Immunolocalization of P2Y₁ and TPα Receptors in Platelets Showed a Major Pool Associated with the Membranes of α-Granules and the Open Canalicul System


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ABSTRACT

P2Y1 and TPα receptors on platelets belong to the G-protein coupled seven transmembrane domain family. They transmit signals for shape change, mobilization of calcium, and platelet aggregation. Immunogold labeling with a monoclonal antibody (MoAb) to the amino-terminal domain of P2Y1 and a polyclonal antibody to the C-terminal domain of TPα revealed that while present at the platelet surface, both receptors were abundantly represented inside the platelet. Specifically, receptors were found in membranes of α-granules and elements of the open-canalicular system. A similar organization was found in mature megakaryocytes. Activation of platelets by ADP and the TXA2 analog, I-BOP, increased both the labeling of P2Y1 and TPα at the surface and in intracellular pools, suggesting that activation resulted in greater antibody accessibility to the receptor. A return to a platelet discoid shape and to basal values of labeling accompanied receptor desensitization. Platelets lacking the P2Y12 ADP receptor normally expressed P2Y1 and TPα, both before and after activation. Studies with the anti-LIBS MoAb, AP6, confirmed that stored fibrinogen associated with internal pools of αIIbβ3 at the start of secretion in a microenvironment containing agonist receptors. Pharmacological antagonism of ADP or TXA2 receptors in antithrombotic therapy may need to take into account blockade of internal receptor pools.

Key Words: Platelet activation, primary receptors, ADP, thromboxane A2, immunogold labeling
Introduction

Receptors for primary agonists in platelets including protease-activated receptors (PARs) mostly belong to the seven transmembrane domain G-protein coupled receptor (GPCR) family. ADP, important both for haemostasis and thrombosis, possesses two platelet receptors belonging to this family. P2Y1 is coupled to Gq and is responsible for shape change, Ca\(^{2+}\) mobilization and the initiation of aggregation. The more recently cloned P2Y12 is coupled to G\(\alpha\)q2 and is necessary for the formation and stabilization of large aggregates. Apart from platelets, P2Y1 is present in endothelial cells, skeletal muscle cells and placenta. TP constitutes the major thromboxane-prostanoid receptor on platelets. TP is responsible for shape change, Ca\(^{2+}\) mobilization, and platelet aggregation. It is associated not only with Gq protein, but also with G\(\alpha\)12 and G\(\alpha\)13, through which it acts as a modulator of the Na\(^{+}/H^+\) exchanger. TP is present in thymus, lung, kidney, spleen and placenta. TP\(\alpha\) is the form that has been preferentially identified in platelets. Although mRNA from the \(\beta\)-isoform derived from an alternative splicing at the 3rd exon of the TP gene has been reported in platelets, doubts remain about the presence of TP\(\beta\) protein. Both P2Y1 and TP initiate platelet activation through the PLC\(\beta\) pathway. ADP- and TXA\(_2\)-dependent activation pathways are major targets for anti-thrombotic therapy. Both agonists can be released during platelet activation, TXA\(_2\) is produced during arachidonic acid metabolism while ADP is stored in a secretable pool in dense granules.

Many receptors in platelets are found in both surface and intracellular membranes, an example being the \(\alpha_{IIb}\beta_3\) integrin. For GPIb, an adhesion receptor, platelet activation is followed by its transient internalization. The PAR-1 receptor for thrombin, a GPCR, has been shown to traffic during platelet activation. Desensitization of GPCRs has been suggested to involve both sequestration and/or uncoupling of the receptor from G proteins. Little is known about the localization of ADP and TXA\(_2\) receptors on platelets. In this study, we have combined immunogold labeling on ultrathin cryosections with electron microscopy to evaluate the cellular distribution of P2Y1 and TP\(\alpha\). Similarities were found in the distribution of both receptors: i) labeling was greater inside the platelets than on the plasma membrane, ii) pools were present in membranes of \(\alpha\)-granules and those of the open canalicular system (OCS), iii) increased labeling was seen after platelet activation with ADP and TXA\(_2\), and iv) for each receptor prolonged exposure to agonist returned labeling density to baseline. By using the MoAb, AP-6, which recognizes a ligand-induced binding site (LIBS) on the integrin, we were able to show integrin activation early in secretion. Secreted ADP or newly formed TXA\(_2\) would be likely to come in contact with these receptors prior to the external pool.
Materials and Methods

Platelet preparations
Peripheral blood was obtained by clean venipuncture from adult human volunteers without medication for at least a week. The initial 3 mL of blood was discarded. Blood was collected into acid-citrate-dextrose NIH formula A (ACD-A) (1 vol of anticoagulant : 6 vol of blood). Platelet-rich plasma (PRP) was prepared by centrifugation at 120 x g for 10 min at room temperature. To prepare washed platelets, 0.05 U/mL apyrase grade VII (Sigma Chemical Company, Saint-Quentin-Fallavier, France) and ACD-A (1 vol to 9 vol PRP) were added immediately to the PRP. Platelets were sedimented by centrifugation at 1200 x g for 15 min and resuspended in 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO3, 0.3 mmol/L NaH2PO4, 1 mmol/L MgCl2, 5.5 mmol/L glucose, 5 mmol/L Hepes, 0.1 % (wt/vol) bovine serum albumin (BSA, Sigma), and 0.05 U/mL apyrase, pH 7.4 (Hepes-Buffered Modified Tyrode, HBMT). Studies were also performed on platelets from patient (M.L.) with an inherited disease recently linked to the absence of the second platelet ADP receptor, P2Y12. Platelets from this patient show a reduced and rapidly reversible aggregation to high doses of ADP and they also show a reduced response to thromboxane analogs.

For platelet activation, unstirred suspensions of washed platelets at 2.5 x 10^8/mL were incubated at 37°C for the stated times with 10 µmol/L ADP (Sigma) or 10 nmol/L I-BOP (Caiman Chemical, Ann Arbor, USA) in the presence or not of 400 µg/mL purified human fibrinogen (Fg) (a gift from Dr. K. Boulimez, Biochemistry Department, Hôpital Cardiologique, Pessac; the Fg was >95% pure as assessed by SDS-polyacrylamide gel electrophoresis). Desensitization was achieved by incubating platelet suspensions with 1 mmol/L ADPßS (Sigma), a stable analog of ADP, for 1h at 37 °C as described by Baurand et al, or with 10 nmol/L I-BOP for 1h at 37°C, again in the absence of stirring.

Megakaryocytes
Bone marrow was taken by sternal puncture performed during a cardiovascular intervention in a hematologically normal patient. Informed consent was obtained. A volume (0.5mL) of bone marrow was delicately added to 5 mL PBS without disruption of the marrow structure. The material was fixed in 1.25 % (vol/vol) glutaraldehyde (Fluka AG, Buchs, Switzerland) in phosphate-buffered saline (PBS) and processed according to our standard procedures, and as for platelets as described below.

Electron microscopy and immunogold labeling
Antibodies. A monoclonal antibody (MoAb) recognizing the P2Y1 receptor was obtained by immunizing mice against a 16aa peptide corresponding to the N-terminal domain of the receptor coupled to keyhole limpet hemocyanin. It was used as purified IgG. The polyclonal antibody to TPα was raised in rabbits against a peptide composed of 15aa (327-341) located at the end of the carboxyl-terminal tail which corresponds to a specific sequence of TPα which differs from that of TPβ. The immunoglobulin fraction was isolated using the E-Z-SEPkit (Amersham Pharmacia) and its specificity shown. AP-6 is an IgM MoAb prepared against the 204-227 amino acid sequence of β3. It is an anti-LIBS, binding to its epitope only after Fg has bound to activated αIIbβ3. It was generously provided by Dr. T. Kunicki (Scripps Research Institute, La Jolla, CA).
Sample preparation. Platelets were fixed in 1.25 % (vol/vol) glutaraldehyde diluted in 0.1 mol/L phosphate buffer (pH 7.2) for 1h at room temperature. After washing, pellets were infused with 2.3 mol/L sucrose (Fluka) before being frozen in propane and then in liquid nitrogen with a Reichert KF 80 freezing system (Leica, Vienna, Austria). Ultrathin sections of approximately 80 nm were cut at -120°C with the Ultracut E ultramicrotome equipped with a FC 4E cryokit attachment and placed on collodion-coated nickel grids. Then, the grids were incubated for 10 min on drops of washing buffer consisting of PBS supplemented with 0.5% or 1% BSA before being incubated with antibodies.

Immunolabeling procedures. An amplification procedure was used for localizing P2Y1. The grids were first placed on drops containing 10 µg/mL of the anti-P2Y1 MoAb for 45 min at room temperature, then on drops containing a 1/100 dilution of fluorescein isothiocyanate (FITC)-conjugated affinity-purified F(ab')2 fragments of sheep anti-mouse IgG (Amrad, Eurobio, Paris) for 30 min. This was followed by incubation with a rabbit antibody to FITC (Dako S.A., Trappes, France) at a 1/1000 dilution in PBS-0.5% alb. Finally, the sections were incubated for 30 min with a goat anti-rabbit antibody adsorbed onto 10 nm gold particles (1/100 dilution of AuroProbe EM G10; Amersham, Les Ulis, France). For TPα, the sections were incubated directly with a 1/100 dilution of the polyclonal antibody to TPα in PBS-1% alb and then with a goat anti-rabbit antibody adsorbed onto 10 nm gold particles as described above. The anti-LIBS MoAb, AP-6, was used at a dilution of 1/10000 and its binding assessed using an anti-IgM antibody associated with gold particles (1/100 dilution of AuroProbe EM GAM IgM G10; Amersham, Les Ulis, France). In double staining, the anti-P-selectin used corresponded to a mixture of 3 MoAbs (VH10, 2.5 µg/ml; S12, 2.5 µg/ml; AK6 1 µg/ml) as described previously. P-selectin was detected using AuroProbe EM GAM G5.

Controls included the absence of primary antibody or its substitution with an irrelevant IgG or IgM of the same species and at the same concentration. The MoAb anti-CD56 (Dako S.A., Trappes, France) was used instead of P2Y1 at the same concentration, and isolated IgG of a rabbit antibody directed against S100 protein (Dako S.A., Trappes, France) was used instead of the anti-TPα. We also performed blocking experiments by preincubating the P2Y1 MoAb with 200 µg/mL of the peptide used for the immunization and likewise the rabbit antibody with 50 µg/mL of the peptide used for immunization as described by Habib et al. P-selectin was then incubated with the sections on the grids as described above.

Electron microscopy and quantitative analyses. The grids were floated several times on PBS and then on water. The cryosections were stained by uranyl acetate and osmium according to our standard procedures and embedded in a thin film of methylcellulose prior to observation with a Jeol JEM-1010 transmission electron microscope (Jeol, Croissy-sur-seine, France) at 80 KV. For quantitative analyses of immunogold labeling, the mean surface area of each platelet section was calculated for at least 50 sections using the Metamorph software Universal Imaging, Paris, France) and a Pentium III computer. The gold particles were counted visually for each platelet section. The results are expressed as mean values ± SD for a minimum of 50 sections. Statistical analysis was performed using the Student’s t-test.

Flow cytometry

Unstimulated platelets or those activated with 10 µmmol/L ADP for 10 min were fixed in 1% (wt/vol) paraformaldehyde (PFA) as described previously. To permit access to the internal compartment, platelets were
treated with 0.1% (vol/vol) Triton X-100 for 30 min, washed, and then incubated overnight at 4°C with the anti-P2Y1 MoAb (10 µg/mL). After further washing, platelets were incubated with phycoerythrin (PE)-labeled F(ab')2 fragments of a sheep anti-mouse IgG (Silenus Laboratories Ltd, Hawthorn, Australia). Negative controls were performed in the presence of 10 µg/ml of a MoAb to CD56 instead of P2Y1. Samples were analyzed FACScan (Becton Dickinson, SA, Le Pont de Claix, France). Gating to select the majority of platelets was based on preliminary determinations of forward and wide-angle light scatter. Fluorescence was measured after passage through a 530 nm long pass interference filter. Histograms were generated from measurements of 10,000 cells and data were analyzed using the LYSYS II software of the FACScan system.
Results

Immunolocalisation of P2Y1

Resting platelets. We first examined the distribution of P2Y1 within the membrane systems of unstimulated platelets. Using a single MoAb, an amplification procedure involving successive incubations with a FITC-labeled anti-mouse IgG and a polyclonal antibody to FITC proved necessary for optimal visualization of the receptor. Labeling of ultrathin sections showed a majority of gold particles localized to membrane systems within the interior of the cells although surface labeling was observed (Fig 1a). Note the typical discoid shape of this unstimulated platelet. Details of intracellular structures containing P2Y1 are illustrated in Fig 1 (b-d). Gold particles were associated with thin channels (panel b). Previously, we have reported that these can link the platelet surface to the granules and may represent routes of trafficking for proteins and receptors (see ref27 and Discussion). Labeling was also observed in more dilated elements of the OCS as shown in panel (c). Finally, there was occasional labeling of the membranes of α-granules. There was no labeling of multivesicular bodies sometimes observed in α-granules28 and clearly visible in the granule to the right of panel (d).
Control experiments performed with an equivalent amount of an irrelevant mouse IgG resulted in virtually no labeling (see legend to Table I). Also, preincubation of the MoAb to P2Y<sub>1</sub> with blocking amounts of the peptide used for immunization resulted in minimal background labeling and none of the features highlighted above (not shown).

**Effects of platelet activation and receptor desensitization.** The distribution of P2Y<sub>1</sub> was next examined on platelets activated by ADP. Washed platelets were resuspended at 37°C and incubated with 10 µmol/L ADP for 10 min without stirring (Fig. 2). Typical activated platelets are illustrated in panels (a) and (b), note their more spherical shape and the presence of pseudopods (PS). The granules are also centralized. Globally the labeling was increased after activation. Pseudopods were often labeled with gold particles showing that P2Y<sub>1</sub> was present. Thin channels were mostly identified by lines of gold particles and often appeared orientated towards the surface. In panel (c), gold particles were clearly present in a channel surrounding the centralized granules. This channel has a localization resembling that of the microtubular ring, leaving open the possibility of an association between these structures.
Homologous desensitization experiments were performed at 37°C by incubating platelets without stirring with 1mmol/L ADPβS, chosen because of its stability (see23). Platelet function testing showed that platelets initially aggregated with ADPβS when stirred, while electron microscopy and immunogold labeling showed similar changes at 10 min of unstirred incubation to those seen with native ADP (not illustrated). However, after incubation with ADPβS for 1h the platelets were unable to aggregate even to freshly added 10 µmol/L ADP and were desensitized. Immunogold labeling of ultrathin sections showed that these platelets now had a discoid shape and that the distribution of P2Y₁ within the different membrane systems was similar to that of unstimulated platelets.
Fig 3: Detection of P2Y₁ in ADP-desensitized platelets

Semi-quantitative analyses. Table 1 shows the values of a semi-quantitative analysis performed by counting gold particles on a minimum of 100 sections of unstimulated platelets from a pool of 6 control donors. Results confirmed that approximately fivefold more gold particles were associated with membrane pools inside the platelet than with the platelet surface. On sections of platelets activated by ADP, the density of gold particles increased both at the platelet surface ($p < 0.001$) and also in the internal membrane systems ($p < 0.02$). At the same time there was an increase in surface area ($p < 0.02$). The values for desensitized platelets were close to those obtained for unstimulated platelets for both the surface and the internal compartment. The surface area also returned to values close to those of unstimulated platelets.
Table 1 Semi-quantitative analysis of P2Y$_1$ in the membrane systems of platelets before and after stimulation with ADP or desensitization with ADP$_{i}$S.

A) Controls

<table>
<thead>
<tr>
<th></th>
<th>Number of gold particles per platelet section*</th>
<th>Number of gold particles per platelet section*</th>
<th>Number of gold particles per platelet section*</th>
<th>Mean surface area per platelet section (µm$^2$)</th>
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<tr>
<td></td>
<td>Surface</td>
<td>Internal membranes</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>9.48±7.31</td>
<td>53.30±25.43</td>
<td>62.77±29.54</td>
<td>2.36±0.92</td>
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<td>ADP</td>
<td>17.37±12.91</td>
<td>62.8±31.10</td>
<td>80.20±37.34</td>
<td>2.77±0.91</td>
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<tr>
<td>Desensitized</td>
<td>9.49±7.38</td>
<td>48.8±14</td>
<td>58.30±31.96</td>
<td>2.22±1.1</td>
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B) Patient (M.L)

<table>
<thead>
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<th></th>
<th>Number of gold particles per platelet section*</th>
<th>Number of gold particles per platelet section*</th>
<th>Number of gold particles per platelet section*</th>
<th>Mean surface area per platelet section (µm$^2$)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Surface</td>
<td>Internal membranes</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>11.22±7.35</td>
<td>44.59±16.30</td>
<td>55.81±55.81</td>
<td>2.58±0.93</td>
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<tr>
<td>ADP</td>
<td>14.85±11.13</td>
<td>59.02±32.36</td>
<td>73.87±40.07</td>
<td>2.8±1.12</td>
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</table>

*Immunogold labeling using a MoAb to the amino terminal of P2Y$_1$ was as described in the Methods. Gold particles were counted visually on the sections. Control values for irrelevant antibody were < 3 particles per 100 sections. Surface area was calculated by computer analysis. Results are ± SD.

**Platelets lacking P2Y$_{12}$** Platelets of the patient (M.L.) lack P2Y$_{12}$, therefore only P2Y$_1$ can assure their activation by ADP. Semi-quantitative analysis showed that P2Y$_1$ was normally distributed in the patients platelets and that total particle counts were not significantly different from the values for normal platelets ($p > 0.05$). Interestingly, the increase in platelet surface section area after ADP activation was no longer significant ($p > 0.05$). Immunogold labeling of P2Y$_1$ in unstimulated and stimulated platelets from the patient was unchanged from that of the control platelets as illustrated in Figs 1 and 2. After a 10 min incubation with 10 µmol/L ADP, shape change had occurred and granules centralized. Labeling concerned the plasma membranes, the membranes of α-granules and thin channels, showing that the deficiency of P2Y$_{12}$ has no consequences on the distribution of P2Y$_1$ (not illustrated).

**Flow cytometry analysis of P2Y$_1$**

Flow cytometry was used as a second approach to confirming the presence of internal pools of P2Y$_1$. Histograms corresponding to the binding of the anti-P2Y$_1$ MoAb to the surface of PFA-fixed unstimulated platelets showed weak labeling (Fig. 4 panel A), confirming previous results.$^{25}$ A slight increase in mean fluorescence intensity (MFI) was observed after ADP stimulation. A clearly increased MFI for permeabilized platelets with Triton X-100 showed an internal pool, thus agreeing with the results found by electron microscopy and the semi-quantitative analysis (panel B).
Immunolocalization of TPα

In resting, activated and desensitized platelets. Preliminary experiments showed that when using the polyclonal antibody specific to the TPα receptor, amplification steps in the labeling procedure were not necessary. The labeling of unstimulated control platelets is shown in Fig. 5. Panel (a) shows a typical discoid unstimulated platelet. While gold particles were present at the surface, labeling was in the majority inside the platelet. Because the antibody recognized the cytoplasmic part of the receptor, labeling often appeared to be closely associated with the membrane or even just below. Higher magnification illustrations showing details of labeled intracellular structures are shown in panels (b-d). Thin channels within the OCS can be distinguished and are seen in panel (b). Some particles were in lines. Labeling of α-granule membranes is shown in panel (c). In panel (d) is shown an α-granule with a Weibel-Palade-like structure.
Platelets were also incubated with I-BOP, a stable analog of TXA₂ for 10 min without stirring. The morphology of the now activated platelet resembled that of ADP-treated platelets with a rounded shape, centralization of granules and the presence of pseudopods. Labeling was again not only increased on the surface, but was also greater in the internal pools (Fig. 6).
Fig 6: Detection of TPα in the membrane systems of I-BOP-activated platelets.

After desensitization by incubation for 1 h with I-BOP, platelet shape returned to a discoid form (not illustrated) and labeling values close to those of unstimulated platelets were obtained (see semi-quantitative analyses). Control experiments where the polyclonal antibody was replaced by an equivalent amount of an irrelevant rabbit antibody showed a much decreased labeling (see legend to Table 2). Furthermore, preincubation of the antibody to TPα with blocking amounts of the peptide used for immunization also resulted in a loss of the labeling highlighted above (not illustrated).

**Semi-quantitative analyses.** Results in Table 2 show that for this antibody between three- to fourfold more gold particles were associated with the internal membrane pools than with the platelet surface. The total number of gold particles increased significantly for both the surface membrane ($p < 0.001$) and the internal pool ($p < 0.001$) after I-BOP treatment. The surface area of the platelets also increased significantly ($p < 0.001$). All values returned to initial levels after desensitization. For the patient (M.L.), I-BOP stimulation also induced a significantly greater antibody labeling ($p < 0.001$), for both the surface and the internal compartment. In contrast, the increase in surface area was not significant ($p > 0.05$).
Table 2: Number of gold particles per platelet section after labeling with the anti-TPα receptor

A) Controls

<table>
<thead>
<tr>
<th></th>
<th>Number of gold particles per platelet section*</th>
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<th>Number of gold particles per platelet section*</th>
<th>Mean surface area per platelet section (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface</td>
<td>Internal membranes</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>16.60 ±7.94</td>
<td>51.71±18.67</td>
<td>68.32±22.92</td>
<td>2.36±0.92</td>
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<td>I-BOP stimulated platelets</td>
<td>32.84±16.90</td>
<td>85.69±49.93</td>
<td>118.53±61.36</td>
<td>3.19±1.27</td>
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<tr>
<td>Desensitized platelets</td>
<td>15.89±6.16</td>
<td>59.17±22.71</td>
<td>75.06±23.54</td>
<td>2.52±1.12</td>
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</table>

B) Patient M.L.

<table>
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<tr>
<th></th>
<th>Number of gold particles per platelet section*</th>
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<th>Mean surface area per platelet section (µm²)</th>
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<tbody>
<tr>
<td></td>
<td>Surface</td>
<td>Internal membranes</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Unstimulated platelets</td>
<td>18.53 ±11.16</td>
<td>51.85±29.78</td>
<td>70.38±37.84</td>
<td>2.58±0.93</td>
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<tr>
<td>I-BOP stimulated platelets</td>
<td>32.67±11.18</td>
<td>103.92±50.46</td>
<td>136.59±58.68</td>
<td>2.8±1.12</td>
</tr>
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</table>

*Experimental procedures were as for Table I. Results are ± SD. For controls done with an irrelevant rabbit antibody, the number of gold particles evaluated for 100 platelet sections constituted respectively 0.74% (unstimulated) and 0.48% (stimulated) of the total number of gold particles seen with the anti-TPα antibody.

Intraplatelet activation of α₁bβ₃ associated with secretion Since receptors for two primary agonists have been revealed to be associated with thin channels and α-granule membranes, we looked closely at the activation of internal pools of α₁bβ₃ using a MoAb, AP-6, recognizing this integrin after Fg has bound. In agreement with our initial report,²⁷ with unstimulated platelets occasional labeling with AP-6 is mostly confined to the α-granule membrane (Fig 7, panel a). After incubating platelets for 10 min with 10 µmol/L ADP, labeling with AP-6 increased on the granule membrane and also in closely associated thin channels (panel b). In panel (c), double labeling performed with AP-6 (10 nm gold particles) and P-selectin (5 nm gold particles) showed their concomitant presence in a thin channel apparently connecting with an α-granule showing that trafficking of P-selectin and the integrin-bound Fg were occurring simultaneously thereby ruling out visualization of Fg uptake. This was also confirmed by performing experiments in the absence of added Fg (not shown).

Fig 7 Detection of activated α₁bβ₃ by the anti-LIBS MoAb, AP-6.
Immunogold labeling of P2Y₁ and TPα in megakaryocytes We finally looked to see how P2Y₁ and TPα were distributed in mature human MK obtained from a control donor. MK were identified in a marrow aspirate by their typical morphology and the presence of a polylobular nucleus. Labeling was detected on surface membranes, but was relatively sparse (not illustrated). Significantly, both P2Y₁ and TPα were present along the demarcation membrane system (DMS). Both receptors were also detected in membranes of α-granules (Fig 7). Thus both P2Y₁ and TPα are expressed during megakaryocyte maturation and are therefore likely to be present in the different membrane systems of platelets at the time of their release.

**Fig 8 Immunolocalization of P2Y1 and TPα receptors in the membrane systems of mature human megakaryocytes.**
Discussion

We have compared the distribution within the platelet of two receptors, P2Y1 and TPα, belonging to the GPCR family. Although many members of this family have been identified in platelets, apart from PAR-1, a receptor for thrombin,1,18,19 little is known about their distribution within the different membrane systems. As well as having a plasma membrane, platelets have a well-developed OCS and several types of storage organelle. Major changes are seen in the distribution of these membranes during platelet activation and secretion (reviewed in17). Interestingly, although P2Y1 and TPα were found as expected in the plasma membrane, the bulk of the immunogold labeling on ultrathin sections concerned internal membrane pools. These included the membranes of α-granules and those of the OCS, including an important network of thin channels that ramify from the surface into the interior of the platelet. The presence of major intracellular pools of these receptors for primary agonists in platelets can have important implications for platelet physiology.

P2Y1 and TPα are both associated with Gα proteins and generate signals inducing platelet activation.3,7-9,15 ADP is the specific ligand for P2Y1 in platelets. ADP also has a second receptor, P2Y12, only recently cloned.5,29 The sparse labeling observed at the platelet surface with the anti-P2Y1 MoAb is in accordance with the low binding previously observed with the same antibody in flow cytometry25 and also confirmed here. ADP receptors have been quantified using radiolabeled 2MeS-ADP, which binds to between 500 and 1000 sites per platelet.5,30-32 This stable ADP analog binds to both P2Y1 and P2Y12. Evaluation of the specific contribution of P2Y1 is allowed by binding studies performed under conditions where one receptor is blocked or absent. In the two well-characterized patients lacking P2Y12, the number of 2MeS-ADP binding sites fell to about 30 for patient (M.L.)5 and to about 170 for a second patient.30 This low range of values explains why an amplification procedure was required to detect P2Y1 using the single MoAb available to us. Since it is unlikely that 2MeS-ADP can reach all of the internal receptor pools identified by us, the number of sites found using radiolabeled ligand almost certainly underestimates the total number of copies per platelet. Intriguingly, in the SP1999 mouse model where P2Y12 was deleted, no specific binding with 2MeS-ADP could be measured.29 Since our results for patient (M.L.) show a relatively normal distribution of P2Y1 between the surface and internal pools, the hypothesis of a downregulation of P2Y1 in the absence of P2Y12 can be excluded, at least in man. Surprisingly, the surface area of platelets increased less for the patient than for the controls during ADP activation suggesting that although shape change is present, and pseudopods form, an associated volume change is absent.

When using a rabbit antibody to the carboxyl-domain of TPα, sufficient labeling was obtained for a classic detection procedure to be used. Yet, labeling of the surface membrane again remained sparse. Once more, greater numbers of gold particles were seen inside platelets, with the membranes of α-granules and those of the thin channels of the OCS again labeled. Previous binding studies with radiolabeled I-BOP, showed close to 1500 sites of TPα per platelet.33,34 I-BOP binds to both high and low affinity sites. With doubts expressed over the presence of TPβ, it was suggested that TPα can represent the two affinity states recognized by TXA2 mimetics in platelets.26 It is probable that our MoAb recognizes both forms. As P2Y1 and TPα show a similar distribution in the different membrane systems of the platelet, it will be interesting to extend these studies to other GPCR receptors, and particularly to P2Y12, to see if this is a common finding. Platelets from patient (M.L.) have a much decreased aggregation response to I-BOP.22 This was interpreted as showing that ADP (via P2Y12) was a major
cofactor in this response. The normal distribution of TPα in the platelets of (M.L.) and the normal response to activation and desensitization with I-BOP, would be in line with this conclusion.

After platelet activation, the labeling of P2Y1 and TPα significantly increased both at the platelet surface and also inside the cell although this increase was lower. Since the capacity for protein synthesis in platelets is very low, an explanation is that their reactivity and/or accessibility to the antibodies on the platelet sections is increased. Trafficking of receptors from the internal pool to the periphery of the cell may also contribute. Interestingly, desensitization was accompanied by a return to a discoid shape and basal surface area of platelets and reactivity with the antibodies on platelet sections, which is in favor of a reversible change in receptor accessibility and/or conformation associated with activation. Another GPCR, the β2-adrenergic receptor, is known to modify its conformation after stimulation through removal of a constraint imposed by an ionic lock located in internal cytoplasmic domains. Events such as this could potentially influence the binding of antibodies.

Baurand et al observed a decreased number of 2MeSADP binding sites on desensitized platelets and concluded that internalization was responsible although it was not excluded that the receptors remained refractory to further contact with ligand. Internalization of P2Y1 was shown in transfected Jurkat cells on incubation with ADP but this model is very different from platelets where a large proportion of receptors were already present in the internal compartment. Even if internalization is frequently associated with desensitization of GPCRs, there may be large differences in the responses of different cell types. In our results, the receptor partition between the internal and surface pools after desensitization was similar to that on resting platelets and we found no evidence of coated pits, endosomes or receptor accumulation in lysosomal granules. The presence of both receptors in mature MK resembled that in unstimulated platelets, a fact that argues against downregulation during platelet isolation.

A major question purports to the possible functional significance of the internal pools, present not only on the membranes of the OCS and thin channels, but also in the membrane of the α-granules. Platelets possess a storage pool of ADP within the dense granules. This pool is secreted into the channels of the OCS before being released to the external medium. TXA2 is produced in platelets following arachidonic acid release and activation of the prostaglandin synthesis pathway. Liberation of arachidonic acid from membrane phospholipids can be induced by many agonists including ADP and collagen. It can be hypothesized that during the activation that follows platelet attachment to subendothelial constituents such as collagen or vWF, the internal pools of receptors for primary agonist would be in contact with TXA2 before their homologues present at the platelet surface. Similarly, ADP released from the granules can have access to P2Y1 receptors inside the platelet before the external pool. Using a mathematical model, Fogelson and Wang have shown that following diffusion of ADP through the channels, the near-surface concentration is very low compared to the internal concentration. In δ-storage pool disease (SPD), where ADP is absent or severely decreased in dense granules, or in aspirin-treated platelets where TXA2 cannot be formed, no second wave of aggregation is seen after activation by ADP while collagen-induced platelet activation is also much reduced. The ability of ADP to induce secretion from platelets is controversial, and may depend on the presence or not of Ca2+ in the medium. Notwithstanding, incubation of platelets with ADP in our studies resulted in an increased amount of ligand-bound αIIbβ3 on α-granule membranes and in associated thin channels presumably representing initiation of secretory pathways for they contained P-selectin. The extent of the changes was nonetheless very different from
the maximal secretion that occurred after activation of platelets with thrombin, where Fg associates rapidly with internal pools of αIIbβ3 prior to being translocated to the exterior.27

Definitive proof for a functional role of the intracellular receptors is not easy to obtain. Pharmacological inhibitors of ADP receptors such as A3P5P (P2Y1) and AR-C69931MX (P2Y12) or ADP eliminating enzymes such as CP/CPK, while blocking surface receptors and removing extracellular ADP, would enter the OCS to unknown extents. Thrombus build up on collagen under flow was markedly affected by a combination of A3P5P and AR-C69931MX with both adhesion and platelet to platelet interactions affected.43 Yet, addition of CP/CPK with total ADP removal was more complete, leaving open a partial inhibition of receptors in the OCS by the antagonists. Similarly, we have shown that a combination of A3P5P, AR-C69931MX and CP/CPK leads to a more effective inhibition of TRAP-induced platelet aggregation than the individual antagonists alone or in pairs perhaps suggesting that total inhibition of the receptor pools is difficult to achieve (P. Nurden, unpublished data).

In summary, we have observed that platelets possess in addition to the plasma membrane pool of receptors for primary agonists, ADP and TXA2, an internal pool present in membranes of α-granules and in a network of channels connected to the surface membrane, with in proximity integrin effectors and ligands. These constitute a microenvironment that becomes interlinked when exocytosis occurs. A subcellular localization of α-subunits of trimeric G-proteins to α-granule membranes has been previously shown,44 so another important part of the signaling machinery is also in place for agonists that could act alone or in synergy.45 Our results therefore raise the question of the role of this internal compartment of agonist receptors and its contribution in platelet activation. At the present time, ADP receptors are a major target for antithrombotic therapy as is TPα.3,16,38 Although thienopyridines such as clopidogrel target P2Y12,46 whose platelet distribution has yet to be established, recent results for P2Y1 null mice,47 suggest that it too is an appropriate target for antithrombotic therapy. Future antithrombotic strategies may well need to take into account less accessible internal pools.
References


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Figure Legends

Figure 1: Detection of P2Y1 in unstimulated platelets by immunogold labeling of frozen ultrathin sections using a MoAb directed against the amino-terminal domain. Panel (a) shows a typical distribution of the labeling within the different membrane systems of the platelet. Surface labeling is highlighted (arrows). Panels (b-d) show higher power magnifications of labeled intracellular structures: (b) thin channels of the OCS (arrow head) (c) a more dilated element of the OCS, and (d) the delimiting membranes of α-granules (arrows). The presence of multivesicular bodies (MVB) inside the granule is indicated. Bars = 0.1 µm.

Figure 2: Immunolocalization of P2Y1 in platelets activated with ADP. Unstirred suspensions of washed platelets were incubated at 37°C for 10 min with 10 µmol/L ADP in the presence of 400 µg/mL Fg. Panels (a) and (b) show surface labeling for P2Y1 which now includes pseudopods (PS). Intracellular labeling remains, lines of gold particles (arrows) can be seen. Also recognized by the MoAb is a clear zone in continuity with a thin channel (arrow heads). Panel (c) shows P2Y1 within a thin channel (arrows) circulating round the granules. Bars = 0.2 µm.

Figure 3: Detection of P2Y1 in ADP-desensitized platelets. Desensitization reverses the changes seen during ADP induced platelet activation. Platelets were incubated at 37°C with the stable ADP analog, ADPβS (1 mmol/L) for 1h without stirring in the presence of Fg. Immunolocalization on ultrathin sections of the now discoid platelets showed that the P2Y1 distribution resembled that seen on unstimulated platelets with labeling (arrows) of both surface and internal membrane systems. Bar = 0.2 µm.

Figure 4: Flow cytometric analysis of the binding of an anti-P2Y1 MoAb to normal platelets. In (A), experiments were performed using unstimulated PFA-fixed platelets and platelets incubated for 10 min with 10 µmol/L ADP before fixation. There was no permeabilization step. In panel (B), the histograms were obtained after permeabilization of PFA-fixed platelets with Triton-X 100 and show the intensity of the internal pool of P2Y1 in the internal compartment of unstimulated platelets. The control histograms (neg) were obtained in the presence of irrelevant antibody instead of P2Y1.

Figure 5: Detection of TPα in the membrane systems of unstimulated platelets using a rabbit polyclonal antibody to the carboxy-terminal domain. Panel (a) shows a typical platelet section. Surface labeling is highlighted (arrows) as is the labeling of thin channels within the OCS (arrow heads). Panels (b) and (c) are higher power magnifications showing abundant labeling within the thin channels of the OCS and labeling of the membranes of α-granules (arrows). In panel (d) is shown a labeled α-granule containing a Weibel-Palade-like structure. Bars = 0.1 µm.

Figure 6: Detection of TPα in the membrane systems of I-BOP-activated platelets. Platelets were incubated with I-BOP for 10 min at 37°C without stirring. The illustrated platelet shows both surface and intracellular labeling. Pseudopods are present and express TPα. Bars = 0.2 µm.
Figure 7: Detection of ligand-bound $\alpha_{\text{IIb}}\beta_3$ by the anti-LIBS MoAb, AP-6. Panel (a) corresponds to unstimulated, washed, platelets. Only a few gold particles were present per platelet and located in the vicinity of $\alpha$-granule membranes (arrow). In (b), a similar illustration is shown but now for unstirred platelets incubated with 10 $\mu$mol/L ADP for 10 min. Note the increased labeling in the vicinity of the $\alpha$-granule membrane. In (c) double staining has been performed for platelets incubated with ADP as above, an initiation of secretion is shown by the concomitant labeling with P-selectin (5 nm gold particles) and AP-6 (10 nm gold particles) in a thin channel extending from an $\alpha$-granule. Bars = 0.1 $\mu$m.

Figure 8: Immunolocalization of P2Y$_1$ (a, b) and TP$\alpha$ receptors (c, d) in the membrane systems of mature human megakaryocytes. In a) gold particles are seen associated with $\alpha$-granule membranes (arrow heads), in b) they are present in the DMS (arrows). In c) and d) the labeling observed with an anti-TP$\alpha$ is also associated with $\alpha$-granule membranes and the DMS. Bars = 0.2 $\mu$m.
Immunolocalization of P2Y₁ and TPα Receptors in Platelets Showed a Major Pool Associated with the Membranes of α-Granules and the Open Canalicular System

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