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Analysis of GTP-Binding Proteins, Phosphoproteins, and Cytosolic Calcium in Functional Heterogeneous Human Blood Platelet Subpopulations

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ABSTRACT. The biochemical basis for the functional heterogeneity of human blood platelets was investigated in terms of protein phosphorylation, cytoplasmic calcium ($[Ca^{2+}]_i$), the ratio of 46 and 50 kDa vasodilator-stimulated protein (VASP), and GTP-binding proteins (G-proteins). Platelets were fractionated by density. Comparing resting low-density platelets (LDP) to high-density platelets (HDP) revealed higher phosphorylation of proteins in the 47, 31, and 24 kDa ranges. A higher phosphorylation of the 20 kDa protein in LDP compared to HDP was related to an enhanced $[Ca^{2+}]_i$, an increased ADP-ribosylation of the inhibitory G-protein ($G_{i\alpha 1-3}$) and rhoA, and a decreased ADP-ribosylation of the stimulatory G-protein ($G_{s\alpha}$). The differences in the ribosylation patterns of the subpopulations were not influenced by thrombin stimulation or exposure to prostaglandin E_1 (PGE_1). An 18 kDa phosphoprotein was more highly phosphorylated in resting HDP than in LDP. Thrombin exposure caused dephosphorylation of the 18 kDa phosphoprotein in the HDP, but generally increased phosphorylation of both HDP and LDP in the 47, 31, 24, and 20 kDa bands. Preincubation with prostaglandin E_1 or sodium nitroprusside diminished the subsequent thrombin-induced increase in phosphorylation, particularly in HDP. In unstimulated HDP, the 50 kDa VASP phospho form was enhanced, whereas in unstimulated LDP the 46 kDa VASP dephospho form was increased. Our findings suggest that the functional heterogeneity of platelets is partly derived from differences in signal transduction mechanisms reflected in varying phosphoprotein patterns and G-protein properties of platelet stimulatory and inhibitory pathways. BIOCHEM PHARMACOL **54**;9:1027–1035, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. platelet, density, functional heterogeneity, phosphoproteins, GTP-binding proteins, signal transduction

The functional heterogeneity of platelets and changes in their density distribution pattern are involved in pathophysiological events concerning the cardiovascular system [1–4]. In previous studies, we have shown that low-density platelets (LDP)‖ are more sensitive than high-density platelets (HDP) to low concentrations of platelet-activating agonists and less sensitive to aggregation-preventing agents [5, 6]. However, the biochemical basis for the functional heterogeneity of platelets in terms of signal transduction mechanisms is still unclear. We have described the modulatory effect of membrane fluidity on the regulation of the second messengers cyclic AMP (cAMP) and inositol phosphate after thrombin stimulation [7]. More recently, we reported that variations in adenylate cyclase, guanylate cyclase, and phosphodiesterases also contribute to the functional heterogeneity of platelets [6]. A balance between the stimulatory and inhibitory pathways is responsible for the overall cellular response. Little is known about how phosphoproteins regulate platelet activation and inhibition [8, 9]. An example of recent research links the association of the cytoskeleton with the Ca^{2+}-dependent phosphorylation of the 20-kDa myosin light chain (MLC) involved in platelet shape change, release reaction, and aggregation [10]. Thrombin, a potent platelet activator, increased the phosphorylation of several proteins [11–16]. Furthermore, thrombin induces the formation of inositol phosphates, the liberation of Ca^{2+}, granule secretion, and platelet aggregation [8, 17–19]. Inhibitory GTP-binding proteins (G_i-proteins) are involved in these processes [20–23]. Disturbance of the balance between these two signal

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‖ *Abbreviations:* $[Ca^{2+}]_i$, cytosolic calcium; ECL, enhanced chemiluminescence; fura-2-AM, 1-[-2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′methyl-phenoxy)-ethan-N,N,N′,N′-teraacidic acid penta acetoxy methylester; G-protein, GTP-binding protein; G_i-protein, inhibitory GTP-binding protein; G_s-protein, stimulatory GTP-binding protein; HDP, high-density platelets; IDP, intermediate-density platelets; LDP, low-density platelets; MLC, myosin light chain; PGE_1, prostaglandin E_1; PRP, platelet rich plasma; SNP, sodium nitroprusside; VASP, vasodilator-stimulated protein.

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transducing mechanisms in platelets may contribute to one platelet population being more sensitive than another. Platelet activation by thrombin can be inhibited by the stimulation of an opposing pathway, by increasing cAMP and/or cyclic GMP (cGMP) via stimulation with prostaglandin E_1 (PGE_1) and vasodilators [24–26]. We have shown that LDP compared to HDP contain a lower amount of cAMP and cGMP [5, 6]. The ability of PGE_1 or sodium nitroprusside to inhibit platelet aggregation was more potent in HDP than in LDP [6]. Therefore, platelet functional heterogeneity may well represent a variation in the balance between the regulation of pathways from more sensitive to less sensitive platelets. In order to gain insight into how the heterogeneity of platelets is regulated, we investigated platelet activation at the level of protein phosphorylation, including the detection of phosphorylated and dephosphorylated vasodilator-stimulated protein (VASP), and cytosolic calcium ($[Ca^{2+}]_i$) in platelets separated according to density. Because of the potential of GTP-binding proteins (G-proteins) to mediate and amplify cell signals from the receptor to the effector systems, we examined the role of G_s- and G_i-proteins in the functional heterogeneity of platelet subpopulations. RhoA contributes to platelet activation involving the organization of the cytoskeleton during shape change and aggregation [27–29]. Consequently, we also analyzed rhoA in the platelet subpopulations.

MATERIALS AND METHODS

Chemicals

[^{32}P]nicotinamide-adenine dinucleotide (NAD), 200 mCi/mmol (7400 Mbq/mmol) and [^{32}P]orthophosphate, 10 mCi/mmol (370 Mbq/mmol), were purchased from ICN. Pertussis toxin (*Bordetella pertussis*), cholera toxin (*Vibrio cholerae*), prostaglandin E_1 (PGE_1), and sodium nitroprusside (SNP) were obtained from Sigma, Deisenhofen, Germany. C3 exoenzyme (*Clostridium botulinum*) was a gift from Dr. I. Just, University of Homburg/Saar, Germany. Thrombin was from Behringwerke, Marburg, Germany, and 1-[-2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′methyl-phenoxy)-ethan-N,N,N′,N′-teraacidic acid penta acetoxy methylester (fura-2-AM) from Molecular Probes. Alkaline phosphatase conjugate (goat anti-rabbit) was from Biorad. The enhanced chemiluminescence (ECL) Western-blotting analysis system containing peroxidase conjugate (sheep anti-rabbit) was from Amersham. Antibodies against $Gi_{1-3\alpha}$, $G_{s\alpha}$ and rhoA were obtained from Santa Cruz. Percoll was purchased from Pharmacia. All other chemicals and reagents were of analytical grade.

Platelet Rich Plasma (PRP)

Blood from volunteers (healthy male donors, mean age: 26) was drawn from the anticubital vein. Citrate was used as an anticoagulant, and PRP was obtained by centrifugation of the blood at 350 × g_{max} and 15° for 15 in and immediately used for the analyses described below. The experiments were single determinations from the platelets of different individuals.

Platelet Subpopulations

Density-dependent platelet subpopulations were obtained from PRP essentially as described previously [7]. This involved centrifugation at 3500 × g_{max} for 15 min at 4°C on a Percoll density gradient (ϱ: 1.08 g/cm^3, 1.07 g/cm^3, 1.065 g/cm^3, and 1.04 g/cm^3) in a Heraeus centrifuge (Omnifuge, 2.0 RS; Heraeus Christ). Three platelet subpopulations were collected from the gradient: LDP (ϱ = 1.04–1.065 g/cm^3); intermediate-density platelets (IDP) (ϱ = 1.065–1.07 g/cm^3); and HDP (ϱ = 1.07–1.08 g/cm^3). A total platelet population, obtained by the same procedure but only with gradient densities of 1.08 g/cm^3 and 1.04 g/cm^3, was used for control experiments (ADP-ribosylation). The subpopulations were washed twice with modified Gaintner's solution (103 mM NaCl, 40 mM Na_2HPO_4, 4.7 mM KH_2PO_4, 5 mM glucose × H_2O, 129 mM citrate, pH 6.4) [30], resuspended in buffer (modified Tyrode: 137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4 × H_2O, 5.5 mM glucose, 3.5 mg/mL BSA, pH 7.35; or modified HEPES: 10 mM $K_2C_2O_4$, 100 mM KCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.55), and diluted to the final concentration. Cell counts were determined in a Sysmex F 800 cell counter (Digitana).

Measurement of Protein Phosphorylation

Washed platelets (1.5 mg platelet protein/mL) of each subpopulation in HEPES buffer, pH 7.4, were incubated with 750 μCi (27.75 Mbq) [^{32}P]-orthophosphate for 1 hr at 37°. Six hundred μL of this cell suspension were stimulated for 2 min with thrombin at a final concentration of 0.1 U/mL. Inhibition of thrombin activation was carried out by incubation of platelets for 2 min with PGE_1 (1 μM) or SNP (100 μM) prior to thrombin stimulation. After centrifugation at 12,000 × g_{max} for 3 min, platelet pellets were suspended in 50 μL buffer (20 mM HEPES, 1% Triton X-100, 2.5 mM phenylmethane sulfonyl fluoride, 800 U/mL trasylol, 8 mg/mL bacitracin, 8 mM EDTA, 10 mM sodium phosphate, 10 μM leupeptin, 2 μM pepstatin A). Platelets were disrupted by ultra sonication for 20 sec at 40 W with a Branson sonifier B-12 (Branson). After addition of electrophoresis buffer (IEF: 2.4 g urea, 430 μL Servalyt, 100 μL Triton X-100, 50 μL 2-mercaptoethanol, 200 μL 1%-phenol blue, and 2.1 mL bidest. water) solubilized platelet proteins were separated by first-dimensional gel electrophoresis and then transferred to the Investigator 2-D electrophoresis system from Millipore [31] for separation in the second dimension (2-D-buffer: 54 g urea, 4 mL Nonidet P-40, 2 mL ampholyte, 1 mL 2-mercapto-ethanol, and 93 mL bidest. water). The phosphoproteins were visualized by exposure of a Kodak X-AR film to the gels at −80°, without

intensifying screen. The specific activity of the [^{32}P]ATP in the platelet subpopulations was controlled using an FPLC method [32] to resolve the ATP from other ^{32}P-labelled molecules. The ATP content was determined by 254 nm absorbance and the [^{32}P]ATP by scintillation counting.

Characterization of $G_{i\alpha}$- and $G_{s\alpha}$-Subunits by Immunoblotting

Platelet proteins (100 μg/lane) were acetone-precipitated, dissolved in sample buffer according to Laemmli [33] and loaded onto a slab gel composed of 10% (w/v) acrylamide, 0.21% (w/v) bisacrylamide, and 4 M urea. Proteins resolved by SDS-PAGE were transferred to nitrocellulose and stained with Ponceau S (5% w/v). After blocking with 3% (w/v) ovalbumin in TBS (200 mM NaCl, 50 mM Tris-base) pH 7.6, nitrocellulose filters were incubated for 1 hr with antiserum (diluted 1:1000 in TBS). After extensive washing in 0.05% (v/v) Tween 20 (in TBS, pH 7.6), filters were incubated for 1 hr with a secondary antibody (sheep anti-rabbit antibody, peroxidase-conjugated, 1:4000 in TBS). After a further washing with Tween 20 (0.05%) in TBS, proteins were visualized using the Amersham ECL Western blotting analysis. Protein was determined according to the method of Lowry et al. [34]. Immunoblots were quantified by a digital scanning method [35] using the SigmaScan software (Jandel Scientific). The linearity of the ECL system was tested for the investigated proteins. The obtained intensity units of this method were used for the statistical analysis.

Measurement of the ADP-Ribosylation

ADP-ribosylation was induced with Pertussis toxin (*Bordetella pertussis*), Cholera toxin (*Vibrio cholerae*), or C3 exoenzyme (*Clostridium botulinum*) and carried out as described elsewhere [36, 37] with the following modifications. Washed platelets from each subpopulation (1.5 mg platelet protein/mL) were resuspended in Tyrode buffer. Platelets were stimulated for 2 min with thrombin (0.1 U/mL) alone or 2 min with PGE_1 (1 μM) prior to thrombin stimulation. Sixty-five microliters of the platelets were then added to 45 μL reaction mixture (final concentration: 20 μg saponin/mL, 1 mM ATP, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM thymidine, 1 mM dithiothreitol, 20 μM NAD, 0.65 μCi [^{32}P]-NAD, 0.65 μg pertussis toxin or cholera toxin, 0.13 μg C3 exoenzyme). For controls (C), platelets (a total platelet population) were incubated with the reaction mixture, but without the toxins. After incubation at 37° for 15–60 min, the reaction was terminated by addition of sodium deoxycholate (2%, w/v) containing TCA (24%, w/v). Sixty-minute experiments were most common. The pellet obtained after centrifugation for 30 min, 4000 × g_{max}, was resuspended in 50 μL Tris-base (1 M) and added to 50 μL sample buffer according to Laemmli [33]. Samples were heated at 95° for 5 min and separated on 10% SDS-PAGE. Gels were dried after electrophoresis and exposed in the range of 12 to 165 hr to Europium sheets. Radioactivity was quantified with a phospho imager SI (Molecular Dynamics) and the degree of ADP-ribosylation was calculated by Microsoft Image QuaNT software.

Immunoblotting of the Vasodilator-Stimulated Phosphoprotein (VASP)

Five hundred microliters of washed platelets (1.5 mg platelet protein/mL) of each subpopulation were centrifuged at 350 × g_{max} for 10 min at 4°. The pellet was resuspended in sample buffer (9 M urea, 2% (v/v) Nonidet P40, 2% (w/v) 2-mercaptoethanol, 0.8% (v/v) ampholyte pH 3–10) and sonicated. Proteins were separated by two-dimensional gel electrophoresis as described above and transferred to nitrocellulose. VASP was characterized by incubation with a specific antiserum (dilution 1:1500) and detection by ^{125}I-protein A labelling as described elsewhere [25].

$[Ca^{2+}]_i$ in Platelet Subpopulations

Two milliliters of washed platelet subpopulations were incubated with fura-2-AM (4 μM) at 37° for 30 min and centrifuged at 350 × g_{max} for 15 min. The resulting platelet pellets were resuspended in HEPES buffer, pH 7.4, and diluted to a concentration of 5 × 10^8 cells/mL. Fluorescence was measured with a Perkin–Elmer LS 5 spectrofluorometer. The excitation and emission wavelengths were 340 nm and 500 nm, respectively. Basal $[Ca^{2+}]_i$ in the platelet subpopulations were calculated as described elsewhere [38].

Data Analysis

For statistical evaluation of the phospho imager quantification, a two factor analysis of variance (ANOVA) was carried out with factor HDP vs. LDP and a second factor unstimulated vs. stimulated situations. The Duncan multiple range test was used after ANOVA to locate significant differences between the ADP-ribosylation pattern. For the immunoblot and $[Ca^{2+}]_i$ quantification, a one-way ANOVA followed by the Duncan test was used.

RESULTS

Protein Phosphorylation in Resting Human Blood Platelet Subpopulations

Figures 1a and 1b illustrate the two-dimensional separation of platelet phosphoproteins before thrombin stimulation (control). Several proteins in LDP exhibited higher phosphorylation levels when compared to HDP (intermediate-density platelets are not shown, since the intensity was between that of LDP and HDP). The same amount of platelet protein was applied for each subpopulation. The specific activity of the [^{32}P]ATP and ATP content did not differ in the platelet subpopulations.

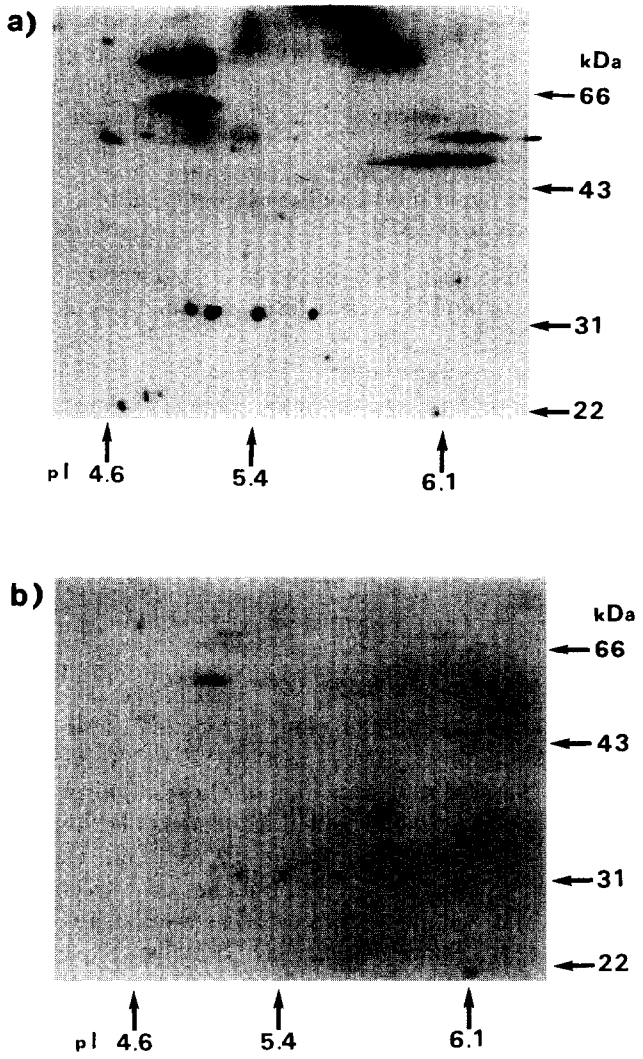

FIG. 1. Autoradiography of [^{32}P]-labelled platelet proteins of LDP and HDP. a) Two-dimensional gel of platelet proteins (LDP) before stimulation with thrombin. b) Two-dimensional gel of platelet proteins (HDP) before stimulation with thrombin. Solubilized platelet proteins (1.5 mg platelet protein/mL) were separated by first-dimensional gel electrophoresis and transferred to the 2-D electrophoresis system (for details see "Materials and Methods"). The phosphoproteins were visualized by exposure of a Kodak X-AR film. Autoradiography of IDP is not shown, because the level of phosphorylation was intermediate between that for LDP and HDP. The pictures shown are representative of 6 experiments. Apparent molecular masses and the pI-range are indicated on the right and bottom, respectively.

Effect of PGE_1 and SNP on Thrombin-Stimulated Protein Phosphorylation of Human Blood Platelet Subpopulations

Thrombin stimulation increased phosphorylation of the 20, 24, 31, and 47 kDa protein over basal (control) levels (Fig. 2); however, the increase was more pronounced in HDP compared to LDP. The thrombin-induced phosphorylation was differently reduced in the two subpopulations by PGE_1 and SNP. Both substances affected the thrombin-induced phosphorylation of the 20-, 24-, 31-, and 47-kDa phosphoproteins more efficiently in HDP than in LDP (Fig. 2).

Dephosphorylation after Thrombin-Stimulation

Following thrombin stimulation, dephosphorylation of an 18-kDa protein (pI 6.2) was observed in HDP which was not influenced by PGE_1 or SNP (Fig. 2). In resting platelets, this protein was more phosphorylated in HDP than in LDP.

Immunoblotting of $G_{i\alpha}$-, $G_{s\alpha}$-subunits and RhoA

Incubation with the $G_{i\alpha 1-3}$ sensitive antibody (Fig. 3a) revealed a significantly higher staining of 40 kDa and 41 kDa proteins in LDP and IDP compared to HDP (intensity units: LDP = 48.483 ± 2.106; IDP = 45.973 ± 2.033; HDP = 22.069 ± 1.589; N = 6, ANOVA $P < 0.01$; Duncan: LDP vs. HDP significantly different, $P < 0.01$).

After incubation with the $G_{s\alpha}$ sensitive antibody (Fig. 3b), a similar staining of the 40 kDa protein was found in the three subpopulations (intensity units: LDP = 12.029 ± 1.439; IDP = 13.563 ± 1.331; HDP = 13.988 ± 1.227; N = 6). The immunoblotting of the rhoA-protein (Fig. 3c) showed a significantly higher staining in LDP than in HDP (intensity units: LDP = 139.100 ± 14.589; IDP = 123.911 ± 13.649; HDP = 76.794 ± 8.731; N = 6, ANOVA $P < 0.001$; Duncan: LDP vs. HDP significantly different, $P < 0.001$).

Pertussis Toxin-Induced ADP-Ribosylation

Figure 3d shows the ADP-ribosylation of a 40 kDa protein by Pertussis toxin. The quantification with the phospho imager (Fig. 4a) revealed a significantly higher level of ADP-ribosylation in LDP than in HDP. Thrombin decreased the level of ADP-ribosylation significantly in the two subpopulations. PGE_1 antagonized the effect of thrombin. Neither thrombin nor PGE_1 + thrombin altered the differences between the two subpopulations.

Cholera Toxin-Induced ADP-Ribosylation

The cholera toxin-induced ADP-ribosylation of a 40-kDa protein (Fig. 3e) as quantified with the phospho imager (Fig. 4b) showed a higher level of ADP-ribosylation in HDP than in LDP. Thrombin reduced the level of ribosylation significantly in HDP. PGE_1 + thrombin reduced the level of ribosylation similarly in the two subpopulations.

C3 Exoenzyme-Induced ADP-Ribosylation

Figure 3f shows the ADP-ribosylation of a 21 kDa protein induced by C3 exoenzyme. The quantification with the phospho imager (Fig. 4c) yielded a significantly higher ADP-ribosylation in LDP compared to HDP (Fig. 4c). Thrombin decreased the level of ADP-ribosylation only in LDP, whereas PGE_1 inhibited the thrombin effect. Neither thrombin nor PGE_1 + thrombin altered the differences between the two subpopulations.

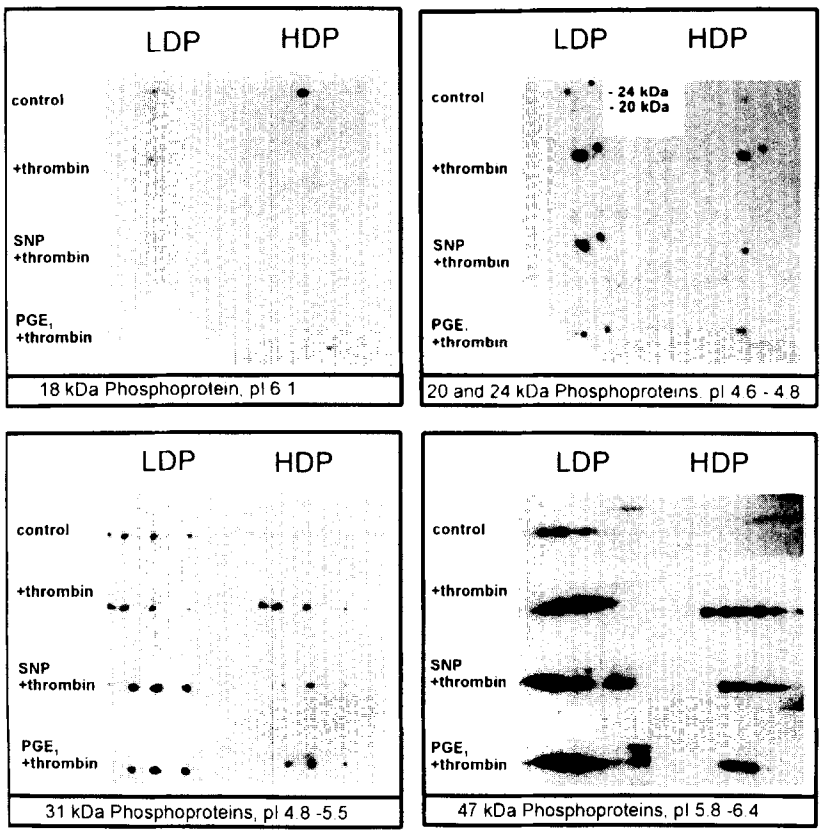

FIG. 2. Autoradiography of [^{32}P]-labelled platelet 18 kDa–47 kDa proteins of LDP and HDP after thrombin or thrombin + PGE_1/SNP stimulation. Two-dimensional gel after incubation with 0.1 U/mL thrombin, 1 μM PGE_1 followed by 0.1 U/mL thrombin stimulation, and 100 μM SNP followed by 0.1 U/mL thrombin stimulation. For further information see legend to Fig. 1. The figures shown are representative of 6 experiments.

Detection of the 46/50 kDa VASP

Figure 5 shows the Western blot of the 46 and 50 kDa VASP. In HDP, the phosphorylated 50 kDa protein represented virtually 100% of the total. LDP contained more of the dephospho 46 kDa protein of VASP. For IDP, the ratio between 46 and 50 kDa VASP was approximately 1:1.

$[Ca^{2+}]_i$ in Platelet Subpopulations

A significantly higher $[Ca^{2+}]_i$ was observed in LDP (211 ± 29 nM) than in HDP (122 ± 32 nM) and IDP (143 ± 35 nM). ANOVA: $N = 6$, $P < 0.001$; Duncan: LDP vs. HDP significantly different, $P < 0.001$).

DISCUSSION

The aim of our study was to test the hypothesis that variations in signalling efficiency, as reflected in changes in phosphoprotein labelling, contribute to known functional differences between platelet subpopulations. In LDP, aggregation is enhanced and the inhibitory effect of SNP on thrombin-mediated aggregation is weaker than in HDP [6]. Furthermore, in LDP compared to HDP, PGE_1 increases cAMP significantly less and thrombin induces significantly more release of [^{3}H]inositol phosphates [6, 7]. The kDa and pI ranges of the analyzed phosphoproteins parallel data from the literature [8, 11, 13, 39–41]. The 47 kDa protein (pleckstrin) is a substrate for protein kinase C and contains variable phosphorylated forms, pI range 5.8 to 6.2 [39]. The higher degree of phosphorylation of this protein in LDP prior to thrombin stimulation may reflect an enhanced preactivation of this platelet subpopulation. Furthermore, the finding that in comparison to HDP the 50 kDa VASP phospho form is lower and the prephosphorylation of the 47 kDa protein in LDP is higher may be explained by a lower level of cyclic nucleotides in resting LDP and the poorer ability to increase these nucleotides in LDP [6]. Elevated levels of cAMP and cGMP result in a phosphorylation of VASP which correlates with the inhibition of inositol phosphate liberation and phosphorylation of the 47 kDa proteins [24–26]. High levels of these cyclic nucleotides have been found in HDP compared to LDP [6, 7]. In both platelet populations, the levels of cyclic nucleotides correlate with the degree of VASP phosphorylation. Interestingly, in IDP, where cyclic nucleotide levels between HDP and LDP have been observed [6, 7], similar amounts of phosphorylated and dephosphorylated VASP are found.

The high prephosphorylation of the 31 kDa protein in LDP compared to HDP might be a hint for the participation of this protein in the difference of the preactivation in the subpopulations. This protein was described as a platelet membrane protein [16]. The situation after stimulation with thrombin and PGE_1 or SNP + thrombin was similar to that of the 47 kDa protein in both LDP and HDP. This finding suggests a role for this protein in platelet activation.

It was previously shown that the 24 kDa protein is phosphorylated by protein kinase A [42] and is identical with thrombolambdan [43, 44]. The phosphorylation of this

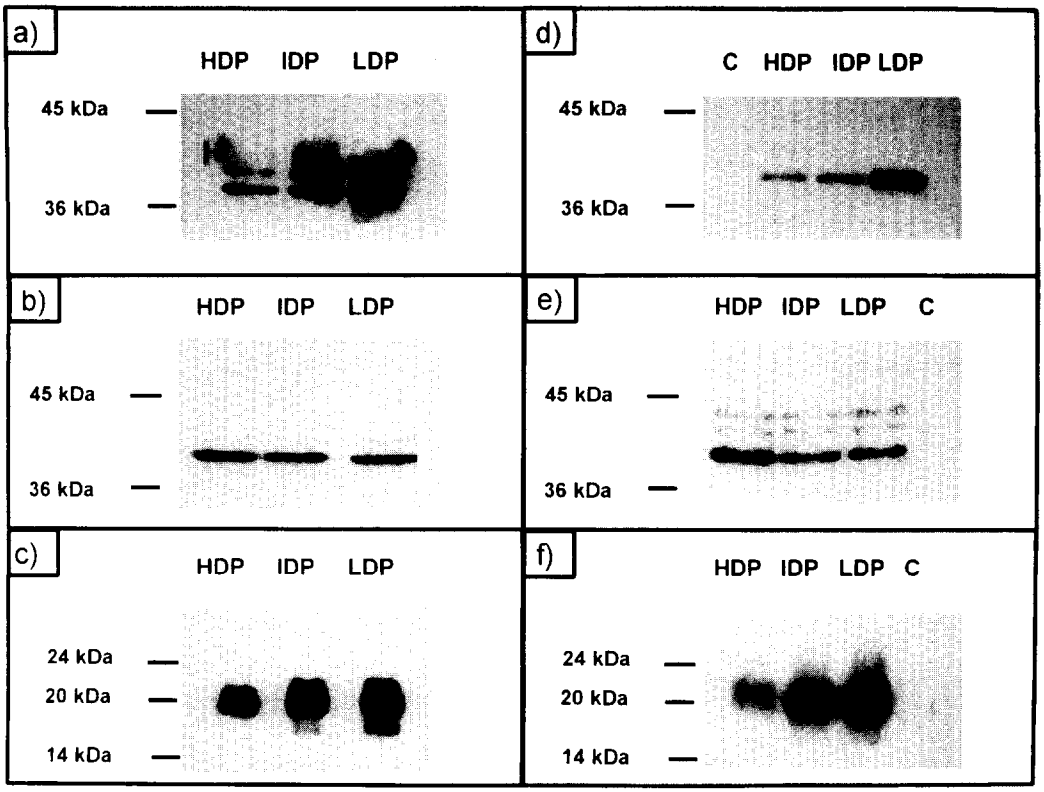

FIG. 3. Immunological identification of $G_{i\alpha}$-, $G_{s\alpha}$-subunits (a, b), and RhoA protein (c); ADP-ribosylation of $G_{i\alpha}$-, $G_{s\alpha}$-subunits (d, e), and RhoA (f). Immunoblotting: 100 μg platelet protein/lane was applied for each subpopulation. Proteins were visualized using the Amersham ECL Western blot analysis. ADP-ribosylation: Washed platelets from each subpopulation (1.5 mg platelet protein/mL) were used for the ADP-ribosylation experiments. Control experiments (without the toxins) are indicated by C. Radioactivity was quantified with a phospho imager. Experiments were also carried out after stimulation with thrombin and PGE_1 + thrombin and quantified (quantified data are shown in Fig. 4). Apparent molecular weights (kDa) are indicated on the left. The figures shown are representative of 6 experiments.

protein in erythroleukemia cells causes its translocation to the cytosol and an impaired ability of thrombin to activate phospholipase C [45]. Interestingly, in our experiments with platelets this protein was more phosphorylated by thrombin and PGE_1 or SNP + thrombin in LDP, the platelet subpopulation with a higher inositol monophosphate formation after stimulation with thrombin and a lower increase of cAMP after stimulation with PGE_1 [5].

The 20 kDa phosphoprotein (pI 4.9) most likely represents the MLC [46]. The phosphorylation of the MLC is associated with the shape change and aggregation of platelets [10]. The observation that the phosphorylation of MLC in resting platelets is higher in LDP than in HDP may help to explain the higher aggregability of LDP [5]. In addition, since the MLC kinase is calcium-dependent, the higher level of $[Ca^{2+}]$ in the LDP may be responsible for the enhanced phosphorylation of MLC in this platelet population.

Dephosphorylation of a 18 kDa protein in platelets by thrombin was described by Davidson and Haslam [47]. These authors observed a dephosphorylation of the 18 kDa protein during later phases of platelet aggregation. In addition, we found that PGE_1 and SNP enhanced the dephosphorylation of a 18 kDa protein in HDP compared to LDP. This finding may be related to the higher ability of vasodilators to prevent aggregation in HDP [6]. The differences found in the phosphorylation pattern of the subpopulations are not a result of differences in the specific activity of $[^{32}P]ATP$ in the subpopulations. Furthermore, we have found no differences in the ATP content or in the energy charge between the subpopulations [32].

Western blotting for the major inhibitory G-protein subunit ($G_{i\alpha 1-3}$) in platelets revealed a higher amount in LDP and IDP compared to HDP. The enhanced ADP-ribosylation by pertussis toxin in LDP compared to HDP might rationalize previously published data of the lower basal and thrombin-induced decrease in cAMP level in LDP [7]. $G_{i\alpha}$-subunits also play a role in the control of thrombin action on phospholipase C [22, 48], and thrombin induces a significantly higher release of $[^3H]$inositol phosphates in LDP than in IDP or HDP [7]. This is of special interest in view of the interaction between adenylate cyclase and phosphoinositide metabolism [49]. Such an interaction may be different for the subpopulations, leading to the greater tendency of LDP for aggregation [6].

On the other hand, cholera toxin-induced ADP-ribosylation was greater in HDP than in LDP despite a similar immunostaining in the subpopulations. The higher cholera

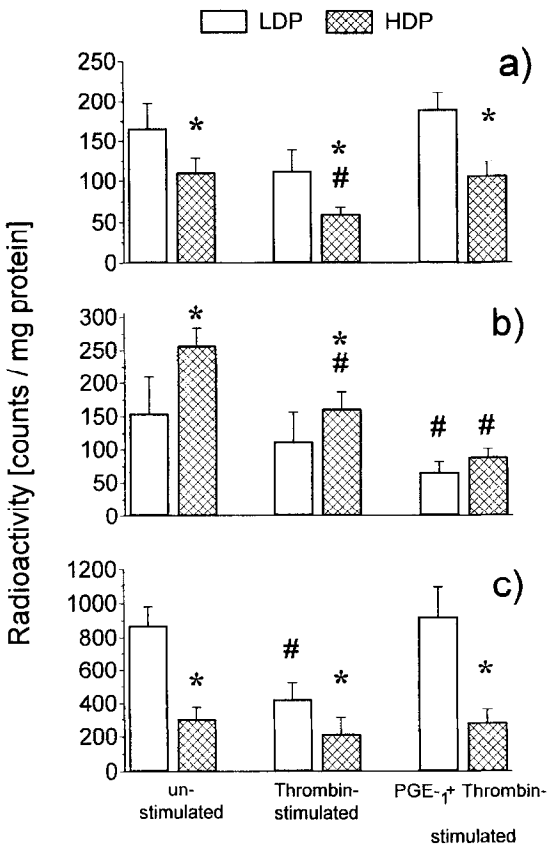

FIG. 4. Quantification of the ADP-ribosylation experiments in LDP and HDP. Counts of the control experiments (see Fig. 3) were used as basal levels. Data are means of 6 experiments ± SD. ANOVA: a) Pertussis toxin-induced ADP-ribosylation, LDP vs. HDP, $P < 0.001$; unstimulated vs. stimulated, $P < 0.05$. b) Cholera toxin-induced ADP-ribosylation, LDP vs. HDP, $P < 0.05$; unstimulated vs. stimulated, $P < 0.05$. c) C3 exoenzyme-induced ADP-ribosylation LDP vs. HDP, $P < 0.001$; unstimulated vs. stimulated, $P < 0.001$. * Significant differences (Duncan test) between LDP and HDP. # Significant differences (Duncan test) between unstimulated and stimulated platelets.

toxin-induced ADP-ribosylation in HDP suggests a more potent ability of these platelets to produce cAMP and may therefore prevent aggregation in this subpopulation as described earlier [6]. Thrombin induced a similar decrease in pertussis toxin-induced ADP-ribosylation in the investigated subpopulations. The substrate for the pertussis toxin-induced ADP-ribosylation is the heterotrimeric $G_{i\alpha\beta\gamma}$-complex and thrombin induces a decrease in this complex [50–52]. The $G_{i\alpha}$-subunit is not a substrate for the pertussis toxin [52]. PGE_1 was able to inhibit the thrombin-induced decrease of pertussis toxin-induced ADP-ribosylation in the subpopulations. This effect was more pronounced when stimulated with PGE_1 before thrombin as was observed earlier [53].

The thrombin-induced decrease in the cholera toxin ADP-ribosylation was found in HDP. One explanation for the decrease after PGE_1 + thrombin could be the effect of PGE_1 on the dissociation of the heterotrimeric complex of the G-protein as shown by Molina y Vedia et al. [53].

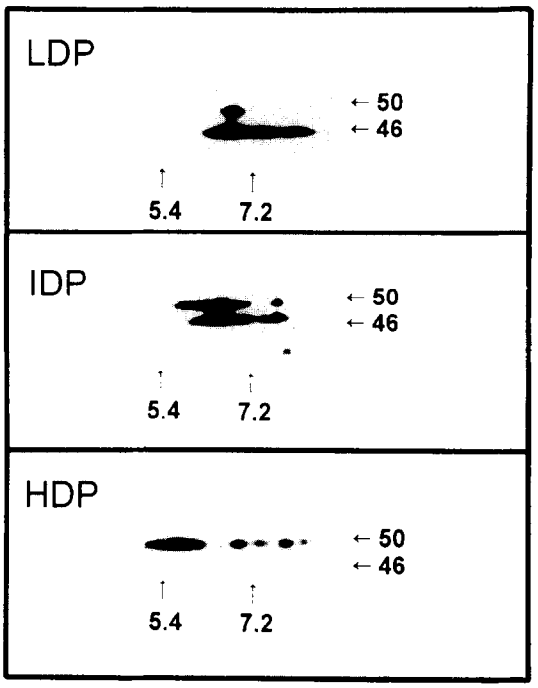

FIG. 5. Immunoblot of the vasodilator-stimulated protein (VASP). Two-dimensional gel of unstimulated cells of LDP, IDP, and HDP. Immunoblotting and detection were carried out as described in "Materials and Methods." Five hundred µL washed platelets (1.5 mg platelet protein/mL) were separated by two-dimensional gel electrophoresis (see "Materials and Methods"). Apparent molecular masses and pI-markers are indicated on the right and on the bottom for each subpopulation. The blots shown are representative of two experiments. The pI-range of the used ampholyte (1st-dimension gel) was 3–10.

RhoA, a 22 kDa substrate protein for botulinum C3 ADP-ribosyltransferase (C3 exoenzyme), has recently been identified in platelets [27, 28] and may be involved in cytoskeletal organization [29]. Furthermore, the ADP-ribosylation of rhoA inhibits thrombin-induced platelet aggregation and may regulate the interaction of actin with the fibrinogen receptor and its activation [28]. It has been suggested that the ADP-ribosylation of rhoA leads to inhibition of the phosphatidylinositide-3-kinase [54]. The ADP-ribosylation of rhoA in HDP was less compared to LDP, and thrombin decreased the ADP-ribosylation only in LDP. This finding suggests that rhoA may also be involved in functional heterogeneity of human blood platelets. In summary, our data suggest that the biochemical basis of functional heterogeneity depends on, besides other parameters, differences in the properties and quantities of G- and phosphoproteins in density-dependent subpopulations.

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