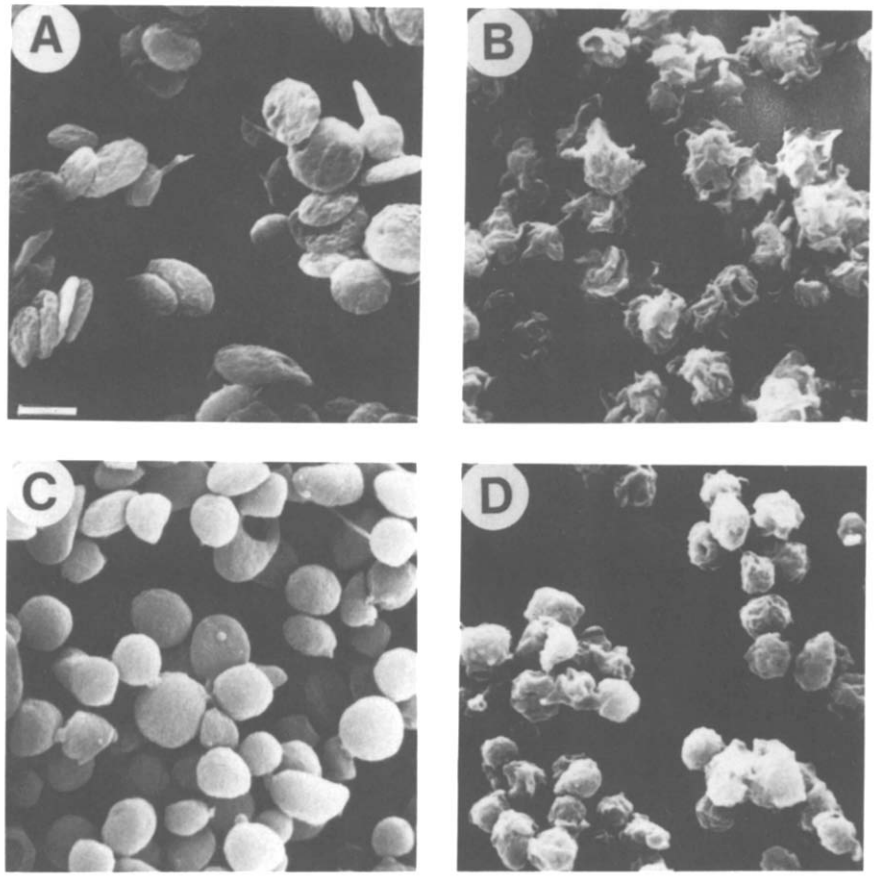

Fig. 3. Effect of DLPS on platelet morphology. Platelets were treated with either PBS or 25 μM DLPS for 20 min. Thrombin was added and the incubation continued for an additional 3 min. Scanning electron micrographs of control platelets before (A) and after (B) the addition of thrombin and of DLPS treated platelets before (C) and after (D) the addition of thrombin. Bar: 1 μm.

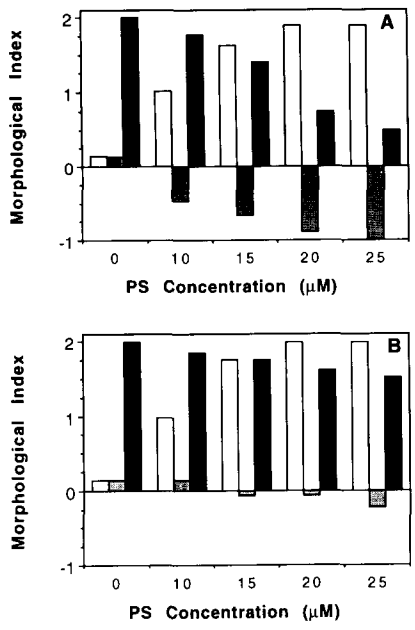

Fig. 4. Effect of vanadate on DLPS-induced platelet shape change kinetics. Platelets were treated with PBS (A) or 500 μM sodium orthovanadate in PBS (B) for 30 min at 37°C. DLPS was added and the incubation continued for 20 min, after which thrombin was added and the cells allowed to incubate for 3 min. Samples were fixed with glutaraldehyde after 5 (open bars) and 20 min (shaded bars) DLPS incubation, and after subsequent treatment with thrombin (filled bars). Morphologies were assessed by phase microscopy.

12:0) induced a transient spiculation; after a 5 min exposure, cells were rounded with numerous fine filopodia (Fig. 4A; open bars). On further incubation, platelets reverted transiently to discs, then adopted a sphered and sometimes indented morphology (Fig. 4A; shaded bars). Both the transient spiculation and eventual sphering increased with increasing concentrations of DLPS. The degree to which DLPS inhibited the thrombin morphology response was likewise dependent on DLPS concentration (Fig. 4A; filled bars).

PS with longer acyl chains also induced platelet sphering, but higher concentrations and longer exposure to the exogenous vesicles were required (Fig. 2B). DMPS (di 14:0) required higher lipid concentrations and more prolonged incubations to induce significant sphering. Incubation with PS of greater chain length (DOPS (di 18:1) or POPS (18:1, 16:0)) promoted sphering, but only in samples exposed to high lipid concentrations (> 1000 μM) for prolonged periods (data not shown). The differing time-courses of the responses to both PC and PS are consistent with the different rates with which phospholipids of varying chain lengths pass between membranes through hydrophilic phases [13].

Morphological alterations of PC- and PS-treated platelets in response to thrombin

PC-spiculated platelets changed shape further on exposure to thrombin; cell body sphering and filopod formation appeared to be superimposed on the amphipath response (Fig. 1D, F). Thrombin-induced filopod formation in PC-treated platelets was inhibited only in cells previously treated with PC concentrations (e.g., 30 μM) sufficient to induce loss of spicules (Fig. 1H).

In contrast, thrombin-induced filopod formation was reduced significantly in platelets sphered via pretreatment with PS (Fig. 3D) or CPZ (Fig. 1J). This reduction in spiculation was evident even at low lipid concentrations (e.g., 10 μM DLPS); the degree of suppression of thrombin-induced filopod formation (Fig. 4A; filled bars) correlated with the extent of amphipath-linked sphering (Fig. 4A; shaded bars).

Equilibration of platelets with sodium orthovanadate (an inhibitor of erythrocyte aminophospholipid translocase activity [12]) prior to the addition of DLPS had a significant effect upon the progress of DLPS-linked shape change, both prior to and after addition of thrombin. Vanadate treatment slightly increased filopodia formation during the initial PS-linked spiculation phase (Fig. 4B; open bars). More significantly, vanadate markedly inhibited the sphering that resulted from longer incubations with DLPS (Fig. 4B; shaded bars). DLPS inhibition of the thrombin response was reduced markedly by vanadate (Fig. 4B; filled bars). Treatment with vanadate alone had no significant effect upon platelet resting morphology or the morphology of thrombin-stimulated cells.

Membrane vesiculation and shape change

Platelets exposed to spiculating agents such as DLPC exhibited a concentration-dependent progression of morphological changes, beginning with surface ruffling, development of increasing numbers of spicules, and cell body sphering, and ending with the apparent disappearance of spicules (expressed in terms of morphological index in Fig. 2A). This result suggests that amphipath incorporation induces shedding of plasma membrane, perhaps similar to membrane budding that accompanies platelet aging in vitro [14]. Membrane vesiculation in response to spiculating agents was quantified. Platelets were labeled with [^{14}C]DMPC (in amounts insufficient to induce significant changes in the cellular morphology), and then incubated with lysopalmitoylphosphatidylcholine (lyso-PC) or DLPC. After 5 min, cells were pelleted and radiolabel released to the supernatant was measured. At concentrations high enough to induce disappearance of spicules (≥ 15 μM DLPC, ≥ 20 μM lyso-PC), both spiculating agents caused release of radiolabeled membrane (Fig. 5A). Brief incubation of platelets with longer chain PC (DOPC), which failed to elicit shape change in the cells, did not induce release of radiolabel. Sphering agents such as PS (data not shown) and CPZ (Fig. 5A),

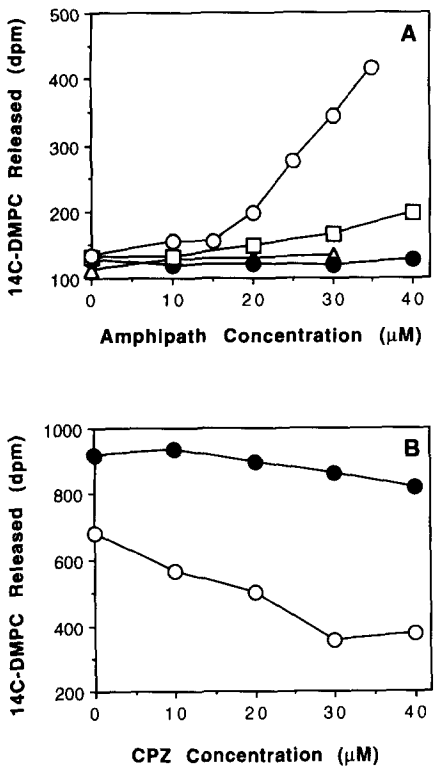

Fig. 5. Vesiculation of platelet membranes. Platelets were radiolabeled with [^{14}C]DMPC and then incubated with amphipaths for 5 min. Cells were then pelleted and the amount of radiolabel released quantified. (A) Release of [^{14}C]DMPC from labeled platelets incubated for 5 min with DLPC (open circles), DOPC (triangles), CPZ (closed circles), or lyso-PC (squares). (B) Release of [^{14}C]DMPC from labeled platelets treated with various concentrations of CPZ in the presence of either 25 μM DLPC (open circles) or 35 μM DLPC (closed circles).

did not induce shedding of [^{14}C]DMPC from labeled platelets.

The role of lipid bilayer balance changes in the release of membrane marker was investigated by treating platelets with a sphere-forming amphipath, CPZ, in addition to a spiculating agent, DLPC (Fig. 5B). Cells treated with both DLPC and CPZ released less membrane marker than those treated with DLPC alone; the mitigating effect of CPZ on DLPC-induced release of label increased with increasing CPZ concentrations.

Effect of amphipaths on dense granule release

The effects of platelet spiculators on activation-linked secretion were determined. Serotonin secretion was measured in PC-treated cells both before and after the addition of thrombin (Fig. 6A). DLPC alone did not induce serotonin secretion, even at the highest concentrations employed. Incubations of platelets with DLPC at concentrations sufficient to result in spiculation of all cells in the sample (e.g., 2.5–5 μM for DLPC) did not inhibit thrombin-induced secretion. Secretion was, however, inhibited in cells pretreated with greater DLPC concentrations. In the case of DMPC, inhibition was evident only if the pretreatment time was long enough (e.g., 100 min) to induce spiculation and then loss of spicules; brief DMPC pretreatment (e.g., 3–5 min) did not inhibit subsequent thrombin-induced serotonin secretion (data not shown).

The effects of PS on activation-linked secretion were examined similarly. Platelets were treated with several PS acyl chain homologues, followed by thrombin; serotonin release was monitored both before and after the addition of thrombin. Treatment with PS alone did not cause significant release of serotonin (data not shown), but PS-treated platelets showed a marked reduction in thrombin-linked serotonin release (to 40% of controls with DLPS; Fig. 7). This inhibition developed at different rates for different PS homologues; DOPS and POPS (2 mM) required more prolonged incubation than DMPS (250 μM) or DLPS (25 μM) to effect significant inhibition. DLPS inhibition of thrombin-linked serotonin release was dependent on the concentration of lipid (Fig. 6B). These data, along with the corresponding PC results, are consistent with the different rates of transfer of lipid from lipid vesicles to platelets.

Prior exposure of platelets to vanadate greatly reduced the inhibitory effects of PS. Platelets treated

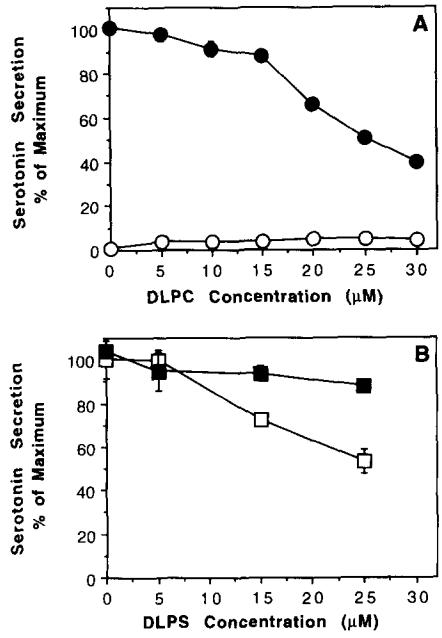

Fig. 6. Effect of PC and PS concentration on serotonin release. Platelets prelabeled with [^{14}C]serotonin were treated with lipid, then stimulated with thrombin. Release of serotonin was determined as a function of lipid concentration. (A) Serotonin secretion from labeled platelets measured after 5 min of pretreatment with DLPC (open symbols) and after subsequent stimulation with thrombin (closed symbols). (B) Serotonin release from labeled platelets equilibrated for 30 min with either PBS (open symbols) or 500 μM sodium orthovanadate (closed symbols), followed by a 20 min incubation with DLPS and subsequent 3 min exposure to thrombin.

first with vanadate, then with PS followed by thrombin, secreted serotonin at 90–100% of control levels (Fig. 6B). Reversing the order of treatment, incubating first with DLPS followed by addition of vanadate, resulted in only a slight reduction in the PS-linked inhibiton of secretion (data not shown).

Effect of amphipaths on protein phosphorylation

Activation of platelets with thrombin results in an increased phosphorylation of myosin light chain (p20) and a 47 kDa protein (p47). To investigate the relationship between lipid-induced shape change and protein phosphorylation, platelets labeled with ^{32}P were treated with PC or PS and subsequently stimulated with thrombin. In some samples, platelets were treated with 500 μM sodium orthovanadate prior to the addition of amphipath or agonist. Incorporation of ^{32}P into proteins, both before and after the addition of thrombin, was quantified. As shown in Table I, treatment of control platelets with thrombin resulted in the expected increase in phosphorylation of both p20 and p47. DLPC pretreatment decreased the extent of phosphorylation of p20 and p47, but only in platelets treated with PC at concentrations sufficient to induce significant membrane vesiculation (data not shown). A 20 min pretreatment of platelets with DLPS, however, significantly reduced the incorporation of ^{32}P into both p20 and p47 upon subsequent addition of thrombin. DLPS inhibition of this phosphorylation was significantly reduced in platelets treated with lipid for shorter periods of time (e.g., 3 min) or equilibrated with 500 μM sodium orthovanadate prior to the addition of lipid. Vanadate alone had little effect upon activation-linked phosphorylation. Treatment with DLPS alone did not have a significant effect upon phosphorylation in resting platelets.

TABLE I

Effects of PS and vanadate on protein phosphorylation

Platelets radiolabeled with ^{32}P were treated for 30 min with PBS or 500 μM sodium orthovanadate in PBS. Platelets were then treated with DLPS, after which thrombin was added and the incubation continued for an additional 3 min. The incorporation of radiolabel into p47 and p20 was determined. These data represent the average of quadruplicate SDS polyacrylamide gels from a typical experiment; error is expressed as ±S.D.

Treatment	p47 (dpm incorporated)	p20 (dpm incorporated)
No DLPS 20 min Thrombin 3 min	792±37	338±34
20 μM DLPS 3 min Thrombin 3 min	731±37	318±16
20 μM DLPS 20 min Thrombin 3 min	501±33	216± 8.4
Vanadate pretreatment DLPS 20 min Thrombin 3 min	798±52	306±28
Vanadate pretreatment 20 μM DLPS 20 min Thrombin 3 min	632±18	253±13
No DLPS 20 min	256±28	154±15
20 μM DLPS 20 min	244±30	148±32

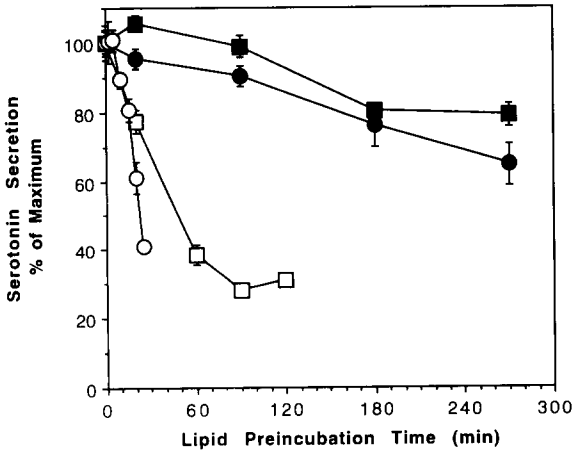

Fig. 7. Effects of PS homologues on thrombin-stimulated serotonin release. Serotonin release from labeled platelets pretreated with PS and subsequently stimulated with thrombin, determined as a function of lipid preincubation time. Platelets were treated with either 25 μM DLPS (open circles), 250 μM DMPS (open squares), 2000 μM DOPS (closed circles), or 2000 μM POPS (closed squares) prior to the addition of thrombin.

Discussion

Morphological responses to amphipath and thrombin

Platelets incubated with short acyl-chain phospholipids display striking changes in their morphology. Platelets treated with PCs develop spicules [2], finer and more elongated than those formed in response to thrombin (Fig. 1) but similar to projections induced by natural agonists like platelet activating factor or ADP. Platelets incubated with PS display a biphasic morphology change (Figs. 3 and 4), first developing similar fine, elongated spicules. This spiculation is followed by reversion to discs and ultimately to spheres and indented forms (Figs. 2–4). These data are consistent with incorporation and translocation of lipids as predicted by the bilayer couple model [4].

The incorporation of exogenous lipid into the platelet membrane affects subsequent thrombin-linked shape changes in complex ways (Figs. 1, 3, 4). Thrombin-induced spiculation is inhibited by PC pretreatment, but only in platelets treated with PC under conditions that result first in spiculation and then in loss of spicules (Fig. 1). The kinetics of these shape

changes vary substantially depending on the acyl chain composition of the PC; DLPC induces spiculation within min of addition to platelets, while longer acyl chain PCs require more prolonged incubation to induce spicule formation (Fig. 2A).

Treatment with exogenous PS produces platelet sphering, resulting in a decrease in subsequent thrombin-linked spiculation (Figs. 3 and 4). If PS treatment is carried out under conditions that reduce the degree of sphering, either by limiting the duration of PS treatment or by preequilibration with vanadate, inhibition of subsequent thrombin-linked spiculation is similarly reduced. As in the case of PC-induced shape changes, the kinetics of PS-induced morphological responses are acyl chain length dependent. DLPS (di-12:0) induces sphering within minutes, while DMPS (di-14:0) requires over an hour of incubation to elicit a similar response (Fig. 2B). PS with still longer fatty acyl chains, such as DOPS (di-18:1) or POPS (16:0, 18:1), require very high lipid concentrations and prolonged incubation times (i.e., approximately 1 mM for 4–5 h) to induce significant platelet shape changes (data not shown). The kinetics of the platelet response to both PC and PS are consistent with the rates of vesicle-to-cell lipid transfer described for red blood cells [13]. These results indicate that simple physical interaction (transient collisions between platelets and vesicles) is not sufficient to induce shape changes, and suggest that these lipids elicit their respective responses by intercalation of phospholipid monomers into the membrane bilayer.

Vesiculation of platelet membranes

At higher concentrations of PC, platelets first form filopodia and then lose them, adopting rounded irregular shapes (Fig. 1). Radiolabeling experiments reveal loss of membrane lipid to the suspending medium when platelets are exposed to DLPC (30 μM) or lyso-PC (40 μM) (Fig. 5A). Such release is not observed when radiolabeled cells are treated with DOPC liposomes, and thus represents release of plasma membrane fragments rather than [^{14}C]DMPC transfer between membranes. These results are consistent with a published report [15] that rabbit platelets shed membrane fragments when incubated with DLPC. Nearly 50% of the lipid present in these fragments is DLPC, and the fragments are highly enriched in acetylcholinesterase, a plasma membrane marker found in rabbit, but not human, platelets [16]. Shedding of acetylcholinesterase-enriched plasma membrane also has been demonstrated in the human erythrocyte [13,17,18].

CPZ and other calmodulin antagonists have been demonstrated to inhibit PC-induced vesiculation in rabbit platelets [19] and in human erythrocytes [20], suggesting an involvement of calmodulin in membrane vesiculation. These results can also be interpreted within the framework of the bilayer-couple model. CPZ induces invagination and sphering in red blood cells [18] and in human platelets [2]. Incubation of DLPC-spiculated platelets with CPZ reverses spiculation; if the conditions are chosen appropriately, these shape changes compensate for each other and produce platelets that display a 'resting' (i.e., discoidal) morphology [2]. Similar compensatory shape changes have been obtained using other sphering agents, such as PS (data not shown). In the current study, spiculation and subsequent shedding of spicules is inhibited in platelets treated with CPZ prior to the addition of DLPC (Fig. 5B). These results indicate that CPZ counteracts the effect of DLPC, presumably by expanding the membrane inner monolayer.

Effects of phosphatidylcholine on platelet activation sequelae

Incubation of rabbit platelets with DLPC has been demonstrated to inhibit platelet-activating factor or thrombin-induced aggregation and secretion [16]. The concentrations of lipid required to inhibit these responses correlate well with the concentrations required to induce exfoliation of the acetylcholinesterase membrane marker from the cells. In the present study, incubation of human platelets with DLPC, even at concentrations that induce membrane vesiculation, fails to elicit release of serotonin (Fig. 6A). Similarly, DLPC treatment decreases protein phosphorylation only slightly and fails to induce aggregation in the absence of other agonists (data not shown). Spiculation of the cells, therefore, can be induced by exogenous manipulation of the bilayer under conditions that do not result in such activation-linked processes as secretion, aggregation, and protein phosphorylation.

PCs do have a concentration-dependent effect on platelet responses to thrombin. At low DLPC concentrations (2.5–5 μM), which induce nearly 100% spiculated forms, neither secretion (Fig. 6A) nor protein phosphorylation is inhibited. At greater DLPC concentrations (> 15 μM), where the cells begin to shed spicules, the thrombin responses are diminished. In the case of the more slowly transferring lipid DMPC, inhibition of cellular responses to thrombin occurs only if the cells are pretreated with liposomes for a period of time sufficient to result in transfer of lipid to the cellular membrane and subsequent vesiculation. Cells pretreated with DMPC vesicles for a short period of time (e.g., 5 min) exhibit normal activation in response to thrombin. These results indicate that the PC-linked inhibition of platelet stimulation by thrombin is not due to thrombin adsorption to vesicles and suggest, instead, that reduction in response to agonist may be due to a loss of membrane components.

Effects of phosphatidylserine on platelet activation sequelae

Incubation of platelets with PS fails to elicit release of serotonin (data not shown) or significantly modify the protein phosphorylation pattern in resting cells (Table I). However, platelets incubated with PS, under conditions that permit the translocation of the lipid across the bilayer, show a marked reduction in the degree of shape change (Figs. 3 and 4), serotonin secretion (Figs. 6B and 7), and protein phosphorylation (Table I) triggered by subsequent exposure to thrombin. This inhibition is significantly reduced if the cells are incubated with PS for a period of time insufficient to allow the lipid to be intercalated into the bilayer and translocated. Similarly, inhibition of translocator activity by preequilibration with sodium orthovanadate also reduces the effect of PS on thrombin activation sequelae. These findings indicate that PS is capable of inhibiting platelet responses to thrombin only if the lipid has translocated to the cytofacial surface of the bilayer.

This conclusion is supported by the results of earlier studies of the effects of PS on platelet activation [21]. These workers examined inhibition of secretion and aggregation by synthetic PS homologues, and found that such inhibition was minimal for PS containing short acyl chains (e.g., di 2:0, di 4:0, di 6:0). PSs containing such short chains display a rapid rate of passive transverse diffusion or 'flip-flop' [22]. Although these lipids would be expected to transfer rapidly, they would not necessarily be sequestered in the inner leaflet of the bilayer. While this same study also demonstrated inhibition of secretion and aggregation due to intermediate chain PS (e.g., di 10:0, di 12:0), it failed to show significant effects employing longer chain PS (e.g., di 14:0, di 16:0); the conditions of time and lipid concentration used in these experiments, however, were not sufficient to allow significant incorporation of lipid into the platelet bilayer [21]. In the present study, long acyl chain PSs similarly failed to inhibit subsequent agonist-induced secretion unless extended incubation times were employed.

While required incubation conditions may vary, the inhibitory activity of PS is evident regardless of the PS homologue tested. The observation that homologues possessing either fully saturated acyl chains (e.g., DLPS and DMPS), unsaturated acyl chains (e.g., DOPS) or a combination of the two (e.g., POPS) are all capable of inhibiting platelet response to thrombin argues against a mechanism involving mere modification of membrane fluidity. A number of studies have demonstrated that manipulation of cholesterol content (and, by implication, alteration of membrane fluidity) has a significant effect on subsequent sensitivity to agonist [23–25]. However, a fluidity-based mechanism would not account for the lack of PS-effect in cells whose translocase activity has been compromised, or in cells treated with PS for periods of time insufficient to allow significant translocation to take place.

Conclusions

These findings demonstrate that PCs can induce platelet spiculation without inducing secretion or other activation sequelae. When present at the minimum concentrations sufficient to induce spicule formation, PCs neither potentiate nor inhibit subsequent responses to thrombin. At higher concentrations, PCs induce membrane shedding, seen morphologically as a loss of spicules and biochemically as a loss of [^{14}C]-DMPC labeled membrane. These results suggest that these amphipaths inhibit thrombin-linked activation by inducing shedding of membrane components as plasma membrane vesicles.

The mechanism of PS-linked inhibition of thrombin-induced activation sequelae is less apparent. Incorporation of PS into platelet membranes does not induce membrane vesiculation. Although a small degree of endocytosis cannot be ruled out, it is unlikely that this event would diminish thrombin responses by 40% (protein phosphorylation) to 60% (serotonin secretion). An indirect effect upon signal transduction, however, may be at play in this system. Possible mechanisms include alteration of bilayer membrane skeleton associations or disruption of receptor coupled responses. PS has been demonstrated to associate directly with platelet cytoskeletal elements [26]; thus, manipulation of the PS content of the cytofacial membrane leaflet could modify lipid-protein interactions, but the amount of lipid incorporated is small [27]. Alternatively, alteration of the curvature of the cytofacial surface of the membrane might physically disrupt required interactions of the signal transduction machinery. Further experiments to distinguish among these and other alternatives are in progress.

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