Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen

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Phosphatidylserine exposure and microvesicle release give rise to procoagulant activity during platelet activation. We have previously shown that whereas the Ca^{2+} ionophore A23187 and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone, a Ca^{2+} -ATPase inhibitor, induce phosphatidylserine exposure, only the former triggers microvesicle release. We now report that microvesicle formation with ionophore A23187 is specifically associated with μ -calpain activation, increased protein tyrosine phosphatase (PTP) activity and decreased tyrosine phosphorylation. The degree to which calpain and individual PTPs were activated in response to A23187 depended on the extent of bivalent cation chelation in the external medium. EGTA (2 mM) blocked or severely retarded their activation, and addition of extracellular Ca^{2+} in excess (2 mM) resulted in virtually immediate tyrosine dephosphorylation. Dephosphorylation was correlated with an increase in total PTP activity in platelet lysates. In platelets stimulated by a

combination of thrombin and collagen, only the subpopulation undergoing microvesicle release and isolated by their binding to annexin-V-coated magnetic beads exhibited protein tyrosine dephosphorylation. Detection of PTP activity in an 'in-gel' assay showed the Ca^{2+} -dependent appearance of active low-molecular-mass bands at 38, 36 and 27 kDa. Individual PTPs varied in their protease sensitivity to changes in intracellular Ca^{2+} levels. For example, PTP1B was a more sensitive substrate than SH2-domain-containing tyrosine phosphatase-1 for μ -calpain cleavage. Incubation of platelets with the PTP inhibitors, phenylarsine oxide and benzylphosphonic acid acetoxymethyl ester, led to increased tyrosine phosphorylation and the surface expression of aminophospholipids but little microvesicle formation. Furthermore, microvesicle release in response to ionophore A23187 was inhibited. We conclude that platelet microvesicle formation is associated with extensive protein tyrosine dephosphorylation.

INTRODUCTION

Blood platelets effect a range of haemostatic responses including adhesion, granule secretion, aggregation, and the expression of procoagulant activity through phosphatidylserine (PS) exposure and microvesicle formation (for a review see ref. [1]). Both the development of procoagulant activity and microvesicle formation are considered to be late platelet responses [2]. It is now well accepted that PS exposure is linked to the inhibition of aminophospholipid translocase and/or to the activation of a scramblase [2,3]. The scramblase enzyme in blood cell membranes has recently been purified and cloned [4]. PS exposure is often but not always followed by microvesicle formation [5,6]. Both processes are induced by the Ca^{2+} ionophore A23187, which is a powerful inducer, followed in potency by Ca^{2+} -ATPase inhibitors such as thapsigargin, the complement membrane attack complex C5b-9 and the physiological agonists of platelets, thrombin and collagen. The intracellular Ca^{2+} threshold required for microvesicle formation is greater than for PS exposure, and the inhibition of microvesicle production by membrane-permeant inhibitors of μ -calpain, a Ca^{2+} -dependent thiol protease involved in cytoskeletal reorganization and the regulation of enzyme activities, is well established [7–9]. Nevertheless, much remains to be understood about the complex signalling pathways leading to PS exposure and microvesicle release.

Protein tyrosine phosphorylation is important for signal transduction in platelets [10–12]. Agonist-induced increases in tyrosine phosphorylation are mediated by intracellular protein tyrosine kinases (PTKs) such as members of the Src kinase family (Src, Fyn, Lyn, Hck and c-Yes), Syk/ZAP 70 and Janus kinase families (reviewed in refs. [10,13]). Under resting conditions, levels of tyrosine phosphorylated protein are low in platelets, suggesting that protein tyrosine phosphatases (PTPs) prevent their accumulation [13]. PTPs identified in platelets include PTP1B, SH2-domain-containing tyrosine phosphatases-1 and -2 (SHP-1 and SHP-2) and PTPH1, a PTP with structural similarities to band 4.1 [13–15]. PTP1B is translocated from the endoplasmic reticulum to the cytosol in thrombin-stimulated platelets [16]. Both PTP1B and its μ -calpain-cleaved 42 kDa form associate with the cytoskeleton in an aggregation-dependent manner. SHP-1 and SHP-2 both contain SH2 domains, permitting their association with tyrosine-phosphorylated proteins in the cytoskeleton of activated platelets [17]. SHP-1, which is tyrosine phosphorylated in response to thrombin via a pertussis-toxin-sensitive G_i protein [18], has been reported to interact with Src [19]. SHP-2, which has a wide tissue distribution, was recently described as associating with the membrane glycoprotein, PECAM-1, in aggregating platelets [20]. PTPH1 (also called PTPMEG), identified in cloning experiments using megakaryocyte cDNA [15], is also a known μ -calpain substrate [21]. Other

Abbreviations used: PS, phosphatidylserine; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SHP, SH2-domain-containing tyrosine phosphatase; PAO, phenylarsine oxide; tBuBHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone; mAb, monoclonal antibody; BPA, benzylphosphonic acid acetoxymethyl ester.

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PTPs may also be present, for at least seven bands with PTP activity were detected in an 'in-gel' assay performed on platelet proteins, although the mechanism by which non-receptor PTPs participate in signal transduction is still incompletely understood [22,23].

As PTK inhibition is known to dampen down other platelet responses [24,25], we have investigated the role of tyrosine phosphorylation and dephosphorylation in PS exposure and microvesicle formation. The relevance of μ -calpain activation in the regulation of these events was also studied. Platelets that have exposed PS and shed microvesicles in response to the Ca^{2+} ionophore or to a more physiological stimulation (thrombin and collagen) were seen to have a much lower content of tyrosine-phosphorylated proteins. Dephosphorylation, which correlated with an increase in PTP activity, was dependent on a large increase in cytosolic Ca^{2+} . Overall, our results suggest a role for PTPs in microvesicle release.

EXPERIMENTAL

Reagents

Prostaglandin E_1 , BSA (fatty acid-free), DMSO, apyrase (grade I), *p*-nitrophenyl phosphate, phenylarsine oxide (PAO) and Na_3VO_4 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The Ca^{2+} ionophore A23187 and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (tubHQ) were from Calbiochem (La Jolla, CA, U.S.A.). Reinforced nitrocellulose membrane was from Amersham (Les Ulis, France). The murine monoclonal antibody (mAb) B_{27}D_8 , against the catalytic subunit of μ -calpain, was provided by Dr. T. Kunicki (Scripps Research Institute, La Jolla, CA, U.S.A.). An affinity-purified goat antibody (N-19) raised against the N-terminus of PTP1B was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). PY20, a murine mAb recognizing phosphotyrosyl proteins, was purchased from Transduction Laboratories (Lexington, KY, U.S.A.) as was the mAb (P17320) specific for the C-terminal domain of SHP-1. Peroxidase-labelled affinity-purified secondary antibodies specific for the Fc region of goat and mouse IgG were from Jackson ImmunoResearch (West Grove, PA, U.S.A.). MicroBCA reagents were from Pierce-Interchim (Montluçon, France). Benzyl-phosphonic acid acetoxymethyl ester (BPA) was from Tebu (Le Perray-en-Yvelines, France). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Isotopchim (Ganagobie-Peyruis, France). Dynabeads M-280 were obtained from Dynal (Oslo, Norway). Recombinant annexin V was a gift from Bender-Co (Vienna, Austria). Ionophore A23187, tubHQ, PAO and BPA were dissolved in DMSO in a stock solution such that the final concentration of DMSO in the platelet suspension did not exceed 1.0 %.

Platelet isolation and induction of microvesicle release

Blood was obtained from control subjects by venepuncture using acid/citrate/dextrose formula A as anticoagulant [26]. Platelet-rich plasma was prepared by centrifugation at 120 *g* for 10 min at room temperature; 9 vol. was acidified by further addition of acid/citrate/dextrose formula A (1 vol.), supplemented with 100 nM prostaglandin E_1 and 25 $\mu\text{g}/\text{ml}$ apyrase and centrifuged at 1200 *g* for 15 min. Platelets were washed three times in washing buffer, pH 6.5, containing 36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 100 mM NaCl, 100 nM prostaglandin E_1 and 25 $\mu\text{g}/\text{ml}$ apyrase. The platelets were resuspended at $(1\text{--}5) \times 10^8/\text{ml}$ in Tyrode's buffer, pH 7.4, containing 137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO_4 , 1 mM MgCl_2 , 5 mM Hepes, 12 mM NaHCO_3 and 5 mM glucose (reaction buffer). Incubations were performed at 37 °C without

stirring after the initial mixing. Depending on the experiment, the resuspension buffer contained 2 mM CaCl_2 , 0.1 mM EGTA or 2 mM EGTA as indicated in the text.

Isolation of procoagulant platelets and microvesicles

Platelet suspensions ($10^8/\text{ml}$) were incubated with 100 μl of annexin-V-coated beads for 5 min in the presence of 2 mM Ca^{2+} as described [7]. Beads were collected by magnetic attraction (Dynal MPC Magnetic Collector) and the supernatant discarded. The beads were gently resuspended in the presence of 2 mM EGTA for 2 min to allow the dissociation of the procoagulant platelets and microparticles. Magnetic separation of the beads allowed the recovery of the supernatant containing the platelets and microparticles with exposed aminophospholipids. Aliquots were labelled with annexin V-FITC after the addition of 2 mM Ca^{2+} (in excess of the EGTA) for flow cytometric analysis or solubilized directly for SDS/PAGE (see below). Only a small number of annexin V-FITC-positive cells were isolated from unstimulated platelet suspensions showing that incubation with the beads does not activate platelets.

Flow cytometry

Recombinant annexin V was labelled with FITC as described [5,26]. In the standard assay, samples (5×10^6 platelets in reaction buffer containing 2 mM CaCl_2) were incubated with annexin V-FITC (150 nM) for 10 min at room temperature [5]. After a fivefold dilution in reaction buffer containing 2 mM CaCl_2 , samples were analysed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France). The light scatter and fluorescence signals were set in logarithmic gain. Fluorescence emission was monitored using a 530/30 band pass filter for FITC. Gating was performed as already described and 10000 particles were acquired from each sample [5,26]. Briefly, the forward light scatter histogram of ionophore A23187-stimulated platelets shows two populations: a major one corresponding to activated platelets (panel R2) and a second one, smaller in size, which corresponds to microvesicles (panel R1) and which is only present after stimulation (see Figure 1). Although there is some overlap, the gate designated to delimit the microvesicles distinguishes elements that fail to sediment when the activated platelet suspension is centrifuged at 2000 *g* for 10 min [27].

Tyrosine phosphatase activity

Unstimulated or activated platelet suspensions from five donors were lysed by sonication in reaction buffer containing 2 mM EGTA, 1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin. The homogenate was centrifuged for 5 min at 10000 *g* to remove intact cells and large particles. Protein concentration was determined using the microBCA kit according to the manufacturer's instructions. Phosphatase activity was measured spectrophotometrically at 37 °C using 5 mM *p*-nitrophenyl phosphate as substrate in 10 mM Mes buffer, pH 5.5. EDTA (1 mM) was added to the reference cuvette, together with 1 mM Na_3VO_4 and 1 mM *N*-ethylmaleimide to establish the baseline. The appearance of the cleaved product was monitored at 410 nm with $\epsilon = 1.78 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Total platelet PTP activity was measured in the presence of 0.5 % (v/v) Triton X-100.

Tyrosine phosphatase activity assessed by an 'in-gel' assay

SDS-soluble platelet proteins (25 μg) prepared as described below

were loaded on to SDS/polyacrylamide gels [8 or 10% (w/v) polyacrylamide]. For the detection of PTP activity, $^{33}\text{PO}_4$ -labelled poly(Glu/Tyr) was preincorporated into the separating gel at 10^5 c.p.m./ml by the procedure of Burridge and Nelson [22]. All incubations were at room temperature. The gels were first incubated overnight in 50 mM Tris/HCl, pH 8.0, containing 20% propan-2-ol to remove the SDS. They were then washed twice for 1 h in 50 mM Tris/HCl, pH 8.0, containing 0.3% (v/v) 2-mercaptoethanol followed by 90 min in the same buffer containing 6 M guanidinium chloride and 1 mM EDTA. The gels were then washed three times for 1 h in renaturation buffer [1 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.3% (v/v) 2-mercaptoethanol, 0.04% (v/v) Tween 40]. Finally, the gels were incubated for 6 h in renaturation buffer containing 4 mM dithiothreitol. They were then stained with Coomassie Brilliant Blue and destained before being dried for autoradiography. As a control, duplicate gels were analysed in which the renaturation step was performed in the presence of 5 mM Na_3VO_4 . Gels were exposed to autoradiographic film. Photographic records present the negative image of the autoradiograph, PTP activity being shown as dark bands.

SDS/PAGE and immunoblotting

Platelets were solubilized in SDS buffer and heated at 100 °C for 5 min. On occasion, disulphides were reduced by heating for a further 5 min at 100 °C in the presence of 10 mM dithiothreitol. Samples (50 µg of protein) were analysed on 7–12% polyacrylamide gradient gels and proteins were visualized by staining with Coomassie-R Brilliant Blue [26]. For immunoblotting, proteins were electrophoretically transferred to reinforced nitrocellulose membrane during 1.5 h at 24 V using a Bio-Rad semidry transfer apparatus (Bio-Rad SA, Ivry-sur-Seine, France) as previously described by us [7]. When we studied high-molecular-mass proteins, transfer was for 2.5 h at 20 V. After saturation of non-specific protein-binding sites, membranes were incubated for 1 h with primary mAb: B27D8 (ascites fluid, dilution 1:10000), PY20 (1 µg/ml), anti-SHP-1 (0.2 µg/ml) or anti-PTP1B (0.2 µg/ml). On occasion, the two anti-PTP antibodies were added together. After five washes, nitrocellulose membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (dilution 1:10000) as required. Bound IgG was revealed by the enhanced chemiluminescence kit from Amersham with exposure to Hyperfilm TM (Amersham). When required, blots were stripped of bound antibody by incubating in 10 mM Tris buffer containing 2% SDS and 100 mM 2-mercaptoethanol at 70 °C for 40 min. Membranes were washed and probed with a different primary antibody.

Effect of platelet PTP inhibitors on microvesicle formation

Washed platelets (5×10^8 /ml) in reaction buffer containing 2 mM CaCl_2 were preincubated with vehicle alone (DMSO), with 1 µM PAO [28] for 10 min, or with 5 mM BPA [29] for 15 min. They were then diluted to 10^8 /ml and further incubated with or without 3 µM ionophore for 10 min at 37 °C. Aliquots were incubated with annexin V-FITC, diluted fivefold and analysed by flow cytometry as described above. The concentrations of PAO and BPA and the lengths of the incubations were determined in preliminary experiments, and were selected so as to obtain about 50% of the platelets labelled with annexin V-FITC in flow cytometry before the addition of the ionophore (see the Results section).

Statistical analysis

Results are expressed as mean $\pm$ S.D. Student's paired *t* test was used and differences were considered significant when $P < 0.01$.

RESULTS

Changes in protein tyrosine phosphorylation and evidence for PTP activation in platelets that have shed microvesicles

Incubation of platelets with 3 µM ionophore A23187 in the presence of 2 mM extracellular Ca^{2+} led to PS exposure and microvesicle formation. Both processes were monitored through the use of annexin V-FITC, the binding of which resulted in a shift to the right of the fluorescence (Figure 1A, panels 1 and 2). Incubation with the Ca^{2+} -ATPase inhibitor, tbuBHQ, led to PS exposure but little microvesicle formation (Figure 1A, panel 3) in confirmation of previous results from our laboratory [5]. Tyrosine phosphorylation was analysed on parallel samples by immunoblotting using the anti-phosphotyrosine antibody, PY20. Unstimulated platelets gave a major band at 60 kDa, corresponding to the constitutive phosphorylation of Src, and other minor bands (Figure 1B, lane 1). Levels were extremely low after platelets had been incubated with ionophore A23187 for 10 min, and only bands of 190 and 55–60 kDa were present (Figure 1B, lane 2). A band at 42 kDa was given by the second antibody alone and represents non-specific binding. In contrast, platelets incubated with tbuBHQ for 10 min showed increased tyrosine phosphorylation of several proteins (Figure 1B, lane 3). PTP activity was analysed in the 'in-gel' assay after renaturation. Results for unstimulated platelets revealed activities at 110, 68 and 53 kDa as well as in the 40–45 kDa range (Figure 1C, lane 1). A weak band at 90 kDa was also present. It should be underlined that the photographic records present the negative image of the autoradiograph, PTP activity being shown as dark bands. Platelet stimulation by the Ca^{2+} ionophore A23187 was accompanied by the loss of the activity at 110 kDa and the appearance of a major band at 42 kDa along with low-molecular-mass bands at 38, 36 and 27 kDa (Figure 1C, lane 2). In contrast, tbuBHQ-stimulated platelets showed none of these changes (Figure 1C, lane 3).

We next studied the time course of changes in tyrosine phosphorylation induced by the ionophore A23187. The role of Ca^{2+} mobilization was assessed in parallel by performing the studies on platelets resuspended in the presence of 0.1 mM or 2 mM EGTA. The extracellular medium was made 2 mM with respect to Ca^{2+} after 10 min. Samples were taken at different intervals for SDS/PAGE and proteins analysed for tyrosine phosphorylation by immunoblotting with PY20. Typical results are illustrated in Figure 2. In the presence of a low external concentration of EGTA, an initial increase in tyrosine protein phosphorylation preceded the dephosphorylation (Figure 2A). The tyrosine phosphorylation peaked at 2 min and dephosphorylation was complete at 5 min. In contrast, in the presence of 2 mM EGTA, dephosphorylation did not begin until after 5 min, yet it occurred rapidly after the addition of Ca^{2+} (Figure 2B). It should be noted that the dephosphorylation of individual proteins did not always follow the same kinetics and that extensive dephosphorylation was accompanied by the appearance of a 190 kDa band.

Isolation of the procoagulant subpopulation after stimulation of platelets with a thrombin and collagen combination

We next investigated whether protein tyrosine dephosphorylation accompanied microvesicle production in response to a more physiological stimulation. Figure 3 shows results from a typical

Figure 1 Microvesicle formation is associated with increased PTP activity in A23187-treated platelets

Platelets in reaction buffer containing 2 mM Ca^{2+} were incubated for 10 min at 37 °C with buffer alone (1), 3 μM ionophore (2) or 100 μM tbuBHQ (3). **(A)** Analysis of PS exposure and microvesicle release by flow cytometry. Illustrated are dot-plots (forward scatter versus fluorescence) of samples incubated with FITC-annexin V (see the Experimental section). **(B)** Detection of tyrosine-phosphorylated proteins by Western blotting. Samples were solubilized in SDS buffer and aliquots electrophoresed on 7–12% gradient gels before protein transfer to nitrocellulose membrane. Anti-phosphotyrosine immunoblots are shown. The migration of molecular-mass markers is indicated on the left. **(C)** Changes in PTP activity revealed in an 'in-gel' assay. SDS-soluble extracts were electrophoresed on a 10% gel containing $^{33}\text{PO}_4$ -labelled poly(Glu/Tyr)-labelled peptide. Proteins were renatured and PTP activity revealed as described in the Experimental section. The apparent molecular masses of the major bands are shown; small hydrolytic products are also highlighted (arrows on the right). The illustrated results are representative of five experiments.

experiment in which platelets in Ca^{2+} -containing buffer were stimulated with a thrombin and collagen mixture. As shown in Figure 3(A) (panel 1), only a subpopulation of platelets had maximally expressed PS and bound annexin V. The subpopulation of procoagulant platelets and isolated microparticles were separated using annexin-V-coated beads. Figure 3(A) (panel 2) confirms that the isolated subpopulation was composed of both procoagulant platelets and microvesicles. In comparison with resting conditions (Figure 1B, lane 1; Figures 2A and 2B, time 0), protein tyrosine phosphorylation levels had increased markedly when samples of the total activated platelet suspension were analysed (Figure 3B, lane 1). Tyrosine-phosphorylated proteins of molecular mass 240, 190, 125, 110–115, 95, 75–80, 55–60 and 42 kDa were seen. In contrast, tyrosine-phosphorylated proteins had virtually disappeared from the isolated procoagulant subpopulation, and the band at 190 kDa was now dominant (Figure 3B, lane 2). The PTP pattern of the isolated subpopulation as visualized in the 'in-gel' assay (Figure 3C, lane d) was quite similar to that seen for Ca^{2+} -ionophore-stimulated platelets of the same donor (Figure 3C, lane b). Although the unfractionated thrombin-plus-collagen-stimulated platelet suspension showed each of the major bands (Figure 3C, lane c),

lower-molecular-mass bands were more readily detected than in the unstimulated platelet suspension (Figure 3C, lane a), a result compatible with the presence of a subpopulation of procoagulant platelets and microvesicles.

Total tyrosine phosphatase activity in platelets

The diminished content of tyrosine-phosphorylated proteins in both A23187-stimulated platelets and the procoagulant subpopulation of thrombin-plus-collagen-stimulated platelets suggested a stimulus-dependent increase in PTP activity. To attempt to prove this hypothesis, we measured PTP activity in platelet lysates. Whereas the basal PTP activity of unstimulated platelets was 91 ± 17 pmol/mg per min (mean $\pm$ S.D., $n = 5$), that in homogenates prepared from A23187-stimulated platelets from the same donors was 191 ± 44 pmol/mg per min ($P < 0.01$). The phosphatase activity in a homogenate of thrombin-and-collagen-stimulated platelets was 88 ± 26 pmol/mg per min ($n = 5$) and indistinguishable from that of unstimulated platelets, a finding similar to that already reported by others [30]. In contrast, the PTP activity in homogenates prepared from the isolated subpopulation of procoagulant platelets for the same donors was

Figure 2 Time course of changes in tyrosine protein phosphorylation during the incubation of platelets with the ionophore A23187

Washed platelets were resuspended in reaction buffer containing 0.1 mM (A) or 2 mM (B) EGTA and incubated with 3 μ M A23187 as indicated. At 10 min, Ca^{2+} was added to the suspension buffer to a final concentration of 2 mM and the incubation continued. Samples were removed at the selected times and the platelets solubilized for SDS/PAGE followed by Western blotting with mAb PY20. The relative migration of molecular-mass standards is indicated on the left. Results are representative of those obtained in five experiments.

172 ± 48 pmol/mg per min ($P < 0.01$). Significantly, platelet stimulation by 100 μ M tbuBHQ had no effect on the basal PTP activity (80 ± 35 pmol/mg per min, $n = 5$). These results correlate microvesicle formation with an increase in PTP activity.

Calpain autolysis, tyrosine phosphatase activation and protein tyrosine dephosphorylation

Tyrosine dephosphorylation during platelet activation has been previously linked in platelets to μ -calpain activation and proteolysis [16,31]. We therefore used immunoblotting to investigate time-dependent changes in (i) μ -calpain activation and (ii) the cleavage of PTP1B and SHP-1 during the incubation of platelets with ionophore A23187. Results are shown for platelets resuspended in the presence of 0.1 mM EGTA. PTP1B and SHP-1 were detected at 53 and 68 kDa respectively when unstimulated

Figure 3 Microvesicle formation, protein tyrosine dephosphorylation and PTP activation occur in a specific subpopulation of platelets in response to a mixture of thrombin and collagen

Washed platelets were incubated with a mixture of 10 nM thrombin and 10 μ g/ml collagen for 10 min at 37 $^{\circ}\text{C}$ in the presence of 2 mM Ca^{2+} . Then an aliquot was labelled with annexin V-FITC and analysed by flow cytometry (A, panel 1). A second aliquot of the platelet suspension was incubated with annexin-V-coated magnetic beads as described in the Experimental section. After separation from the non-adherent fraction and their release from the beads, the isolated subpopulation was analysed by flow cytometry after a further 10 min incubation with free annexin V-FITC (A, panel 2). Samples identical with the above were also taken for Western-blot analysis with the anti-phosphotyrosine mAb, PY20 (B) and for the detection of PTP activity in the 'in-gel assay' (C). In (B) lanes 1 and 2 correspond to the total activated platelet suspension and to the isolated subpopulation respectively. In (C), lanes c and d correspond to the total activated platelet suspension and the isolated subpopulation. Lanes a and b correspond to unstimulated platelets from the same donor and platelets activated with ionophore A23187 analysed in parallel. Similar results were obtained in five different experiments.

platelets were analysed. PTP1B continued to be detected as a single band at 53 kDa until 3 min after the addition of the A23187, when a second band appeared at 42 kDa. After 4 min, only the band at 42 kDa was present. SHP-1 was detected at 68 kDa until 10 min, after which bands of slightly lower molecular mass were observed. These bands were clearly present 1 min after the addition of 2 mM Ca^{2+} to the extracellular medium, whereas at 5 min after the addition of the Ca^{2+} little reactivity was detected using mAbs to either PTP1B or SHP-1 (Figure 4A). The identity of the bands and the origin of the derived degradation products were confirmed using the antibodies individually.

For the same samples, autolysis of μ -calpain, detected by the presence of the active hydrolytic product at 78 kDa, was observed between 3 and 4 min after the addition of the ionophore (Figure 4B). The 78 kDa band was strong at 10 min, and was the only

Figure 4 Time-dependent changes in PTP1B, SHP-1 and μ -calpain hydrolysis during the incubation of platelets with ionophore A23187

Samples identical with those described in Figure 2(A) were probed with mAbs specific for PTP1B and SHP-1 by Western blotting (A). Arrows identify SHP-1 and PTP1B. μ -Calpain was detected using the mAb, B27D8 (B). Both the native (85 kDa) and the fully autolysed form (78 kDa) of the catalytic subunit are recognized. The results are typical of five different experiments.

band seen after the addition of 2 mM Ca^{2+} to the extracellular medium which leads to a strong Ca^{2+} influx [7]. These results suggest that PTP1B is more sensitive (or more accessible) to cleavage by autolysed μ -calpain than SHP-1 during platelet stimulation by the ionophore A23187. When experiments were performed with 2 mM EGTA in the extracellular medium (as in Figure 2B), μ -calpain autolysis was detected only after the addition of extracellular Ca^{2+} , and cleavage of both PTP1B and SHP-1 and a strong tyrosine dephosphorylation of all proteins occurred concomitantly (results not illustrated).

Time-dependent changes in PTP hydrolysis during platelet stimulation with the ionophore A23187

Again we analysed platelets activated with the ionophore in buffer containing 0.1 mM EGTA. The 110 and 53 kDa bands showed significant decreases in intensity within 3 to 4 min and their decrease was accompanied by the appearance of bands at 42, 58 and 90 kDa (Figure 5A). The 68 kDa band was much more resistant to degradation. The changes associated with the 53 and 68 kDa bands paralleled the kinetics of μ -calpain-linked proteolysis of PTP1B and SHP-1 (see above), suggesting that they represented the same proteins. Purified PTP1B and SHP-1 have previously been shown to appear as active bands in the 'in-gel' assay [22]. The 110 kDa band was also considerably reduced after incubation for 4–5 min with the ionophore but remains to be clearly identified. Interestingly, the first low-molecular-mass product of about 42 kDa was seen after 1 min, suggesting that it was formed with different kinetics from those of the hydrolysis of PTP1B by autolysed μ -calpain.

When platelets were resuspended in buffer containing 2 mM EGTA, PTP1B activity (at 43 kDa) was much less intense (Figure 5B, time 0) and the reason for this is unknown. Platelet activation with 3 μM A23187 resulted in new bands at 42 and 90 kDa, although reductions in the activity of the 110 kDa or other bands

Figure 5 Time course of PTP hydrolysis during Ca^{2+} -ionophore stimulation as determined in the 'in-gel' assay

Experiments were performed using platelets suspended in reaction buffer containing 0.1 mM (A) or 2 mM (B) EGTA as described for Figure 2. At each time interval, an aliquot was solubilized by heating in SDS buffer. Aliquots were loaded on 8% gels containing $^{33}\text{PO}_4$ -labelled poly(Glu/Tyr)-labelled peptide. After electrophoresis, proteins were renatured and the dried gels exposed to X-ray film. Calculated molecular masses of proteins with PTP activity are indicated on the left. Arrows on the right indicate hydrolytic products. Similar results were obtained in three different experiments.

were not apparent during the 10 min incubation. In contrast, addition of 2 mM Ca^{2+} was followed by the rapid disappearance of the activities at 110 and 90 kDa, and the 68 kDa band (SHP-1) decreased in intensity. The low-molecular-mass bands were only strongly present after the addition of the extracellular Ca^{2+} . The experiments shown in Figure 5 reflect the extent to which the PTP profile was sensitive to changes in cytosolic Ca^{2+} .

Inhibition of platelet PTPs was associated with a lack of microvesicle formation

As platelet PTPs appear to exert a positive role in microvesiculation, we studied the effects on platelets of two compounds,

Figure 6 Effect of platelet PTP inhibition on microvesicle formation

Platelets suspended in reaction buffer containing 2 mM CaCl_2 were preincubated with vehicle alone (panels 1 and 4), with 1 μM PAO for 10 min (panels 2 and 5) and with 5 mM BPA for 15 min (panels 3 and 6). Platelets were then further incubated alone (panels 1, 2 and 3) or with 3 μM ionophore for 10 min at 37 °C (panels 4, 5 and 6). Aliquots taken as indicated were incubated with annexin V-FITC, diluted and analysed by flow cytometry as described in the Experimental section. Similar results were obtained in three different experiments.

PAO and BPA, with recognized PTP-inhibitory activity. Incubation of platelets with 1 μM PAO at 37 °C for 10 min was followed by procoagulant phospholipid exposure but without significant microvesicle release as seen by flow cytometry using annexin V-FITC (Figure 6, panel 2). Subsequent stimulation of the platelets by 3 μM ionophore A23187 also resulted in little microvesicle release (panel 5). BPA (5 mM) also induced procoagulant phospholipid exposure (Figure 6, panel 3) with little or no microvesicle formation. Consistent with the finding for PAO, addition of the ionophore A23187 again did not correct the lack of microvesicle release (panel 6). Nevertheless, the ionophore A23187 resulted in total exposure of the aminophospholipids on PAO- and BPA-stimulated platelets. Overall, these results support an involvement of one or more PTPs in the final stages of the pathway leading to microvesicle formation.

DISCUSSION

Our results underline that regulation of the protein tyrosine phosphorylation balance plays a crucial role in platelet function. The importance of aminophospholipid translocation and microvesicle release from blood cells is emphasized by the role of (i) procoagulant activity in thrombosis and (ii) PS expression in apoptosis [32,33]. Information on the intracellular control of PS movements and microvesicle formation can therefore have considerable therapeutic implications. The present work was undertaken to study the effect of protein tyrosine phosphorylation on platelet PS exposure and microvesicle release. Annexin V was used as a probe for PS expression.

The ability of platelets to rapidly expose PS, shed microvesicles and undergo protein tyrosine dephosphorylation when challenged with the ionophore A23187 is already known [16,34]. Using platelets suspended in the presence of 0.1 or 2 mM EGTA,

we showed that the ionophore initially stimulated tyrosine protein phosphorylation. However, once intracellular Ca^{2+} levels had increased sufficiently, tyrosine protein dephosphorylation predominated. When extracellular Ca^{2+} was present, the dephosphorylation was very rapid and tyrosine phosphorylation levels were quickly lower than in unstimulated platelets. Even the constitutive tyrosine phosphorylation of Src (64 kDa) had disappeared. At the same time, an unidentified tyrosine-phosphorylated protein at 190 kDa appeared and its presence was concomitant with the autolysis of μ -calpain as indicated by Western blotting. It appears to be a PTP-resistant protein. The identity of the 190 kDa protein and its possible relationship to platelet cytoskeletal protein substrates for calpain such as talin or actin-binding protein [6] are under investigation.

Membrane vesiculation and PS exposure in response to a thrombin and collagen combination are restricted to a subpopulation of platelets [26]. The reason for this heterogeneity has not been determined, but may relate to the degree to which the intracellular Ca^{2+} concentration varies in individual platelets. We have demonstrated elsewhere that only platelets within the procoagulant subpopulation showed large increases in intracellular Ca^{2+} levels, as well as full μ -calpain activation, exposed PS and shed microvesicles [35]. Heemskerk et al. [36] have recently reported that substantial exposure of PS and microvesicle shedding occurred when platelets adhered to immobilized collagen. These authors also showed that PTK inhibitors decreased PS exposure induced by collagen, suggesting that PS exposure is associated with protein tyrosine phosphorylation.

Previous reports have shown little or no change in PTP activity in total lysates of platelets activated by thrombin, but considerably increased PTP activity in an isolated platelet cytoskeletal fraction [14,30]. We observed increased PTP activities in lysates of platelets stimulated with ionophore A23187 and in the

annexin V-reactive subpopulation of thrombin-and-collagen-activated platelets. However, the total population of thrombin-and-collagen-activated platelets showed little increase. Such results imply that in the latter case the procoagulant platelet subpopulation is in the minority. Nevertheless, our results demonstrate that those platelets that respond to thrombin and collagen through both PS exposure and microvesicle formation exhibit the same behaviour as A23187-stimulated platelets with regard to protein tyrosine dephosphorylation and PTP activity.

One or more PTPs become activated in platelets to account for the dephosphorylation. Newly formed low-molecular-mass bands as observed in the 'in-gel' assay for PTPs may participate in this process. The kinetics of their appearance, like those of the other responses, were highly dependent on the extent of Ca^{2+} chelation in the external medium, implying a key role for Ca^{2+} influx. Our previous studies showed that a 3–8 μM Ca^{2+} threshold level is required for μ -calpain autolysis [7]. The 53 and 68 kDa proteins recognized in Western blotting by the mAbs to PTP1B and SHP-1 showed different Ca^{2+} -sensitivities to hydrolysis. An increase in PTP1B activity has previously been described in Ca^{2+} -ionophore-stimulated platelets after μ -calpain cleavage, and this was linked with its relocalization from the endoplasmic reticulum to the platelet cytosol [16]. SHP-1, which was relatively insensitive to hydrolysis in our studies, is known to become more active when localized in the proximity of anionic phospholipids [37]. Cleavage of PTPH1 by calpain has also been reported to be accompanied by an increased PTP activity [21]. An equivalent 110 kDa protein was sensitive to μ -calpain in our study, suggesting that it may be the same protein. The dephosphorylation wave which began between 2 and 3 min for platelets resuspended in buffer containing 0.1 mM EGTA correlated well with PTP1B cleavage and the detection of a subpopulation of activated μ -calpain. Recently, an 'intermediate' autolysed form of μ -calpain has been reported to cleave PTP1B, although the fully autolysed enzyme was required for microvesicle release [38,39]. Possibly, μ -calpain can act on different steps of the pathway leading to procoagulant activity. Another calpain-dependent step could be the degradation of the cytoskeleton.

For SHP-1, the C-terminal domain which we have detected to be cleaved by μ -calpain (leading to the loss of reactivity with the anti-SHP-1 mAb in immunoblotting) has been shown to play a role in both its functional and ligand-binding properties [40]. Of the seven PTPs detected in the 'in-gel' assay, at least three appear to be modulated by μ -calpain proteolysis [16,21,41]. Nevertheless, we cannot rule out that a Ca^{2+} influx and/or mobilization can lead to the activation of other unidentified phosphatases, such as low-molecular mass or dual phosphatases, which can also be involved in the dephosphorylation of proteins containing phosphotyrosine [23,42]. Interestingly, PTPs may themselves be among the substrates of PTKs and/or serine/threonine kinases; for example, SHP-1 has a decreased activity after serine phosphorylation [43]. The involvement of serine/threonine phosphorylation in the control of platelet PTP activity is another pathway to consider in the regulation of PS exposure and microvesicle formation from platelets [44,45].

The Ca^{2+} -ATPase inhibitor, *thapsigargin*, induces PS translocation without microvesicle formation probably as a result of the low Ca^{2+} mobilization arising from the selective inhibition of SERCA-3 [46]. This result with *thapsigargin* suggests that PTP-dependent dephosphorylation requires a higher Ca^{2+} threshold than the induction of PTK activity. This was confirmed by the finding that PTP activity in homogenates of *thapsigargin*-stimulated platelets was similar to that in homogenates of untreated platelets. Fleming and co-workers [47] have obtained direct evidence that Ca^{2+} plays a role in triggering dephosphorylation via PTPs from

studies with PAO, a thiol-oxidizing compound. Using this compound and BPA, another PTP inhibitor [29], we observed procoagulant phospholipid exposure without significant microvesicle release. Furthermore, both compounds prevented microvesicle formation induced by ionophore A23187. As we have previously shown that the ionophore A23187 can induce membrane vesiculation when added after *thapsigargin* [7], and in this situation rapid dephosphorylation again occurs (results not shown), *thapsigargin* is presumably not acting as a PTP inhibitor. It may even be proposed that PTK activity favours amino-phospholipid exposure but that PTPs play an active role in microvesiculation. Thus controlling the protein tyrosine phosphorylation balance may be a way of modulating the expression of procoagulant activity of platelets. The presence of several tyrosine residues on the recently cloned 'scramblase' enzyme [48] and a reduced agonist-induced tyrosine phosphorylation in platelets of a patient with the Scott syndrome [49], an inherited disorder of the expression of procoagulant activity, are also in favour of this hypothesis.

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