Involvement of Phosphatidylinositol 4,5-Bisphosphate in Phosphatidylserine Exposure in Platelets: Use of a Permeant Phosphoinositide-Binding Peptide†

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ABSTRACT: During platelet activation, phosphatidylserine (PS) exposure on the extracellular face of the plasma membrane is associated with increased procoagulant activity. PS externalization is generally attributed to an increase in intracellular Ca^{2+}. Various phospholipid transporters, such as specific scramblases or proteins from the family of multidrug resistance proteins, and cofactors such as phosphatidylinositol 4,5-bisphosphate (PIP_2) have been proposed to participate in this process. In this study, we used a membrane-permeant polycationic peptide (RhB-QRLFQVKGRR), derived from the PIP_2-binding site of gelsolin (GS 160−169) and linked to rhodamine B, to investigate the role of PIP_2 in PS externalization in whole platelets. The peptide penetrated rapidly into the platelets, specifically bound to PIP_2, and induced PS exposure to a similar extent as thrombin or collagen, but independently of changes in intracellular Ca^{2+} or phosphoinositide 3-kinase activity. A pretreatment of platelets with quercetin, an inhibitor of phosphoinositide metabolism, drastically decreased PS exposure induced by agonists or peptide. In large unilamellar vesicles (LUVs), the presence of PIP_2 was strictly required for the induction of scrambling of NBD-labeled phospholipids (PC and PS) by the peptide. In inside-out vesicles from erythrocytes (IOVs), the peptide also induced redistribution of PC and PS. Our data suggest that, in intact platelets, PIP_2 acts as a target of polycationic effectors, including Ca^{2+}, to promote PS exposure. The use of a membrane-permeant and fluorescent peptide which binds to PIP_2 is a promising tool to investigate the role of PIP_2 in various cellular processes.

In resting cells, phospholipids are distributed asymmetrically over both halves of the cell plasma membrane (*1*). The aminophospholipids, phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE), are essentially located on the inner leaflet of the plasma membrane, whereas the outer leaflet is mainly composed of the choline-headed phospholipids, sphingomyelin (SM) and phosphatidylcholine (PC). This segregation results, in part, from the activity of a Mg^{2+} and ATP-dependent aminophospholipid translocase that transports PS and PE from the outer to the inner monolayer (*2*). Loss of the asymmetric distribution of phospholipids (scrambling) has been reported in a number of circumstances such as cell activation, cell injury, or apoptosis. PS exposure at the cell surface promotes assembly and activation of the coagulation cascade (*3*) and accelerates the removal of injured or apoptotic cells by the reticuloendothelial system (*4*, *5*). A gene encoding a specific receptor recognizing cell surface PS has been cloned from human macrophages (*6*).

Platelet activation is characterized by an increase in cytosolic Ca^{2+}, a signal generally associated with scrambling in various cells. Ca^{2+} inhibits the aminophospholipid translocase and, in parallel, activates phospholipid redistribution. Previous hypotheses, suggesting that phospholipid scrambling resulted from membrane fusion occurring during granule secretion, platelet activation, or vesicle shedding (*7*, *8*), have been countered by demonstration that scrambling and fusion are independent processes in both erythrocytes (*9*) and platelets (*10*, *11*). The role of protein tyrosine kinase activity in scrambling was suggested from the fact that in platelets or erythrocytes from patients affected by Scott syndrome, a rare inherited hemorrhagic disorder, a deficiency in tyrosine phosphorylation was associated with the inability of Scott blood cells to expose aminophospholipids (*12*, *13*). However, the aberrant tyrosine phosphorylation was shown to be a consequence rather than a cause of defective phospholipid scrambling (*13*).

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; LUVs, large unilamellar vesicles; IOVs, inside-out vesicles derived from erythrocytes; TRAF, thrombin receptor agonist fragment (SFLLRN); PH domain, pleckstrin homology domain; BSA, bovine serum albumin; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; FITC, fluorescein isothiocyanate; C6-NBD, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl; RT, room temperature.

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A plasma membrane 37 kDa protein with scrambling activity (scramblase) has been isolated from platelets and erythrocytes, cloned, and sequenced (*14*). During cell stimulation and apoptosis, its activity could be regulated by protein kinase C δ (*15*). When scramblase was reconstituted in liposomes and activated by Ca^{2+}, it facilitated phospholipid redistribution only to a limited extent (*16*, *17*), suggesting that not all vesicles contained a functionally reconstituted scramblase or that, in cells, other processes are involved in the phospholipid redistribution. Proteins of the MDR PgP family are able to transport phospholipids (*18*) and could be implicated in phospholipid movements during cell activation or apoptosis (*19*, *20*). The activation of these transporters is ATP dependent and Ca^{2+}-independent, whereas scrambling is generally ATP independent and Ca^{2+}-dependent.

Effectors other than Ca^{2+} are also able to regulate scrambling. Polycations, such as polyamines, were reported to down- or up-regulate phospholipid scrambling in resealed ghosts from erythrocytes (*21*, *22*) or in apoptotic cells, respectively (*23*). Furthermore, we have recently shown that other polycations (polylysine and neomycin) or polycationic peptides [MARCKS peptide (151-175)] promoted phospholipid redistribution in inside-out vesicles from erythrocyte membranes (IOVs) (*24*). Acidification has been also demonstrated to induce scrambling in IOVs (*24*, *25*).

Several lines of evidence argue in favor of a role for phosphatidylinositol 4,5-bisphosphate (PIP_2) in this process (*22*, *24*, *26*, *27*). External Ca^{2+}, which has no effect on scrambling in intact erythrocytes, induces phospholipid scrambling when erythrocytes are loaded with a physiological concentration of PIP_2, or in large unilamellar vesicles (LUVs) containing PIP_2 (*22*, *27*). Addition of vinblastine or chlorpromazine induces scrambling in erythrocytes and in LUVs only when they contain PIP_2 (*28*). PIP_2 was shown to be a target of Ca^{2+} and other polycations, as scrambling induced by Ca^{2+} or polycations was abolished by a PH domain of PI-specific phospholipase C-δ_1 (*24*), which competitively interacts with PIP_2 with a high selectivity (*29*). We have recently suggested that PIP_2 lateral domain formation could play a critical role in phospholipid scrambling (*24*). Indeed, spermine, polylysine, MARCKS peptide, and Ca^{2+} induced scrambling in erythrocyte membrane vesicles, in parallel to their reported ability to form domains of acidic phospholipids, including PIP_2 (*30*, *31*). Additionally, a PIP_2 antibody was also found to induce scrambling, presumably by a similar mechanism, since phospholipid antibodies are known to promote phospholipid capping (*32*).

Based on the PI-binding sequence of gelsolin (GS 160-169) (*33*, *34*), a rhodamine B-linked peptide (RhB-QRLFQVKGRR) was synthesized (*35*, *36*) and used in the present study to investigate the functional role of phosphoinositides in intact cells. We show that the peptide binds specifically to PIP_2 penetrated into platelets and induced PS exposure. In contrast to thrombin or collagen, peptide-induced PS exposure was independent of changes in both the internal concentration of Ca^{2+} and the PI 3-kinase activity. However, under all conditions, PS exposure was suppressed by quercetin, an inhibitor of phosphoinositide synthesis. The peptide also promoted significant PC and PS redistribution in the absence of Ca^{2+} in IOVs and in LUVs only when they contained PIP_2. We propose that the peptide could mimic the effect of Ca^{2+} to induce PS exposure by interacting with PIP_2.

MATERIALS AND METHODS

Materials. Thrombin (T-6848), calcium ionophore A23187 (C-7522), fatty acid free BSA (A6003), FITC-annexin V (A-9210), apyrase (A-6535), sepharose CL-2B (CL-2B-300), FURA 2-AM (A-9210), BAPTA-AM (A-1076), prostaglandin E_1 (P-5515), wortmannin (W-1628), L-α-phosphatidylinositol 4,5-bisphosphate (P-9763), L-α-phosphatidylcholine (P-6638), L-α-phosphatidyl-L-serine (P-6641), sodium dithionite (S-256), lactate dehydrogenase (LDH) kit (500), and quercetin (Q-O125) were obtained from Sigma. The thrombin receptor agonist fragment (SFLLRN) (TRAF) was from Bachem, and the calf tendon collagen reagent (385) was from Chrono-Log Corporation. C6-NBD-PC (810132) and C6-NBD-PS (810194) were obtained from Avanti Polar Lipids. The set of peptides included QRL, QRLFQVKGRR (GS 160-169), FRVKLKQGQR, and KHVVPNEVVVQRLFQVKGRR (GS 150-169) prepared either by free peptides or conjugated to rhodamine B or to pyrenylbutyrate as an amide link at the N-terminus of the peptide as previously described (*34*, *35*) and is denoted with RhB- or pyrene- as a prefix to the peptide sequence.

Interaction of Peptides with Lipid Vesicles Containing PIP_2. To prepare LUVs, a dry lipid film of PC (1.66 nmol) or PC/7.5% PS was hydrated with 0.5 mL of buffer B (140 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, pH 7.35), containing or not containing 15 mol % PIP_2, and was vortexed. The suspension was freeze-thawed five times and then extruded through polycarbonate filters five times in a 400 nm pore size filter and five times in a 100 nm pore size filter. According to a previous estimation, the yield of PIP_2 incorporation into the LUVs is about 50% (*22*). The final PIP_2 concentration in LUVs was thus about 7.5% of total phospholipids. Fifteen minutes after the addition of various concentrations of LUVs to 1 μM solutions of peptides in buffer B, the fluorescence of RhB- or pyrene-peptides (I_{em} = 590 nm, I_{ex} = 560 nm, or I_{em} = 473/378 nm, I_{ex} = 343 nm, respectively) was measured. The expectation was that if peptides bound to LUVs, their surface concentration would become much higher than their bulk concentration, thereby resulting in either quenching of rhodamine B fluorescence or formation of pyrene-excimers with a shift of fluorescence emission from the monomer wavelength at 378 nm to the excimer emission at 473 nm. Measurements of fluorescence spectra of pyrene-QRLFQVKGRR confirmed the expectation that pyrene excimers formed, and the resulting ratio of emission at 473 to emission at 378 is reported as a measure of the extent of peptide clustering at the LUVs surface.

Preparation of Human Platelets. Human blood was collected from healthy adult donors by venipuncture in 0.1 vol ACD (111 mM dextrose, 85 mM trisodium citrate, 71 mM citric acid). Donors were free of any medication for 2 weeks prior to blood collection. Platelet-rich plasma (PRP) was obtained after centrifugation of the blood supplemented with apyrase (0.5 U/mL) (15 min, 110*g*, room temperature (RT)). PRP was centrifuged (5 min, 300*g*, RT) to eliminate contaminating erythrocytes. Platelets were sedimented by centrifugation (10 min, 1000*g*, RT), suspended in buffer A (139 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, 0.3% albumin, pH 7.35), and filtered on a 50 mL column of Sepharose 2B equilibrated

with the same buffer. Cell count was determined using a Z2 Coulter particle counter and size analyzer.

Microscopy Study. Platelets (1.5×10^8/mL) suspended in buffer A, supplemented with apyrase (1 U/mL) and PGE_1 (5 μM) to prevent activation, were preincubated without or with RhB-QRLFQVKGRR or RhB-QRL peptides for 5 min at RT. At indicated times, they were washed twice in the same buffer. A Delta Vision restoration microscope was used to acquire stacks of optical sections spaced 0.5 μm apart. Observation of fluorescence was performed using a 63× objective lens and rhodamine filters. Image analysis was accomplished using Delta Vision software. These observations indicated that both peptides were present in the cytoplasmic space (data not shown).

Actin Polymerization. The level of actin polymerization was evaluated by the estimation of actin content in the Triton-insoluble cytoskeleton isolated from platelets. Platelets pretreated or not pretreated with 5−20 μM RhB-QRLFQVKGRR peptide or 10 and 20 μM RhB-QRL or RhB−FRVKLKQGQR, as control peptides, were incubated at 37 °C for 5 min, without stirring, in the presence of thrombin (0.5 U/mL). At the end of incubation, platelets were lysed by addition of an equal volume of lysis medium (1.5% Triton X-100, 1 mM PMSF, 120 mM PIPES, 50 mM HEPES, 20 mM EGTA, 4 mM $MgCl_2$, 10 mM glucose, 0.1 mM DTT, 20 μg/mL leupeptin, 156 μg/mL benzamidine, 80 μg/mL aprotinin, pH 7.2). The lysate was kept at 4 °C for 5 min and centrifuged (10 min, 12 000*g*). The pellet, containing the Triton-insoluble cytoskeleton, was dissolved in SDS, and actin content was visualized after protein separation by SDS-PAGE electrophoresis on 10% polyacrylamide gels and Coomassie Blue staining.

PS Exposure. PS exposure on the outer platelet surface was monitored by binding of FITC-labeled annexin V, as described previously (*37*, *38*). Platelet suspensions in buffer A (1.5×10^8/mL) were activated at 37 °C for 20 min, without stirring, by the agonists: thrombin (0.5 U/mL), collagen (20 μg/mL), TRAF (100 μM), and A23187 (2 μM), in the presence of 2 mM $CaCl_2$ or by 5−20 μM RhB-QRLFQVKGRR peptide in the presence of either 2 mM $CaCl_2$ or 0.1 mM EGTA. FITC-annexin V (200 nM) was then added to all samples (with 2 mM $CaCl_2$ also added to the platelets incubated with the peptide in the presence of EGTA). Unbound annexin V was removed by rinsing and centrifugation (3 min, 1000*g*). Bound FITC-annexinV was released from platelets using the same buffer containing 50 mM EDTA. The amount of released FITC-annexin V was quantified by fluorometry (I_{ex} = 495 nm, I_{em} = 519 nm). The increase in annexin V binding was calculated by dividing the fluorescence resulting from release of FITC-annexin V from activated platelets by the corresponding fluorescence from inactivated platelets. Platelet lysis occurring during FITC-annexin V binding assay was determined by LDH activity in the supernatant of activated platelets. Depending on the experimental conditions, lysis amounted to 0.5−8% of total cells. When required, platelet suspensions were preincubated for 90 min, at 37 °C, with 200 μM quercetin before agonist addition. Stock solutions of quercetin were prepared freshly in 95% ethanol for each experiment, and quercetin was present in platelet suspensions during the annexin V binding assay.

Measurement of $[Ca^{2+}]_i$. Platelet suspensions in buffer A (3×10^8/mL) were incubated for 45 min at RT, in the dark, with Fura 2-AM (2 μM), centrifuged (10 min, 1000*g*), washed in the same buffer, and resuspended in buffer A containing 2 mM $CaCl_2$ to a density of 1.5×10^8/mL. After introduction of platelets in a cuvette of a SLM-Aminco MC 200 fluorimeter and addition of agonists, the suspension was stirred (1000 rpm) for 20 s, and transient Fura-2 fluorescence was recorded for 10 min (I_{ex} = 340 nm, I_{em} = 510 nm). The $[Ca^{2+}]_i$ levels were measured using Fura-2 AM loaded platelets resuspended in the presence of 1 mM $CaCl_2$. $[Ca^{2+}]_i$ was calculated from the general formula $[Ca^{2+}]_i = K_d(F - F_{min}/F_{max} - F)$, in which K_d is the dissociation constant of Fura-2 for Ca^{2+} binding (224 nM), and F is the fluorescence intensity of the sample. F_{max} was determined after lysing the cells with 50 μM digitonin, and F_{min} was determined after adjusting the pH of the lysed cells to 8.5 with 20 mM TRIS base, followed by the addition of 10 mM EGTA.

Redistribution of NBD-Phospholipids (LUVs). LUVs were prepared as described above. NBD-PC or NBD-PS were incorporated in the outer leaflet, following addition of the analogue (1% of LUVs lipids) dissolved in ethanol to PC or PC/PIP_2 LUVs with stirring, and vesicles (75 nmol lipid/mL) were incubated for 60 min at RT for equilibration. LUVs were then incubated with peptides for 30 min at 37 °C and placed in a cuvette of a LS-5B spectrofluorometer (Perkin-Elmer). NBD fluorescence was recorded (I_{ex} = 470 nm, I_{em} = 540 nm) until a constant baseline (F_0) was obtained. Detection of NBD-PC present in the LUVs inner leaflet was monitored after reduction of the NBD group of the analogues present in the outer leaflet by dithionite ion, resulting in a loss of fluorescence intensity due to the conversion of the NBD group to its 7-amino derivative. After the decrease in fluorescence emission caused by addition of dithionite (10 mM) to intact LUVs reached a plateau value (F_1), Triton X-100 (1%) was added to reduce the remaining internal signal, providing the basal fluorescence signal (F_2). The fraction of analogues redistributed into the inner leaflet of LUVs was calculated from $F_1 - F_2/F_0 - F_2$.

Measurement of Vesicle Diameter. The LUVs size (hydrodynamic diameter) was determined by dynamic light scattering (DLS) using a DynaPro 99 instrument. Details of this measurement are described elsewhere (*39*). Briefly, the method measures the diffusion constant of the vesicles by measuring the autocorrelation function of scattered light intensity, and the diameter of the vesicle is calculated from the relation $D = kT/6\pi\eta R_h$, where D is the translational diffusion constant, η is the solvent viscosity, and R_h is the hydrodynamic radius. To determine if the phospholipid flip-flop could be caused by vesicle fusion or disruption, vesicles treated with peptides were measured by DLS immediately before determination of lipid redistribution, as described above.

Redistribution of NBD-PC and NBD-PS in Erythrocyte IOVs. Packed erythrocytes were lysed in 40 vol of 5 mM phosphate buffer (pH 8). The hemoglobin-free membranes were resuspended in 40 vol of 0.1 mM EGTA, 0.5 mM phosphate buffer (pH 8−8.2), and incubated for 18 h at 4 °C to extract cytoskeletal proteins. After centrifugation of the membrane suspension, the pellet was washed once in the same medium before homogenization with a 27 gauge needle. The vesicles were further purified through a Dextran

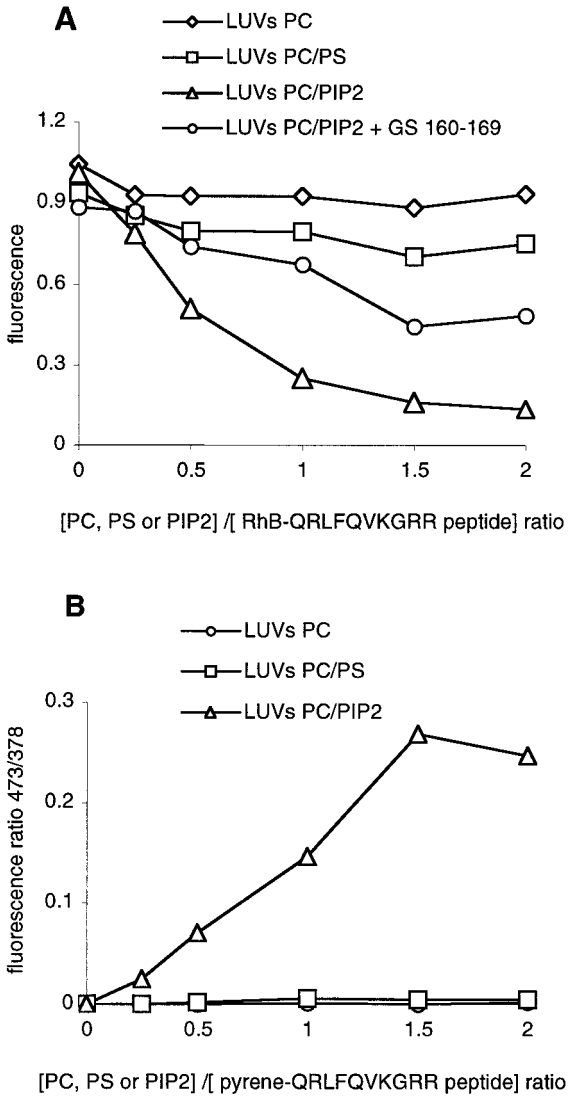

FIGURE 1: Specific interaction of RhB-GS 160-169 with PIP_2. LUVs were prepared in buffer B (140 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, pH 7.35) by lipid extrusion and added to 1 μM peptide also in buffer B. Fluorescence quenching of rhodamine B (I_{em} = 590 nm, I_{ex} = 560 nm) was used as a test of phospholipid binding. (A) Interaction of RhB-QRLFQVKGRR peptide with LUVs containing PC (diamonds), PS (squares), and PIP_2 (triangles) or with LUVs containing PIP_2 in the presence of unlabeled GS 160-169 peptide (dilution 1:2, circles). (B) Similar experiments with a pyrene-QRLFQVKGRR peptide. Data shown were obtained in a single experiment. Two other experiments gave similar data.

barrier (d = 1.03) by centrifugation (120 min, 100 000g). Purified vesicles were resuspended in 10 mM TRIS, 0.1 mM EGTA (pH 7.4). The membrane sidedness and the percentage of sealed vesicles were determined as described previously (*24*). Transbilayer movement of NBD-PC and NBD-PS was determined by the BSA back-exchange method (*24*).

RESULTS

Specific Interaction of Gelsolin-Derived Peptides with PIP_2. As shown in Figure 1A, incubation of the RhB-QRLFQVKGRR peptide with increasing concentrations of PC LUVs containing 7.5% PIP_2 resulted in a dose-dependent loss of rhodamine B fluorescence. The gradual loss of fluorescence is likely to be due to self-quenching of rhodamine B groups, as they come within molecular distances of each other at the vesicle surface, and reflects increased binding of the peptide. This conclusion was confirmed by the observation that dilution of the RhB-QRLFQVKGRR peptide with unlabeled peptide at a ratio of 1:2 labeled:unlabeled peptide markedly reduced the fluorescence quenching. Figure 1B shows the result of similar experiments using the pyrene-QRLFQVKGRR peptide. In this case, incubation with increasing concentrations of PC/PIP_2 LUVs promoted an increase in fluorescence at the excimer wavelength (473 nm) and a loss of monomer fluorescence at 378 nm, as expected if the pyrene groups are juxtaposed within molecular distances on the order of 1 nm at the bilayer surface. In both cases, the effect was specific for PIP_2 LUVs, since no or very little change of fluorescence was observed when RhB- or pyrene-peptides were incubated with LUVs containing only PC or 7.5% PS. Other RhB-linked peptides with randomized positive charges (FRVKLKQGQR) or a shorter sequence with only one charge (QRL), used as a control, did not lose fluorescence after addition to PC/PIP_2 LUVs (results not shown).

RhB-QRLFQVKGRR Peptide Induces PS Exposure in Platelets. Platelet suspensions were incubated at 37 °C for 20 min, with 5−20 μM RhB-QRLFQVKGRR peptide in the presence of 0.1 mM EGTA. The extent of PS exposure on the platelet surface was measured by the binding of FITC-annexin V and expressed as a relative increase over the binding to unstimulated platelets. Figure 2A shows that the RhB-QRLFQVKGRR peptide induced PS exposure in a concentration-dependent manner, whereas the RhB-QRL- or rhodamine B-conjugated peptides with the scrambled sequence (RhB-FRVKLKQGQR) used as the control peptides had negligible effect on PS exposure.

The possibility that PS exposure after addition of RhB-QRLFQVKGRR resulted from platelet activation was addressed by changes in light transmission with a platelet aggregometer. Platelet aggregation induced by TRAF, collagen, or thrombin resulted in a 80−90% increase in light transmission. In contrast, addition of RhB-QRLFQVKGRR peptide (20 μM) to platelets suspended in buffer containing 2 mM Ca^{2+}, did not induce platelet aggregation, providing evidence that, at this concentration, the peptide had no effect on the platelet resting state (data not shown).

The ability of RhB-QRLFQVKGRR to induce PS exposure in platelets, in the presence of 2 mM Ca^{2+}, was compared with that of other agonists (thrombin, TRAF, collagen, collagen + thrombin, and the calcium ionophore A23187) (Figure 2B). A23187 was the most potent inducer giving rise to a 15-fold increase in annexin V binding, consistent with prior observations (*38*). RhB-QRLFQVKGRR caused a 4-fold increase in PS exposure, a level intermediate between the changes caused by thrombin and collagen. The RhB-QRLFQVKGRR peptide (20 μM) had an effect additive with that of thrombin or TRAF on PS exposure, without potentiation (Figure 2C). As none of these inducers was able to promote maximal PS exposure (when compared to the effect of A23187) (Figure 2B), it is not possible to decide whether the peptide and the physiological agonists used the same or different mechanisms to induce PS externalization.

Preincubation of platelets for 90 min with 200 μM quercetin, which is expected to reduce PIP and PIP_2 concentration by inhibiting PI 4-kinase (*40*−*42*), almost

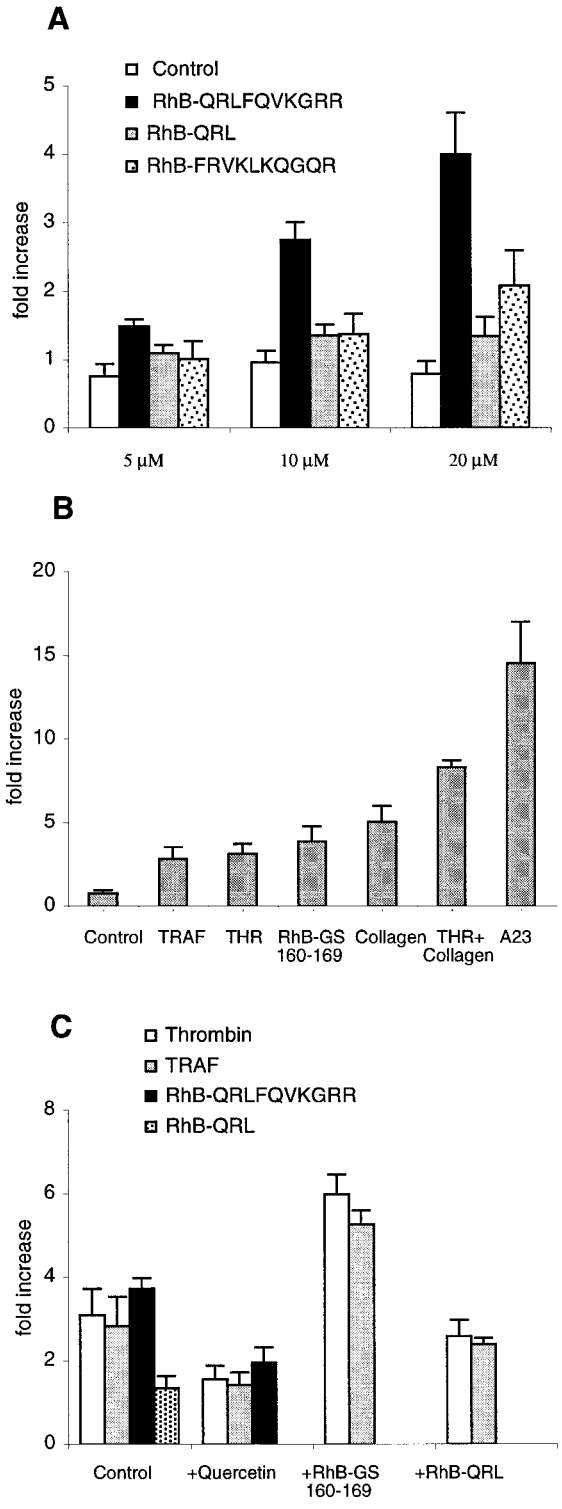

FIGURE 2: Effect of RhB-QRLFQVKGRR or agonists on PS exposure in platelets. Platelet suspensions were incubated for 20 min, at 37 °C, with peptides or platelet agonists. The amount of cell-bound FITC-annexin V (PS exposure on platelet surface) was expressed as a relative increase over the level in unincubated control. (A) Dose-dependent effects of RhB-QRLFQVKGRR and lack of effect of other peptides in a Ca^{2+} free medium. (B) Comparison of the effect of RhB-QRLFQVKGRR (RhB-GS 160-169; 20 μM) and of platelet agonists: TRAF (100 μM), thrombin (THR; 0.5 U/mL), collagen (20 μg/mL), and calcium ionophore (A23) (2 μM) in a medium containing 2 mM $CaCl_2$. (C) Effect of 90 min preincubation with quercetin (200 μM) and of simultaneous addition of thrombin (0.5 U/mL) or TRAF (100 μM) and RhB-GS peptides (20 μM) in a medium containing 2 mM $CaCl_2$. Data shown are means ±SE of 3−6 experiments.

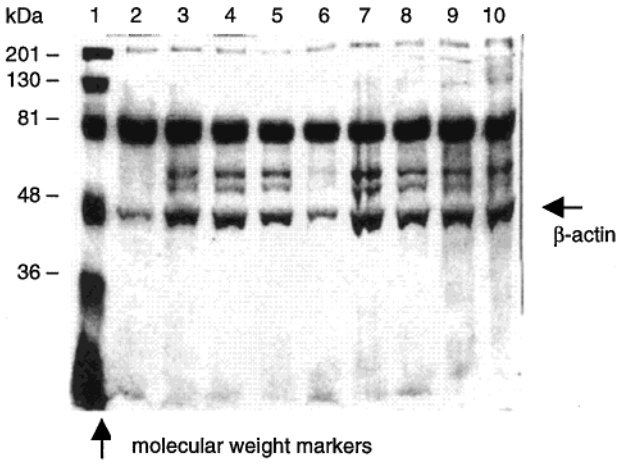

FIGURE 3: Effect of RhB-QRLFQVKGRR on thrombin-induced actin polymerization. Platelets were incubated for 5 min without agonist (lane 2) or with thrombin (0.5 U/mL) in the absence (lane 3) or presence of 5 μM (lane 4), 10 μM (lane 5), and 20 μM (lane 6) of RhB-QRLFQVKGRR peptide, or 10 μM (lanes 7 and 9) and 20 μM (lanes 8 and 10) of RhB QRL or RhB-FRVKLKQGQR peptide, respectively. After platelet lysis and centrifugation, the pellet, containing the Triton-insoluble cytoskeleton, was electrophoresed. Polymerized actin was detected after Coomassie Blue staining (arrow). The large band comigrating with the 81 kDa marker is BSA present at 3 mg/mL in all buffers used.

completely abolished PS exposure induced by RhB-QRLFQVKGRR, thrombin, or TRAF (Figure 2C). However, a 5 min preincubation with the same concentration of quercetin did not prevent scrambling induced by thrombin or RhB-GS 160-169 peptide, indicating that quercetin had no direct effect on scrambling and, consequently, that scrambling inhibition, after 90 min of incubation, would result from inhibition of phosphoinositide metabolism (results not shown). Actin polymerization induced by agonists in permeabilized platelets was reported to be inhibited by the GS 150-169 peptide (*43*). Figure 3 shows that the RhB-QRLFQVKGRR peptide (20 μM) was also able to inhibit actin polymerization induced by thrombin in whole platelets, whereas it produced an effect additive with that of thrombin or TRAF on PS exposure (Figure 2C). The RhB-QRL or RhB-FRVKLKQGQR peptides used as a control had no significant effect either on actin polymerization or on PS exposure induced by thrombin or TRAF (Figures 2C and 3).

Activation of PI 3-Kinase and Increase in $[Ca^{2+}]_i$ Are Not Required for PS Exposure Induced by RhB-QRLFQVKGRR. As PI 3-kinase was reported to be activated by the RhB-QRLFQVKGRR peptide (*39*, *44*) or by agonists in platelets (*45*), we investigated whether PS exposure induced by the peptide was dependent on PI 3-kinase activity. Figure 4 shows that after preincubation of platelets with 100 nM wortmannin, PS exposure induced by thrombin or collagen was significantly inhibited, whereas the inhibitor had no effect on PS exposure induced by the peptide.

To determine whether Ca^{2+} was involved in PS exposure induced by the peptide, platelets were preincubated with BAPTA-AM, to prevent any further changes in $[Ca^{2+}]_i$, before addition of the RhB-QRLFQVKGRR peptide or other agonists. This treatment did not significantly affect PS exposure induced by the peptide, in contrast to the significant inhibition of the redistribution of PS induced by thrombin and collagen (Figure 4).

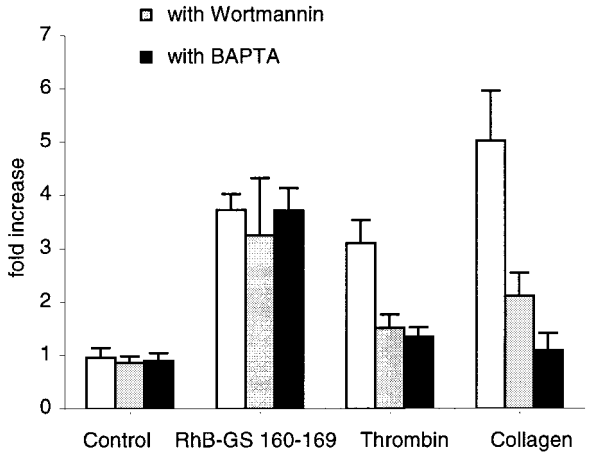

FIGURE 4: Lack of effect of PI 3-kinase and of $[Ca^{2+}]_i$ on PS exposure induced by RhB-QRLFQVKGRR. Platelets suspended in a medium containing 2 mM $CaCl_2$ were preincubated for 10 min with wortmannin (100 nM) or for 30 min with BAPTA-AM (100 μM), before addition of thrombin (0.5 U/mL), collagen (20 μg/mL), or RhB-QRLFQVKGRR peptide (20 μM). The amount of cell-bound FITC-annexin V (PS exposure on platelet surface) was expressed as a fold increase over basal level. Data shown are means ±SE of three experiments.

Changes in Fura-2 fluorescence and in $[Ca^{2+}]_i$ (in the presence of 2 and 1 mM Ca^{2+}, respectively) in response to the RhB-QRLFQVKGRR peptide demonstrated that, in contrast to thrombin and A23187, the peptide (20 μM) did not modify the steady-state level of $[Ca^{2+}]_i$ (Figure 5). These data confirm that the effect of the peptide on PS exposure was not mediated through an increase in $[Ca^{2+}]_i$ even in the presence of 2 mM Ca^{2+} during the annexin V binding assay. However, the peptide at concentrations higher than 50 μM induced a slow increase in $[Ca^{2+}]_i$ (data not shown).

RhB-QRLFQVKGRR Peptide Induces NBD-PC and NBD-PS Scrambling in LUVs. To determine if the effects of RhB-QRLFQVKGRR on PS exposure in intact platelets result from a direct interaction with membrane-bound lipids, an assay for lipid scrambling in vesicles formed by purified lipids was employed. The percentage of NBD-PC and NBD-PS, redistributed into the inner leaflet of LUVs, was evaluated after reduction with the dithionite of the NBD fluorescence group present in the outer leaflet. Figure 6A shows that the RhB-QRLFQVKGRR peptide induced a significant internalization of both NBD-phospholipids only when LUVs contained PIP_2. The RhB-QRL peptide, used as a control, did not induce any redistribution. To eliminate the possibility that lipid scrambling in LUVs was due to peptide-induced vesicle fusion, the effect of peptide binding on the diameter of LUVs was determined by light scattering (Figure 6B). Increased RhB-QRLFQVKGRR peptide binding to PC/1% NBD-PS/PIP_2 LUVs had no effect on LUVs diameter up to a peptide concentration of 5 μM, the maximum used in the experiments of Figure 6A, suggesting that within that range, the peptide did not induce vesicle fusion. At higher concentrations (10, 20 μM), a drastic increase in PC/1% NBD-PS/PIP_2 LUVs diameter indicates that high concentrations of peptide induce fusion or aggregation of LUVs. Increasing concentrations of the RhB-QRLFQVKGRR peptide had no effect on the diameter of PC/1%NBD-PS LUVs, and increasing concentrations of the RhB-QRL peptide had no effect on the diameter of PC/1% NBD-PS/PIP_2 LUVs (Figure 6B).

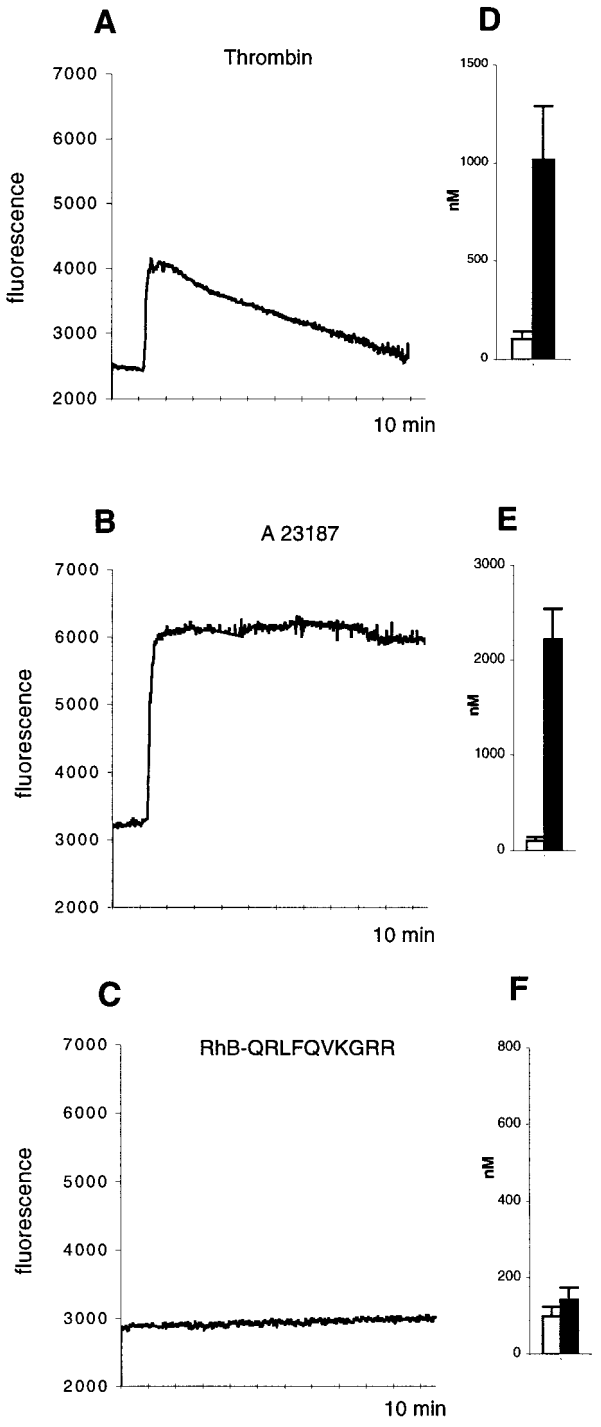

FIGURE 5: Lack of effect of Rhd-QRLFQVKGRR on platelet $[Ca^{2+}]_i$. Fura-2 AM-loaded platelets, resuspended in the presence of 2 mM $CaCl_2$, were stimulated with agonists, and transient Fura-2 fluorescence was recorded for 10 min (A, B, C). $[Ca^{2+}]_i$ was measured in Fura-2 AM-loaded platelets resuspended in the presence of 1 mM $CaCl_2$, before (white bars) and after (black bars) stimulation with the agonists (D, E, F). Data shown are representative of 3−5 independent experiments or means ±SE of 3−4 experiments.

Gelsolin 150-169 Peptide (GS 150-169) Promotes NBD-PC and NBD-PS Scrambling in Erythrocyte IOVs. IOVs were loaded with the analogues, washed once in 10 mM TRIS (pH 7.4) and 10 μM EGTA, and incubated for 30 min at 37

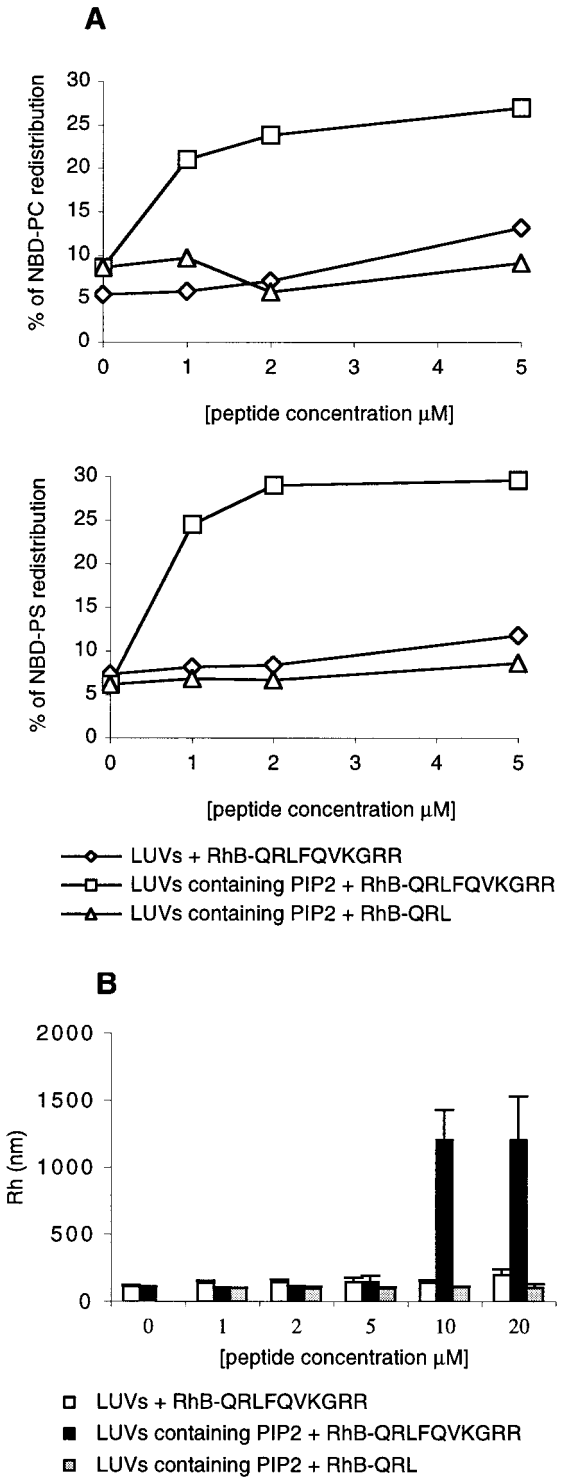

FIGURE 6: Effect of RhB-QRLFQVKGRR on NBD-PC and NBD-PS redistribution in LUVs. PC LUVs, containing or not containing PIP_2, were labeled with 1% NBD-lipids in the outer monolayer. After the addition of peptides, LUVs were incubated for 30 min at 37 °C. The fraction of NBD-PC or NBD-PS flipped to the inner monolayer was determined by the dithionite assay (A). Data shown are representative of two independent experiments. The PC/1% NBD-PS LUVs size (Rh, hydrodynamic diameter) was determined by dynamic light scattering (B). Data shown are means ±SE of three experiments.

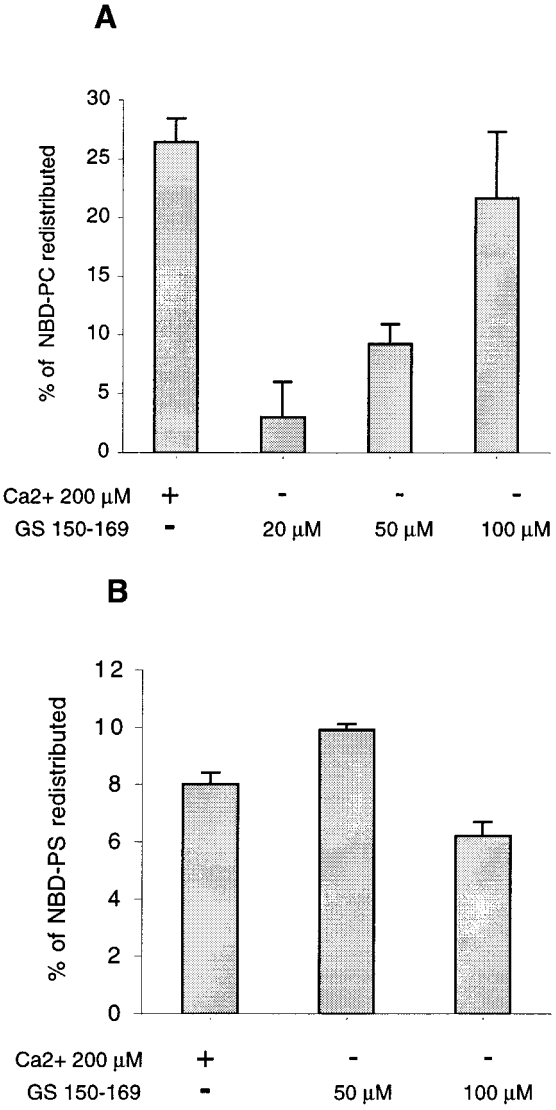

FIGURE 7: Effect of GS150-169 on NBD-PC and NBD-PS redistribution in IOVs. IOVs were loaded with NBD-lipids and incubated for 30 min at 37 °C with 0.2 mM $CaCl_2$ or the indicated peptide concentrations. The fraction of NBD-lipids redistributed was calculated from the percentage of the internalized fraction, determined by the BSA back-exchange method, minus that measured in control condition (EGTA 10 μM). Data shown are means ±SE of 3−5 independent experiments.

°C in the presence of either 200 μM Ca^{2+} or 20−100 μM GS 150-169. The extent of NBD-PC or NBD-PS internalization was measured after BSA extraction of the fraction of the analogue remaining in the external leaflet. The effect of GS 150-169 on NBD-PC internalization was dose-dependent (Figure 7A), reaching a maximal level of 25%, similar to the effect of 200 μM Ca^{2+}. In the same assay, 200 μM Ca^{2+} and 50 or 100 μM GS 150-169 had a significant but smaller effect on NBD-PS redistribution (Figure 7B). These results show that the scrambling effects of the rhodamine B-linked peptide, observed in platelets and in LUVs, do not require the presence of RhB, as long as the peptide has direct access to the membrane leaflet containing PIP_2.

DISCUSSION

In the present study, we have used a gelsolin-derived, rhodamine B-linked, membrane permeant peptide (RhB-QRLFQVKGRR) to investigate the role of PIP_2 on PS externalization in platelets. A specific high affinity phosphoinositide-binding peptide derived from gelsolin (GS 150-169) and a cationic fragment of the same peptide (GS 160-

169) have been both used previously to probe the role of PIP_2 on actin polymerization in detergent permeabilized platelets (*43*), but have no effect on intact cells presumably because they cannot access the intracellular leaflet in which PIP_2 resides. The GS 160-169 peptide (QRLFQVKGRR) linked to rhodamine B is membrane permeant, as evidenced from the internalization of RhB fluorescence in whole platelets incubated with the RhB-labeled peptide. The RhB-GS 160-169 peptide bound to PIP_2 but not to PS, indicating that the binding is specific for phosphoinositides. In addition, the binding of the peptide to PIP_2 depends on peptide structure, since a peptide with scrambled sequence did not bind PIP_2. Furthermore, the binding was not due entirely to the amphiphilic characteristic of rhodamine B, since the RhB-QRL peptide did not bind to PIP_2. The properties of the RhB-GS 160-169 peptide to enter the cells and to bind specifically to PIP_2 make it a convenient tool to probe the role of PIP_2 in various processes (*44*), such as cell motility and actin assembly (*36*).

We found that the peptide induced PS exposure in platelets to a similar extent as did classical agonists (thrombin, collagen, TRAF). This effect was independent of any stimulation of the platelets, as the peptide did not alter the platelet resting state in terms of aggregation or shape change. Peptide-induced PS exposure did not result from unspecific chaotropic membrane perturbations due to the peptide translocation through the membrane, since other RhB-linked peptides penetrated into cells but did not induce any PS exposure (Figure 2). Furthermore, the effect of RhB-GS 160-169 on phospholipid redistribution was not due to a perturbation of the membrane caused by rhodamine B, since the GS 150-169 peptide, without rhodamine B, induced redistribution of NBD-PC and PS analogues in IOVs. The effect was presumably mediated by the specific interaction of RhB-GS 160-169 with PIP_2 located in the inner leaflet of the membrane. This hypothesis was supported by the results obtained in LUVs, in which the presence of PIP_2 was required to allow the effect of the peptide on NBD-PC or PS analogue redistribution.

Some amphipathic peptides have been reported to interact with phospholipids, to penetrate into the bilayer with a perpendicular orientation, and to induce pore formation in a dose-dependent manner. In parallel, these peptides promote a very fast phospholipid flip-flop with half-time in order of seconds (*46*, *47*). The activity of RhB-QRLFQVRKGRR on PS exposure and phospholipid scrambling is unlikely to be induced by pore formation. Pore formation would be expected to allow leakage of small molecules from lipid vesicles (*46*), whereas the peptide, at concentrations below 20 μM, did not induce membrane leakage in platelets (no increase in $[Ca^{2+}]_i$) or in LUVs (reduction of NBD signal by dithionite remained limited to the fraction of the probe in the external leaflet). Some other peptides can induce phospholipid scrambling at a lower rate, more consistent with that observed in the present study. In general, peptides adopt an α helical structure favored by the presence of acidic phospholipids and required for the interaction with the membranes. The peptide could adopt an α helical structure where PIP_2 is present in the membrane (*48*, *49*) accounting for the interaction of RhB-QRLFQVRKGRR with the membrane and resulting in phospholipid redistribution.

The role of the cytoskeleton in phospholipid redistribution in platelets is controversial. Although proteolysis of cytoskeletal proteins by calpain and PS exposure were both induced by effectors such as Ca^{2+}/ionophore or agonists (thrombin and collagen) (*50*, *51*), the inhibition of calpain by calpeptin did not prevent phospholipid redistribution, but inhibited vesicle shedding (*52*, *53*). Other experiments also demonstrated that vesicle shedding, but not PS exposure, depends on calpain activation or cytoskeleton reorganization, when tested with cytoskeleton-disrupting agents such as nocodazole or cytochalasin D (*54*). In contrast to the latter data, it has been recently reported that cytochalasin D inhibited PS exposure induced by Ca^{2+}/ionophore in human erythroleukemia cells (*55*). Gelsolin-derived peptides (GS 150-169 or GS 160-169) have been shown to bind to newly synthesized PIP_2 and to result in inhibition of actin polymerization induced by agonists (thrombin or TRAF) in permeabilized platelets, when added at a concentration higher than 17 μM (*43*). Our data show that the RhB 160-169 peptide, at concentrations of 20 μM, also inhibited actin polymerization induced by thrombin in whole platelets (Figure 3). However, the addition of both thrombin or TRAF and the peptide (20 μM) had only an additive effect on PS exposure, suggesting that, in platelets, actin polymerization and PS exposure are unrelated events.

A drastic, but not physiological, increase in Ca^{2+} concentration induced by the ionophore A23187, expected to produce a maximal PS exposure, resulted in a 15-fold elevation in a FITC-annexin V binding. The increase in PS exposure induced by RhB-QRLFQVKGRR was about 4-fold, similar to that observed with physiological agonists (thrombin, collagen) or with TRAF. The limited PS redistribution with physiological agonists is presumably attributable to a transient increase in the $[Ca^{2+}]_i$, as compared to the sustained elevation induced by the ionophore (Figure 2). However, PS exposure induced by the peptide was not inhibited when intracellular Ca^{2+} concentration was clamped with BAPTA, in contrast to the effect induced by the agonists. We suggest that the peptide might replace Ca^{2+} in the interaction with PIP_2 to induce PS exposure. This possibility is supported by the data obtained in LUVs containing PIP_2 or in IOVs, in which either Ca^{2+} (*22*, *27*) or the peptide (present data) induced phospholipid scrambling.

Platelet activation with agonists leads not only to changes in the levels of PI(4)P and $PI(4,5)P_2$, but also to PI 3-kinase activation, generating D3 phosphoinositides involved in some platelet responses (*45*, *56*). We found that PS exposure, induced by thrombin or collagen, was inhibited by wortmannin. The peptide RhB-QRLFQVKGRR was also reported to activate PI 3-kinase (*39*, *48*). However, PS exposure induced by the peptide was unaffected by wortmannin, ruling out a requirement for D3 phosphoinositides in this process. The involvement of PIP_2 was further confirmed by the observed inhibition of peptide-induced PS exposure by quercetin, an inhibitor of PIP and PIP_2 synthesis, previously used to investigate the role of PIP_2 in various processes (*40*−*42*). As quercetin also inhibited PS exposure induced by thrombin or collagen, PIP_2 would be also implicated in this process during physiological activation of platelets.

In erythrocytes, a special pool of PIP_2, insensitive to PLC, was required for Ca^{2+}-induced phospholipid scrambling (*22*). In a similar way, in activated platelets, two distinct pools of

PIP_2 would play different functional roles; in the first seconds of activation, the first one is a substrate for PLC, leading to IP_3 formation and Ca^{2+}-store release, and, subsequently, a second pool, generated by newly synthesized PIP_2, increases PIP_2 levels by 40% over the resting baseline (*43*) and could serve as a direct target for Ca^{2+} to contribute to PS exposure. We have previously suggested that the formation of lateral domains, enriched in PIP_2 and induced by PIP_2 ligands including polycations, MARCKS peptide, PIP_2 antibody, and Ca^{2+}, could result in phospholipid scrambling in erythrocyte membranes. As there is some evidence that the RhB-QRLFQVRKGRR peptide could induce formation of PIP_2 domains in lipid monolayers (*57*), we propose that, as in erythrocytes (*24*), formation of PIP_2 domains could participate in PS exposure in intact platelets.

By using the gelsolin-derived peptide, we show for the first time that, in intact cells, PIP_2 is involved in phospholipid scrambling, as it is in erythrocyte membranes. A protein of the gelsolin family containing QRLFQVKGRR in its sequence and playing a physiological role in the induction of scrambling would deserve to be investigated.

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