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Differential Regulation and Relocalization of the Platelet P2Y Receptors after Activation: A Way to Avoid Loss of Hemostatic Properties?[S]

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ABSTRACT

In the present study, we investigated the desensitization and trafficking of the $P2Y_1$ and $P2Y_{12}$ receptors after agonist-induced stimulation of platelets or astrocytoma cells transfected with the $P2Y_1$ or $P2Y_{12}$ receptors fused to green fluorescent protein. In platelets and in transfected cells, exposure to 10 μM ADP caused desensitization of the $P2Y_1$ receptor-driven calcium signal, whereas the $P2Y_{12}$ receptor-mediated inhibition of cAMP formation was not affected. Plasma membranes from ADP-stimulated platelets also retained $P2Y_{12}$ activity. Agonist-induced $P2Y_1$ receptor desensitization was accompanied by its internalization in platelets and transfected cells. In contrast, although a substantial fraction of $P2Y_{12}$ receptors was rapidly and transiently internalized, most of the $P2Y_{12}$ receptors remained at the plasma membrane. Activated $P2Y_1$ receptors were internalized through a clathrin-dependent pathway in cells and platelets, whereas the $P2Y_{12}$ receptors seemed to use a distinct, clathrin-independent pathway. Together, these data indicate that the $P2Y_1$ and $P2Y_{12}$ receptors are differentially regulated upon activation. The absence of desensitization of the Gi protein-coupled $P2Y_{12}$ receptor-dependent responses could represent a mechanism to preserve the hemostatic properties of otherwise unresponsive platelets.

ADP plays a central role in hemostasis and in arterial thrombosis. Stored in the platelet dense granules and released upon activation by a number of stimuli, including vessel wall collagen, thrombin, and thromboxane A_2, it participates in all stages of platelet activation and enhances thrombus growth (Gachet, 2001). Two G protein-coupled receptors mediate platelet aggregation in response to ADP. The Gq-coupled $P2Y_1$ receptor is responsible for the mobilization of intracellular calcium stores, shape change, and transient aggregation, whereas the Gi_2-coupled $P2Y_{12}$ receptor is responsible for inhibition of cyclic AMP production, amplification of the response and stabilization of the aggregates. Based on pharmacological studies as well as on studies with P2 receptor knockout animals, it is now well established that both receptors are necessary for normal platelet activation by ADP (Hechler et al., 1998b; Fabre et al., 1999; Leon et al., 1999; Foster et al., 2001). The $P2Y_{12}$ receptor mostly supports the enhancing cofactor role of ADP when platelets are activated by other stimuli through activation of transduction pathways downstream of Gi, including phosphoinositide 3-kinases, rap-1b, and vasodilator-stimulated phosphoprotein dephosphorylation (Hechler et al., 1998a; Schwarz et al., 1999; Trumel et al., 1999; Gachet, 2001; Kauffenstein et al., 2001; Conley and Delaney, 2003; Kim et al., 2004). This receptor is thus an attractive target for potent antiplatelet drugs, which is indeed the case with the thienopyridine compound clopidogrel (Herbert and Savi, 2003) and with several classes of competitive antagonists such as the AR-C compounds (Humphries, 2000; Hollopeter et al., 2001).

To date, little is known concerning the regulation of these

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ABBREVIATIONS: eGFP, enhanced green fluorescent protein; PG, prostaglandin; TRAP, thrombin-receptor-activating peptide; AM, acetoxymethyl ester; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HEK, human embryonic kidney; OCS, open canalicular system; ADPβS, adenosine-5′-*O*-(2-thio)diphosphate; U46619, 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$; 2MeSADP, 2-methylthio-ADP; AR-C69931MX, N^6-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP.

receptors after their activation. It has been known for a long time that platelets can become refractory to activation by ADP in vitro (O'Brien, 1965; Spaet and Lejnieks, 1966) or after major surgical operations (O'Brien et al., 1971), which could represent a postsurgical bleeding risk. ADP accumulates extracellularly in suspensions of washed platelets and is sufficient to desensitize the aggregation response to ADP (Ardlie et al., 1971; Holme and Holmsen, 1975; Fijnheer et al., 1992). Addition of apyrase (ATP-diphosphohydrolase; EC 3.6.1.5), an ATP/ADP degrading enzyme extracted from potatoes, protects the cells from desensitization and enhances their aggregability (Ardlie et al., 1971; Holme and Holmsen, 1975; Fijnheer et al., 1992). Thus, it seems that a nucleotide scavenger mechanism is necessary not only to prevent irrelevant cell activation but also to preserve cell responsiveness. When such a system is present, platelets activated by ADP are only transiently refractory to a second challenge with the agonist, whereas in its absence, their refractoriness is stable. This refractoriness could be mimicked in vitro by the use of nonhydrolyzable agonists such as ADPβS (Poole et al., 1993; Baurand et al., 2000). In vivo, such a scavenger enzyme system has been discovered to be the CD39/ATP diphosphohydrolase or E-NTDPase-1, which is expressed on endothelial cells and is responsible not only for inhibition of platelet activation at the surface of healthy vessels (Marcus et al., 1997; Robson et al., 1997) but also for maintenance of platelet responsiveness, because the response to ADP is desensitized in CD39-deficient mouse platelets (Enjyoji et al., 1999).

In a previous work, we showed that platelet refractoriness to ADP, illustrated by complete absence of shape change and aggregation in response to ADP, was caused entirely by selective desensitization of the $P2Y_1$ receptor, whereas the $P2Y_{12}$ receptor remained functional (Baurand et al., 2000). Our hypothesis, based on functional and radioligand binding studies, was that the $P2Y_1$ receptor could be desensitized and internalized, whereas the $P2Y_{12}$ receptor persisted at the plasma membrane. The aim of the present study was to further investigate the desensitization and the trafficking of the $P2Y_1$ and $P2Y_{12}$ receptors both in platelets and in cells separately transfected with the receptors fused to the enhanced green fluorescent protein (eGFP). We report here that, either in platelets or in the heterologous systems, the $P2Y_1$ receptor-induced intracellular calcium increase could be fully desensitized, whereas the $P2Y_{12}$ receptor-induced inhibition of cAMP production remains functional after either short- or long-term stimulation. Both receptors were found to be internalized, however, with different kinetics and through distinct pathways, resulting in a permanent presence of the $P2Y_{12}$ receptors at the plasma membrane, whereas the $P2Y_1$ receptors remained inside the cells as long as the nucleotide was present in the medium.

Materials and Methods

Materials. ADP, ADPβS, U46619, 2MeSADP, adenylyl 5′-imidodiphosphate, GTP, forskolin, prostaglandin E_1 (PGE_1), adrenalin, isobutylmethyl xanthine, and essentially fatty acid-free human serum albumin were from Sigma (Saint Quentin-Fallavier, France). Thrombin-receptor-activating peptide (TRAP) was purchased from Neosystem (Strasbourg, France). AR-C69931MX was a generous gift from Astra (Charnwood, UK). Human fibrinogen was from Kabi (Stockholm, Sweden), and Fura-2/acetoxymethyl ester (Fura-2/AM) was from Calbiochem (Meudon, France). The cAMP assay kit was from Amersham Biosciences (Les Ulis, France). Apyrase (ATP-diphosphohydrolase; EC 3.6.1.5) was purified from potatoes (Cazenave et al., 1983). Dulbecco's modified Eagle's medium, G418 (Geneticin), and PBS were from Invitrogen (Paris, France), and FuGENE 6 transfection reagent was from Roche Diagnostics (Mannheim, Germany). Texas Red-transferrin conjugates and Lysotracker were from Molecular Probes (Eugene, OR).

Synthesis of eGFP-P2Y Fusion Proteins. eGFP was fused to the C-terminal and N-terminal ends of the human $P2Y_1$ and $P2Y_{12}$ receptors, respectively, to generate the construct $P2Y_1$-eGFP and $P2Y_{12}$-eGFP. The chimera N was constructed by polymerase chain reaction amplification of the coding sequence of the $P2Y_{12}$ and in frame cloning downstream of the eGFP coding sequence into the pCDNA3 vector (Invitrogen, Groningen, The Netherlands), preceded by a signal sequence derived from the α7-5-hydroxytryptamine$_3$. Chimera C construct was obtained by cloning the coding sequence of the $P2Y_1$ receptor into the pEGFP-N3 vector (BD Biosciences Clontech, Palo Alto, CA). Characterization of these cells is shown in supplemental data.

Cell Culture. Human astrocytoma 1321N1 cells (reference no. 80630402, European Collection of Cell Cultures, UK) were cultured as described previously (Kauffenstein et al., 2004). Cells were grown to 50 to 80% confluence overnight in 35-mm culture dishes and transfected with 1 to 2 μg/dish plasmid DNA by non–liposome-mediated transfer using FuGENE 6 transfection reagent at a ratio of 1:3 (micrograms of DNA/microliters of FuGENE6). After 24 to 48 h, the cells were washed and stable transfectants were selected in the presence of G418 (0.8 mg/ml). Twelve hours before the experiment, apyrase (0.5 U/ml) was added to the cell culture to prevent desensitization of the receptors by naturally occurring nucleotide release from cells.

Platelet Preparation. Washed human platelets were prepared as described previously (Cazenave et al., 1983) and resuspended in Tyrode's buffer containing 2 mM $CaCl_2$, at a density of 3×10^5 platelets/μl, in the presence of 0.02 U/ml apyrase, a concentration sufficient to prevent the desensitization of platelet ADP receptors during storage.

Desensitization Procedures. Two different procedures were used to study the desensitization of the P2 receptors. First, the washed platelets or the cells were incubated with ADP (10 μM) for 1, 5, or 15 min, followed by apyrase (0.2 U/ml for 30 s) to remove the ADP. Platelets or cells were then restimulated with ADPβS (10 μM), the nonhydrolyzable analog of ADP. The functionality of the $P2Y_1$ and $P2Y_{12}$ receptors was determined by intracellular calcium measurements and intracellular cAMP assays, respectively, as described below. The second procedure was already reported (Baurand et al., 2000). In brief, the platelets or the cells were incubated with ADPβS (1 mM) or with 2MeSADP (1 mM) for 1 h at 37°C. The agonist was then removed by centrifugation (Baurand et al., 2000). Under these experimental conditions, washed platelet suspensions, with no added fibrinogen and in the absence of stirring, do not aggregate during the ADP-induced desensitization.

Preparation of Human Platelet Membranes. Platelet plasma membranes were prepared essentially as described previously (Barber and Jamieson, 1970; Gachet et al., 1995). Washed platelets were loaded with glycerol by centrifugation through a 0 to 30% glycerol gradient and lysed in a hypotonic 10 mM Tris/HCl buffer, pH 7.5. After lysis, broken platelets were layered onto a 30% sucrose cushion and centrifuged for 4 h at 60,000*g*. The floating plasma membranes were removed and pelleted by centrifugation at 100,000*g* and resuspended in 10 mM Tris/HCl buffer, pH 7.5, and stored at −80°C. For desensitization conditions, washed platelets were first treated with ADP (10 μM) for 15 min, and ADP (10 μM) was added during subsequent centrifugation through the glycerol gradient.

Adenylyl Cyclase Assay on Human Platelet Plasma Membranes. The reaction mixture for adenylyl cyclase assay contained 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM isobutylmethyl xanthine, 2 mg/ml bovine serum al-

bumin (BSA), 30 μM adenylyl 5′-imidodiphosphate (a nonhydrolyzable ATP analog used as adenylyl cyclase substrate), 10 μM GTP, and 30 μg of membrane protein. The following reagents were added: 1 μM PGE_1, 10 μM AR-C69931MX, 1 μM 2MeSADP, or 10 μM adrenalin in a total volume of 250 μl. Incubation was started by addition of platelet membrane suspension to the reaction mixture and carried out in duplicate for 10 min at 37°C. Reaction was stopped by addition of 25 μl of ice-cold 6.6 N perchloric acid, and cAMP was extracted and quantified using the commercial radioimmunoassay kit as described previously (Baurand et al., 2000).

Intracellular Signaling. For intracellular calcium measurements, platelets were loaded with Fura-2/AM as described previously (Hechler et al., 1998b). Suspension of 1321N1 cells (15×10^6 cells/ml) was loaded with 5 μM Fura-2/AM for 30 min at 37°C, and cells were resuspended at a density of 2×10^6 cells/ml for intracellular calcium measurements. cAMP measurements of adherent cells or platelets were performed as described previously (Hechler et al., 1998b).

Confocal Microscopy. 1321N1 cells were grown to about 50% confluence on fibronectin-coated coverslips. The medium was replaced with PBS containing calcium and 0.1% fatty acid-free human serum albumin, and the cells were incubated at 37°C in the presence of the agonist or the vehicle. Reactions were stopped by washing the dishes with ice-cold PBS. After fixation with 2% paraformaldehyde in PBS for 15 min at room temperature, the cells were examined by confocal microscopy using an inverted microscope Zeiss LSM510. The amount of internalized eGFP-receptor was determined by quantifying the intracellular fluorescence using an image analysis software (MetaMorph; Universal Imaging Corporation, Downingtown, PA). At each time point, at least 25 cells were analyzed. In double-labeling experiments, the 1321N1 cells were either pretreated with 50 μg/ml Texas Red-conjugated transferrin for 45 min before addition of ADPβS (1 mM), or they were incubated with 75 nM Lysotracker Red after cell fixation. In some experiments, sucrose (0.45 M) was added during the incubation with the agonist.

Generation of $P2Y_1$ and $P2Y_{12}$ Polyclonal Antibodies. The antibody against $P2Y_1$ was generated by immunizing rabbits with a peptide of 15 N-terminal amino acids of human $P2Y_1$ (GTDAAFLAGPGSSWG). The anti-$P2Y_{12}$ antibody was a rabbit polyclonal IgG raised against the second extracellular domain (TNRQPRGKNVKKC) and the C-terminal domain of human $P2Y_{12}$ (CKKEQDGGDPNEETPM). The characterization of the antibodies was performed by flow cytometry using 1321N1-transfected cells expressing the $P2Y_1$ receptors or HEK-293 cells expressing the $P2Y_{12}$ receptors (kindly provided by Sylvie Reigner-Meyer, Roche Diagnostics, Basel, Switzerland).

Transmission Electron Microscopy and Immunolabeling. For transmission electron microscopy, two types of approaches were used, a post- and a pre-embedding method. In the postembedding method, platelets were fixed at several time points after activation, and immunogold labeling was performed on sections. This approach allows quantifying the distribution of the receptors at defined time points. In brief, washed platelets (1 ml) were fixed with an equal volume of 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer for 1 h. The fixed platelets were infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Ultrathin sections (70 nm) were prepared, incubated in PBS containing 1% BSA for 10 min and then in 0.02 M glycine in PBS for 10 min. Samples were incubated at 25°C for 1 h with anti-$P2Y_1$ (10 μg/ml), anti-$P2Y_{12}$ (3 μg/ml), or the corresponding nonimmune antibodies and then washed in PBS-BSA. After further incubation with protein A-10-nm gold for 20 min at 25°C, the samples were examined under a Philips CM120 electron microscope at 120 kV (Eindhoven, The Netherlands). Immunogold particles on platelets were counted manually and assigned to various subcellular compartments, including 1) the platelet surface, 2) the inner compartments, and 3) the cytosol. At each time point, assignment of 450 to 800 gold particles on about 30 to 50 platelets enabled calculation of the percentage of labeling of each compartment. The total number of particles per platelet obtained with each antibody was considered to be the total labeling (100%). The number of particles corresponding to each antibody was expressed as the percentage of total labeling.

We also used the pre-embedding approach that allows studying the dynamic of the receptor internalization from the external membranes to cellular compartments. In this method, gold-labeled antibodies were directly coupled to gold particles and were incubated with the platelets during activation, and the samples were fixed at indicated time points. In brief, washed platelets (1 ml) were incubated with a mixture of anti-$P2Y_1$ antibodies labeled with 15-nm gold particles (5 μg) and anti-$P2Y_{12}$ antibodies labeled with 10-nm gold particles (5 μg), 1 min before addition of ADP (5 μM). The platelets were fixed for 1 h by adding an equal volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were then incubated at room temperature with 1% tannic acid and processed for transmission electron microscopy as described previously (Eckly et al., 2001).

Results

Desensitization of $P2Y_1$-Dependent ADP-Induced Calcium Increase. Platelets were stimulated with ADP (10 μM) for 1, 5, or 15 min, followed by 0.2 U/ml apyrase treatment for 30 s to remove ADP and subsequent restimulation with 10 μM ADPβS, a nonhydrolyzable ADP analog, under stirring. Under these conditions, the intracellular calcium increase induced by ADPβS (Fig. 1A, a) was abolished at all time points tested (Fig. 1A, b). ADP-pretreated platelets were still responsive to 10 μM serotonin and 1 U/ml thrombin (data not shown), indicating that a general desensitization/refractoriness of the calcium response is unlikely to occur and is not responsible for the absence of response to ADP. Likewise, in $P2Y_1$-eGFP transfected cells, the calcium signal induced by ADPβS (10 μM) (Fig. 1B, a) was completely inhibited after a first challenge with ADP for up to 15 min (Fig. 1B, b). However, the desensitization of the $P2Y_1$-driven calcium signal was transient because the calcium response reappeared after 5 min when ADP was removed by three washes (data not shown). Similar experiments were performed in platelets and in transfected cells pretreated with 1 mM ADPβS or with 1 mM 2MeSADP for 1 h (second procedure, see *Materials and Methods*) with identical results (data not shown). Thus, the $P2Y_1$ receptor-mediated increase in intracellular calcium was desensitized both in platelets and in transfected cells.

Absence of Desensitization of $P2Y_{12}$-Dependent ADP-Induced Inhibition of cAMP Formation. The $P2Y_{12}$-mediated inhibition of cAMP production both in platelets and in $P2Y_{12}$-transfected cells was studied as described above after stimulation with ADP for 1, 5, or 15 min, followed by apyrase treatment (0.2 U/ml, 30 s) and subsequent restimulation with 10 μM ADPβS. As shown in Fig. 2, ADPβS-induced inhibition of cAMP accumulation was not affected by 1, 5, or 15 min ADP pretreatment in either platelets (Fig. 2A) or transfected cells (Fig. 2B). It is noteworthy that forskolin-induced cAMP accumulation in $P2Y_{12}$-transfected cells decreased with the time of ADP-pretreatment (Fig. 2B), whereas cAMP levels induced by PGE_1 stimulation of platelets were not affected by ADP pretreatment (Fig. 2A). This difference between 1 min ADP pretreatment versus 15 min (Fig. 2B, black columns) most probably results from the constitutive release of nucleotides by the cultured cells in the medium. These nucleotides may act on the $P2Y_{12}$ receptor to

inhibit the adenylyl cyclase, thus counteracting the effect of forskolin, because addition of the selective $P2Y_{12}$ antagonist AR-C69931MX 10 μM completely restored the effect of forskolin (Fig. 2B, gray columns). Likewise, AR-C69931MX reversed the inhibitory effect of ADPβS on cAMP accumulation in platelets (Fig. 2A). These results indicated that the $P2Y_{12}$ receptor-dependent inhibition of cAMP production remains functional upon ADP stimulation and is not desensitized. Similar results were obtained with platelets or transfected cells pretreated with ADPβS (1 mM) or with 2MeSADP (1 mM) for 1 h (data not shown), a procedure previously shown to induce a stable desensitization state of the $P2Y_1$ receptor for several hours (Baurand et al., 2000).

To avoid the possibility that resensitization of the $P2Y_{12}$ receptor after ADP pretreatment might be occurring during subsequent adenylyl cyclase assays, plasma membranes from vehicle- or ADP-pretreated platelets (15 min) were prepared (see *Materials and Methods*), and cAMP levels were measured. As shown in Fig. 2C, 2MeSADP-induced inhibition of cAMP accumulation in platelet membranes was not affected by the ADP pretreatment, whereas the $P2Y_{12}$ antagonist AR-C69931MX inhibited the effect of ADP. These data add strong evidence that the $P2Y_{12}$ receptor remains functional upon ADP stimulation and is not desensitized. Together, these results demonstrate both in a native and in a heterologous model of transfected cells that the $P2Y_1$ receptor can be desensitized, whereas the $P2Y_{12}$ is not.

$P2Y_{12}$-Dependent Amplification of Gq-Coupled Responses Are Conserved in ADP-Prestimulated Platelets. To further establish functionality of the $P2Y_{12}$ receptor, we checked for its capacity to potentiate responses to agonists of other Gq-coupled receptors such as the PAR-1 agonist peptide SFFLRN (TRAP) or the TP receptor agonist U46619. As shown in Fig. 3, AR-C69931MX (10 μM) inhibited the PAR-1 and the TP receptor-mediated platelet aggregation to a similar extent in control or ADP-pretreated platelets, demonstrating that the $P2Y_{12}$ receptor activation pathway remained functional. Again, these results were confirmed after pretreatment with 1 mM ADPβS or 1 mM 2MeSADP for 1 h (data not shown).

Internalization of $P2Y_1$- and $P2Y_{12}$-eGFP Receptors in Transfected Cells. Our previous radioligand binding studies suggested that the $P2Y_1$ receptor could undergo internalization, whereas the $P2Y_{12}$ receptor was retained at the plasma membrane (Baurand et al., 2000). The intracellular trafficking was investigated in more detail by confocal microscopy on eGFP-$P2Y_1$ and $P2Y_{12}$ receptors in individually transfected cell lines. The cells have been examined at different time points after agonist incubation. As shown in Fig. 4A, ADPβS (1 mM) induced internalization of the $P2Y_1$ receptor within minutes, and which remained intracellularly located for at least 1 h. By contrast, only a small fraction of the $P2Y_{12}$ receptors was found intracellularly, whereas most of the $P2Y_{12}$ receptors remained at the plasma membrane (Fig. 4A). The intracellular fluorescence (expressed as the percentage of total fluorescence) was quantified using an image analysis software (MetaMorph) with a minimum of 25 cells examined at each time point (Fig. 4B). After a 15-min stimulation of $P2Y_1$-expressing cells with ADPβS, 50% of the total fluorescence was located intracellularly (Fig. 4B) and increased to 75% after 1-h stimulation (Fig. 4B). Concerning the $P2Y_{12}$ receptor, stimulation with ADPβS (1 mM) for 30 s, 60 s, 2 min, or 5 min did not induce intracellular relocation of the $P2Y_{12}$ receptor (Fig. 4B; data not shown). After 15 min, the $P2Y_{12}$ receptor was found to enter the cells transiently, because only a small increase in intracellular fluorescence was observed compared with a longer time point (Fig. 4B). Moreover, clear labeling of the plasma membrane was observed at all time points, indicating that either a large proportion of the $P2Y_{12}$ receptors was not internalized or a very

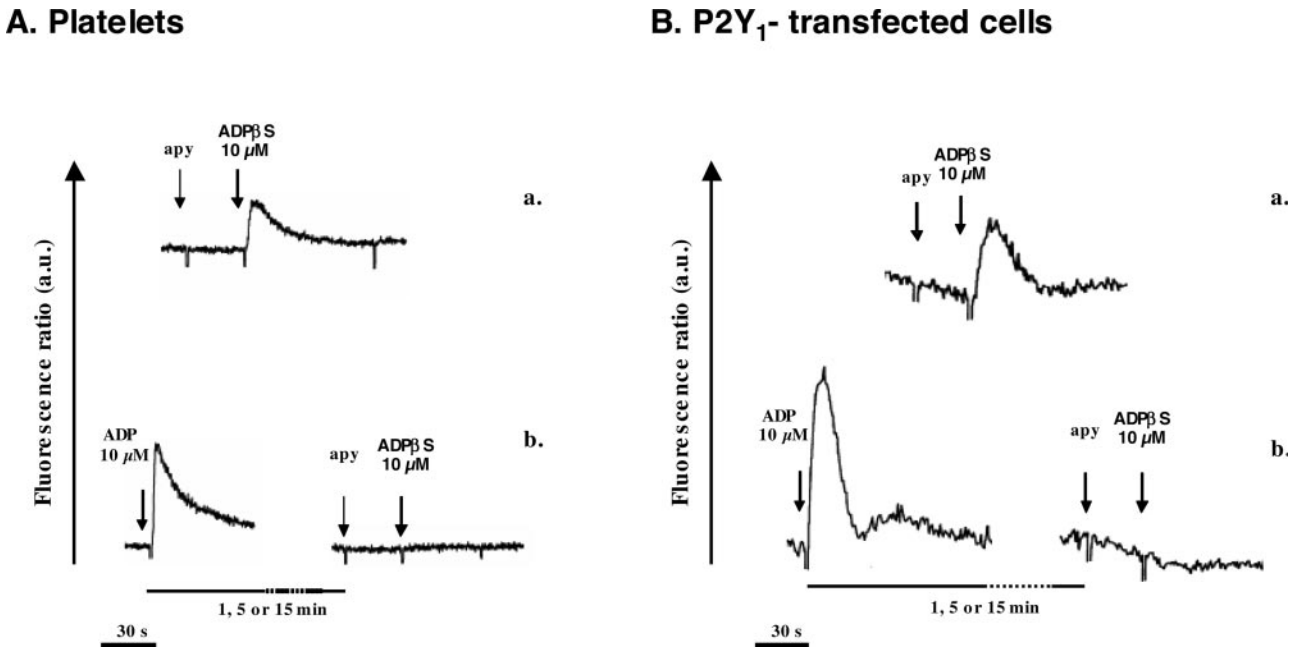

Fig. 1. Desensitization of the $P2Y_1$ receptor after exposure to ADP. Suspensions of washed platelets (A) or of $P2Y_1$-eGFP transfected cells (B) were pretreated with vehicle (a) or 10 μM ADP for 1, 5, or 15 min (b), followed by apyrase (0.2 U/ml) for 30 s to remove ADP, and subsequently with 10 μM ADPβS. Intracellular calcium signals were measured in platelets or cells loaded with Fura-2/AM. Data are representative of three independent experiments.

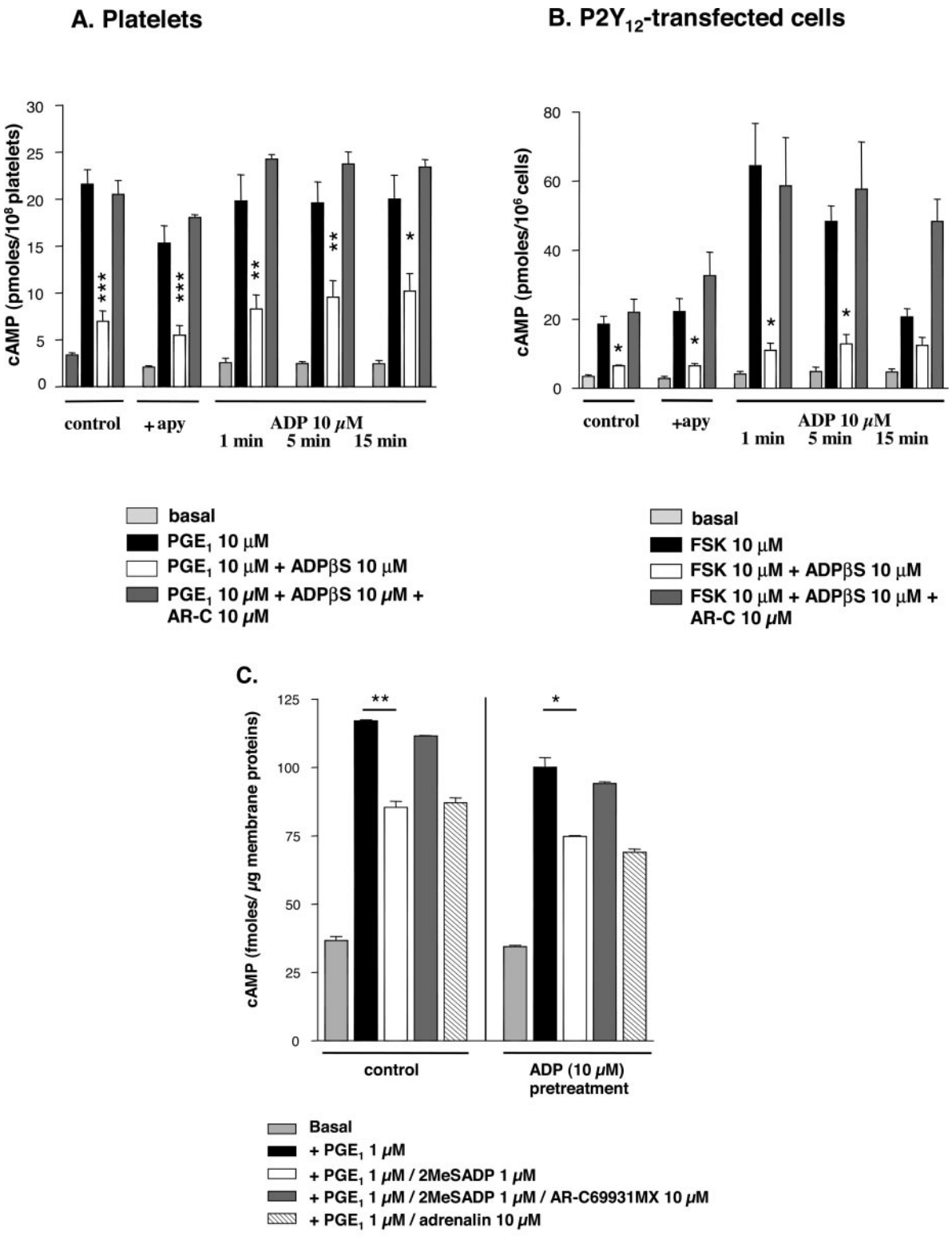

Fig. 2. $P2Y_{12}$ receptor-mediated inhibition of cAMP production remains functional after ADP stimulation. A, measurement of cAMP levels induced by 10 μM PGE_1, 10 μM PGE_1 + 10 μM ADPβS, or 10 μM PGE_1 + 10 μM ADPβS + 10 μM AR-C69931MX in intact platelets pretreated with vehicle (control), with 0.2 U/ml apyrase alone (30 s) or with 10 μM ADP for 1, 5, or 15 min followed by 0.2 U/ml apyrase for 30 s. Data are the mean of three independent experiments, performed in duplicate. B, measurement of cAMP levels induced by 10 μM forskolin, in the presence or absence of 10 μM ADPβS and 10 μM AR-C69931MX. The $P2Y_{12}$-eGFP cells were pretreated with vehicle, 0.2 U/ml apyrase alone (30 s), or with 10 μM ADP for 1, 5, or 15 min followed by 0.2 U/ml apyrase for 30 s. Data are the mean of three independent experiments, performed in duplicate. C, measurement of cAMP levels induced by 1 μM PGE_1, 1 μM PGE_1 + 1 μM 2MeSADP, 1 μM PGE_1 + 1 μM 2MeSADP + 10 μM AR-C69931MX or 1 μM PGE_1 + 10 μM adrenalin in human platelet plasma membranes. Adrenalin (10 μM) was used as a positive control. Membranes were prepared as described under *Materials and Methods* from washed platelets treated with vehicle (control) or with 10 μM ADP for 15 min. Data are the mean of four independent experiments, with two different platelet plasma membrane preparations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus forskolin or PGE1 stimulation, respectively, using paired Student's t test.

rapid turnover of receptor recycling occurred, allowing the maintenance of the receptors at the cell surface.

ADPβS-induced internalization of the $P2Y_1$ receptor was significantly inhibited by treatment with sucrose (0.45 M), an inhibitor of the clathrin-coated endocytic pathway (Fig. 4C). In contrast, sucrose had no effect on the early relocalization of the $P2Y_{12}$ receptors. Double-labeling experiments indicated that the $P2Y_1$-eGFP receptor relocalized into vesicles that overlapped with those stained by Texas Red-transferrin, a marker of early endosomes, contrary to $P2Y_{12}$-eGFP receptors. These results suggested that $P2Y_1$ receptor sequestration, unlike the $P2Y_{12}$ receptor, occurred through a clathrin-endocytotic pathway. Finally, the pattern of $P2Y_1$ and $P2Y_{12}$ fluorescence did not coincide with that of LysoTracker Red, which labels lysosomes, suggesting that these receptors do not enter the lysosomal pathway (Fig. 4C).

Characterization of Polyclonal Antibodies against the $P2Y_1$ and $P2Y_{12}$ Receptors. To study the cellular distribution of $P2Y_1$ and $P2Y_{12}$ upon ADP stimulation, polyclonal antibodies were raised as described in *Materials and Methods*, and characterized by flow cytometry analyses. As shown in Fig. 5A, 1321N1-$P2Y_1$ cells incubated with the $P2Y_1$ antibody exhibited a 10-fold rightward shift of the mean fluorescence intensity compared with cells incubated with nonimmune serum (Fig. 5A, left), whereas HEK-$P2Y_{12}$ cells incubated with the $P2Y_{12}$ antibody exhibited a 9-fold rightward shift of the fluorescence intensity (Fig. 5B, left). No fluorescence shift was detected with nontransfected cells (Fig. 5, A and B, right). In addition, no labeling of anti-$P2Y_{12}$ antibody on 1321N1-$P2Y_1$ cells (Fig. 5A, dotted lines) or of anti-$P2Y_1$ antibody on HEK-$P2Y_{12}$ cells was detected (Fig. 5B, dotted lines), indicating absence of cross-reactivity between the two antibodies.

Localization of the $P2Y_1$ and $P2Y_{12}$ Receptors in Platelets by Electron Microscopy. A first approach was to examine the distribution of both receptors by performing postembedding immunostaining of ADP-stimulated platelets. This method allows the quantification of the receptor distribution at defined time points and was performed on cryosections, a procedure preserving the antigenicity of the proteins and the subcellular structures. In resting platelets and 7 s after ADP stimulation, $P2Y_1$ receptors were detected on the surface of the plasma membrane and in the open canalicular system (OCS) (Fig. 6A). After 45 s, at maximum of aggregation, the $P2Y_1$ receptors occurred in inner compartments where they were located in α-granules (Fig. 6A). After 4 min, the $P2Y_1$ receptors were detected both on the extracellular membranes and in inner compartments, whereas after 15 min, the majority of the $P2Y_1$ receptors were again located at the surface of the platelets. For the $P2Y_{12}$ receptors (Fig. 6B), the receptors were located on the external membranes in resting platelets (Fig. 6B, R). Some of the gold particles that seem apparently intracellular are in fact located on the OCS, which is an involution of, and continuous with, the outer platelet plasma membrane and not an inner compartment (Fig. 6B, arrowheads). After 7 s, a small fraction of the $P2Y_{12}$ receptors was observed in inner compartments (Fig. 6B, arrows). After 45 s, 4 min, or 15 min, most of the $P2Y_{12}$ receptors were located in external membranes (Fig. 6B). The quantification of the immunostainings corresponding to $P2Y_1$ and $P2Y_{12}$ receptors is shown in Fig. 6C (average of 40 platelets for each time point). In resting platelets, 50 $\pm$ 2% of $P2Y_1$ and 50 $\pm$ 2% of $P2Y_{12}$ receptors were located at the external membranes and weakly in inner compartments (10 $\pm$ 2 and 13 $\pm$ 1%, for $P2Y_1$ and $P2Y_{12}$ receptors, respectively). At 45 s and 4 min, the $P2Y_1$ receptors have mostly disappeared from the plasma membrane (Fig. 6C, left, ■) and were found in inner compartments (32 $\pm$ 3% at 45 s, 26 $\pm$ 2% at 4 min) (Fig. 6C, left, ▲), whereas at 15 min, they were observed on the external membranes (40 $\pm$ 2%; Fig. 6C, left, ■). On the other hand, the $P2Y_{12}$ receptors, although transiently internalized at 7 s (25 $\pm$ 2%), rapidly returned to the external membrane (42 $\pm$ 2% at 45 s; Fig. 6C, right, ■). These results indicated, similarly to transfected cells, differential relocation of the $P2Y_1$ and $P2Y_{12}$ receptors upon platelet activation with ADP.

A second approach using a pre-embedding procedure was used to study the dynamic of internalization of the receptors

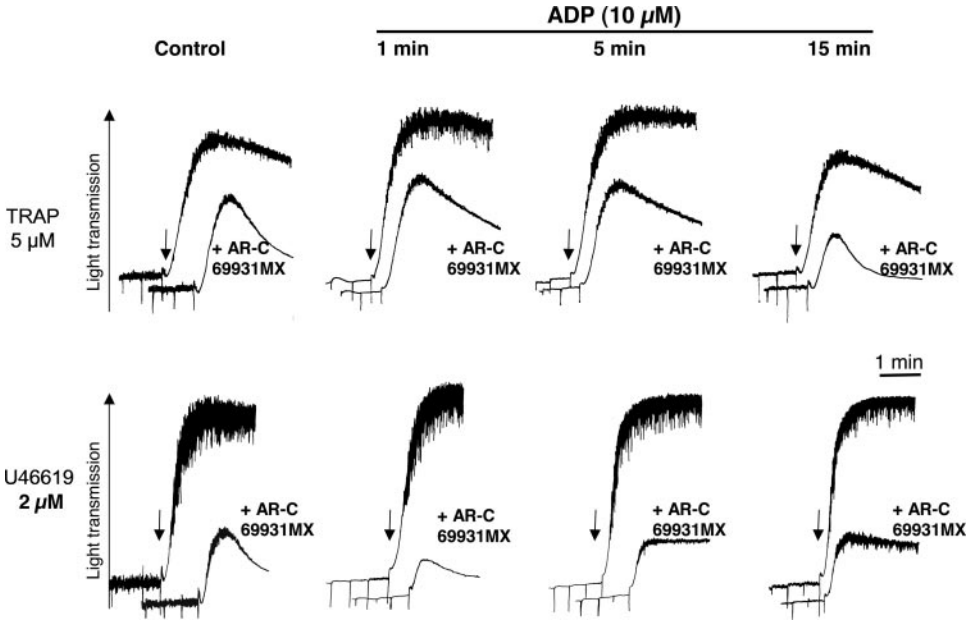

Fig. 3. $P2Y_{12}$ receptor-mediated potentiation of platelet aggregation induced by strong agonists. Platelets were pretreated or not with 10 μM ADP for 1, 5, or 15 min followed by 0.2 U/ml apyrase for 30 s. Aggregation curves were obtained by stimulation of platelets with 5 μM TRAP or 2 μM U46619, in the presence or absence of 10 μM AR-C69931MX, a selective $P2Y_{12}$ receptor antagonist. Data are from one experiment representative of three independent experiments.

from the external membranes to cellular compartments, with fine identification of the organelles. Gold-labeled anti-$P2Y_1$ and anti-$P2Y_{12}$ antibodies were coincubated with the platelets before (T0) or during activation with 5 μM ADP. At the resting state, 15-nm-labeled $P2Y_1$ and 10-nm-labeled $P2Y_{12}$ were localized at the cell wall and the OCS (Fig. 7A). At 45 s, the $P2Y_1$ and $P2Y_{12}$ receptors were still found at the external membranes (Fig. 7B), despite a few gold-labeled $P2Y_{12}$ in inner compartments (Fig. 7C). These observations, which contrast to the internalization kinetic determined with the postembedding method (Fig. 6C), are most probably caused by the difference of technical approaches. Indeed, the conju-

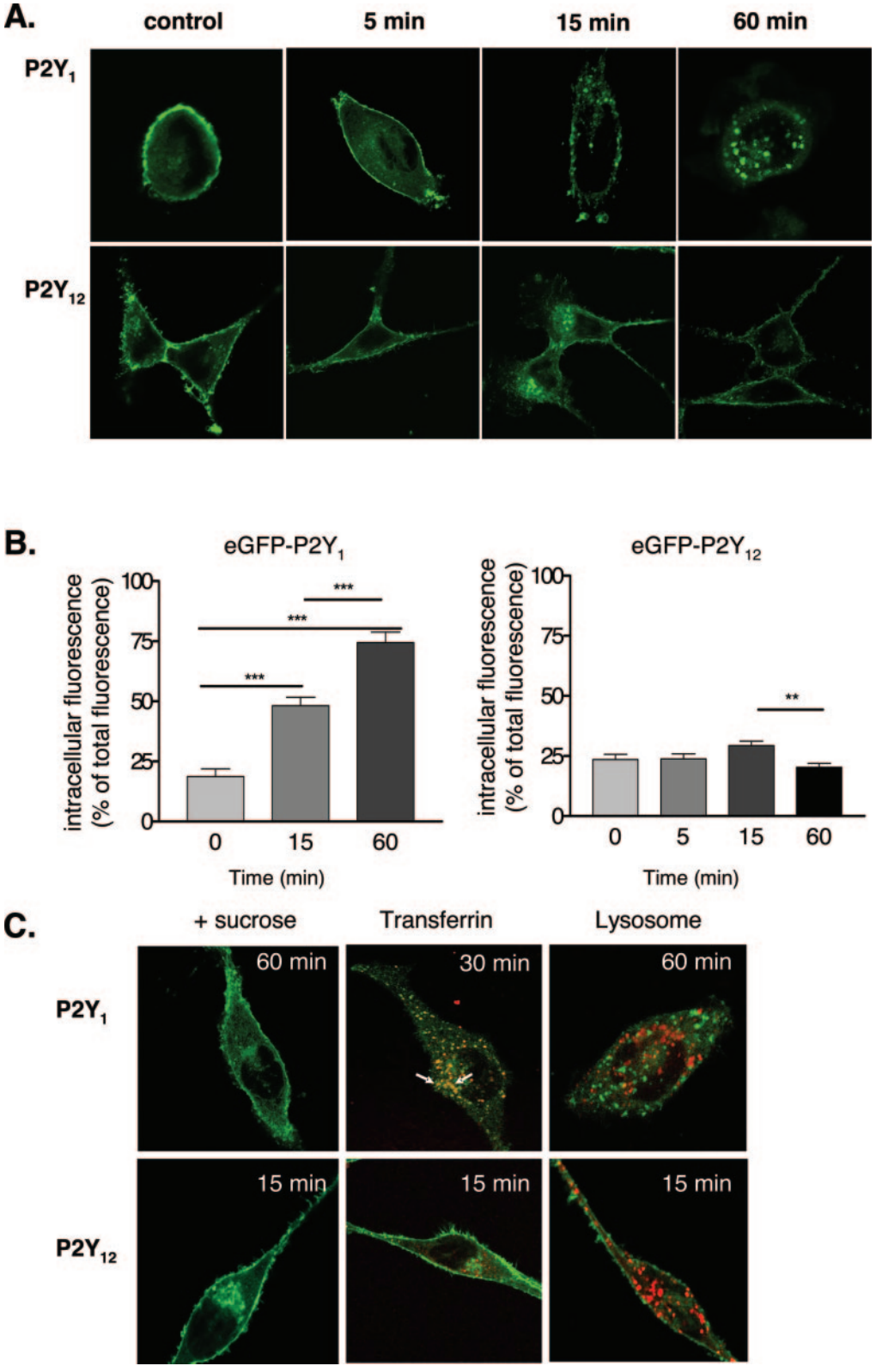

Fig. 4. ADPβS-induced relocalization of the $P2Y_1$ and the $P2Y_{12}$ receptors in astrocytoma cells. A, 1321N1 cells expressing $P2Y_1$-eGFP or $P2Y_{12}$-eGFP were treated with vehicle (control) or ADPβS (1 mM) for increasing periods of time (5–60 min) and observed under a confocal microscope. B, quantification of the internalization of the eGFP receptors. Results are means ± S.E.M. of intracellular fluorescence expressed as the percentage of total fluorescence, after agonist stimulation for 5, 15, or 60 min.**, $p < 0.01$; ***, $p < 0.001$, $n =$ 25–39, using one-way analysis of variance and Bonferroni's multiple comparison test. C, intracellular trafficking of the $P2Y_1$-eGFP and $P2Y_{12}$-eGFP receptors. Effect of 0.45 M sucrose on 1 mM ADPβS-induced internalization of the $P2Y_1$-eGFP (at 60 min) and the $P2Y_{12}$-eGFP (at 15 min). Transfected cells (green) were loaded with Texas Red-transferrin (red) and treated with 1 mM ADPβS for 30 min ($P2Y_1$-eGFP) or 15 min ($P2Y_{12}$-eGFP). The arrows indicate an area of colocalization (yellow). Treatment of transfected cells with 1 mM ADPβS 30 min ($P2Y_1$-eGFP) or 15 min ($P2Y_{12}$-eGFP) did not lead to colocalization of P2 receptors (green) with lysosome marker LysoTracker Red Fluorescence (red). Images are from one experiment representative of three independent experiments.

gation of the antibodies with the gold particles is known to affect their ability to penetrate into the cells (Robinson et al., 2000). The intracellular $P2Y_{12}$ receptors were observed in compartments devoid of clathrin coat, even at 19.5°C, temperature at which the internalization is slowed down (Fig. 7C, inset). Four minutes after stimulation, the $P2Y_{12}$ receptors have returned to the external membranes, whereas almost all the $P2Y_1$ receptors were in inner vesicles (Fig. 7D). These vesicles displayed the classic feature of clathrin-coated vesicles (Fig. 7E, arrowheads), suggesting that the $P2Y_1$ receptors were internalized through a clathrin-endocytic pathway. Ten minutes after stimulation, the $P2Y_1$ receptors were back to the external membrane, with only minor inner labeling (about 14%) (Fig. 7F). Thus, using two different approaches, we found that platelet ADP stimulation induces internalization of the $P2Y_1$ receptors, whereas only one-third of the $P2Y_{12}$ receptors was rapidly and transiently internalized.

Discussion

The aims of the present study were to assess possible differential desensitization and internalization of the $P2Y_1$ and $P2Y_{12}$ receptors in platelets and in heterologously transfected cells. The present results unambiguously confirm that the so-called "refractory state" of platelets to ADP is entirely caused by selective desensitization of the $P2Y_1$ receptor, whereas the $P2Y_{12}$ receptor remains fully functional after either short- (1–15 min) or long-term (1 h) stimulation (data not shown; Baurand et al., 2000). This was the case not only in intact cells but also on membranes prepared from ADP-treated platelets. The functional and physiological consequences are that under conditions where platelets are rendered refractory to ADP, the $P2Y_{12}$ receptor is still able to potentiate aggregation induced by other agonists (Fig. 3). Previous radioligand binding studies suggested that desensitization of the $P2Y_1$ receptor was accompanied by internalization of these receptors but not of the $P2Y_{12}$ receptors (Baurand et al., 2000). In fact, the present study established that both receptors are internalized in platelets or in heterologously transfected cells, but in a different manner. Kinetic analysis using immunoelectron microscopy showed that the platelet $P2Y_1$ receptors were internalized within 1 min and reappeared on the external membranes by 15 min, obviously through recycling, because platelets do not synthesize proteins. This is in line with the fact that desensitization is only transient provided that a scavenger system removes the nucleotide from the medium (Ardlie et al., 1971). In transfected cells, the eGFP-$P2Y_1$ receptor also underwent almost complete internalization. This $P2Y_1$ receptor internalization was transient because 30 min after removal of the agonist the receptors were recycled back to the plasma membrane (data not shown).

On the other hand, the $P2Y_{12}$ receptor was internalized more rapidly, within seconds in platelets, and seemed to recycle rapidly to the platelet membrane. This may explain why the number of $P2Y_{12}$ binding sites was not decreased after agonist pretreatment in our previous study (Baurand et al., 2000). In transfected cells, weak internalization of the fluorescent $P2Y_{12}$ receptor was detected 15 min after agonist stimulation, but the plasma membrane always retained the majority of the fluorescence, suggesting either that only a fraction of the $P2Y_{12}$ receptors was internalized or that the receptor was rapidly recycled to the plasma membrane. Thus, in platelets and in transfected cells, the fraction of the $P2Y_{12}$ receptors present at the plasma membrane did not desensitize, or the rapid turnover of the internalized $P2Y_{12}$ receptors returned to the cell surface in an active form, immediately available for reactivation by agonist, either of which explains the absence of functional $P2Y_{12}$ desensitization. Our results using plasma membranes of ADP-pretreated platelets favor the first hypothesis.

The molecular mechanisms of desensitization and internalization have been extensively studied in several G protein-coupled receptors (Hall and Lefkowitz, 2002). Receptors are initially desensitized by rapid phosphorylation allowing the binding of β-arrestin followed by internalization via clathrin-coated pits. Once internalized into endosomes, the receptors become dephosphorylated and are recycled back to the plasma membrane ready for reactivation. Electron microscopy clearly showed that the $P2Y_1$ receptor was internalized in vesicles coated with clathrin (Fig. 7E), whereas in eGFP-$P2Y_1$ cells internalization was inhibited in the presence of hypertonic sucrose, which leads to abnormal clathrin polymerization. In addition, sucrose prevented internalization of the $P2Y_1$ receptor in intact platelets, as measured by radioligand binding experiments (A. Baurand, unpublished data), adding evidence that the $P2Y_1$ receptor is internalized through a clathrin-dependent pathway toward early endosomes. The internalization mechanisms are very different for the $P2Y_{12}$ receptor because the intracellular compartments were devoid of coat (Fig. 7C), and the endocytic process was

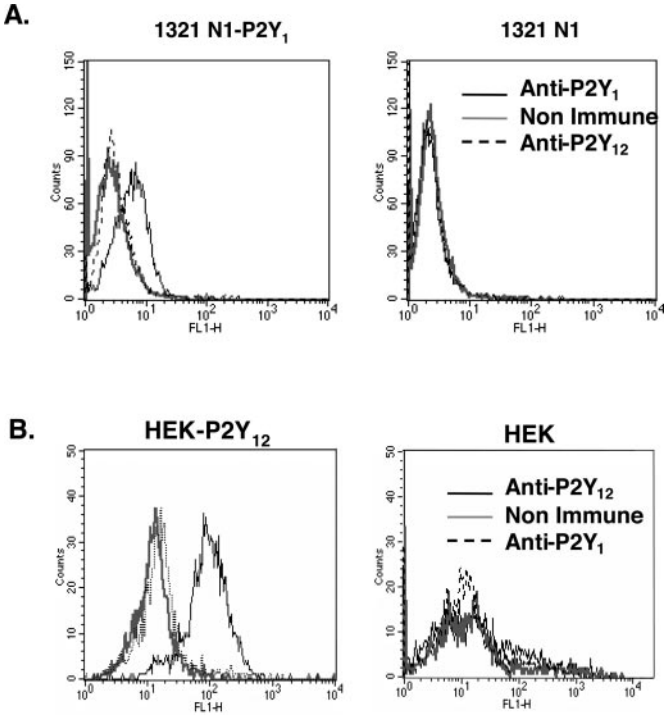

Fig. 5. Characterization of polyclonal antibodies against the $P2Y_1$ and $P2Y_{12}$ receptors. A, flow cytometry analyses of 1321N1-$P2Y_1$ or nontransfected 1321N1 cells incubated with the anti-$P2Y_1$ (black line), anti-$P2Y_{12}$ antibody (dotted line), or control IgG (gray line). B, flow cytometry analyses of HEK-$P2Y_{12}$ or nontransfected HEK cells incubated with the anti-$P2Y_{12}$ (black line), anti-$P2Y_1$ (dotted line), or control IgG (gray line). Data are from one experiment representative of at least five independent experiments.

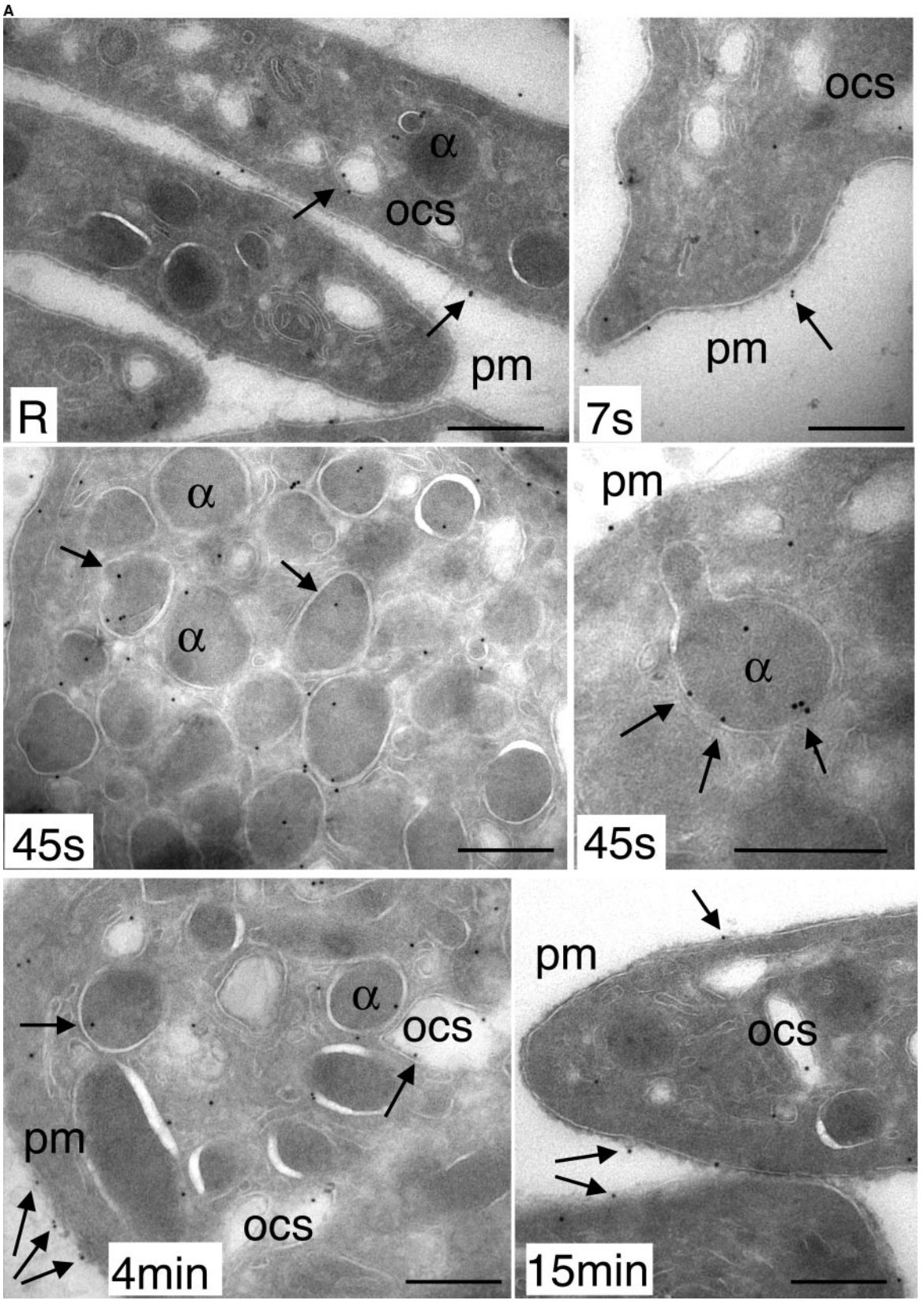

Fig. 6. Redistribution of $P2Y_1$ and $P2Y_{12}$ receptors after platelet activation with ADP. Platelets were activated with 5 μM ADP and fixed at different time points corresponding to the resting state (R): platelet shape change (7 s), the maximum amplitude of aggregation (45 s), disaggregation (4 min), and 15 min where platelets have returned to their discoid state. Cells were then frozen, cut, and immunogold labeling was performed on thin cryosections as described under *Materials and Methods*. A, localization of the $P2Y_1$ receptors. $P2Y_1$ receptors were located on the external membranes in resting platelets (R) and 7 s after ADP stimulation (arrows). After 45-s stimulation, the $P2Y_1$ receptors were mainly found in α-granules (arrowheads). Similar relocation was found at 4 min, whereas after 15 min, the $P2Y_1$ receptors were located on the external surface of the platelets (arrows).

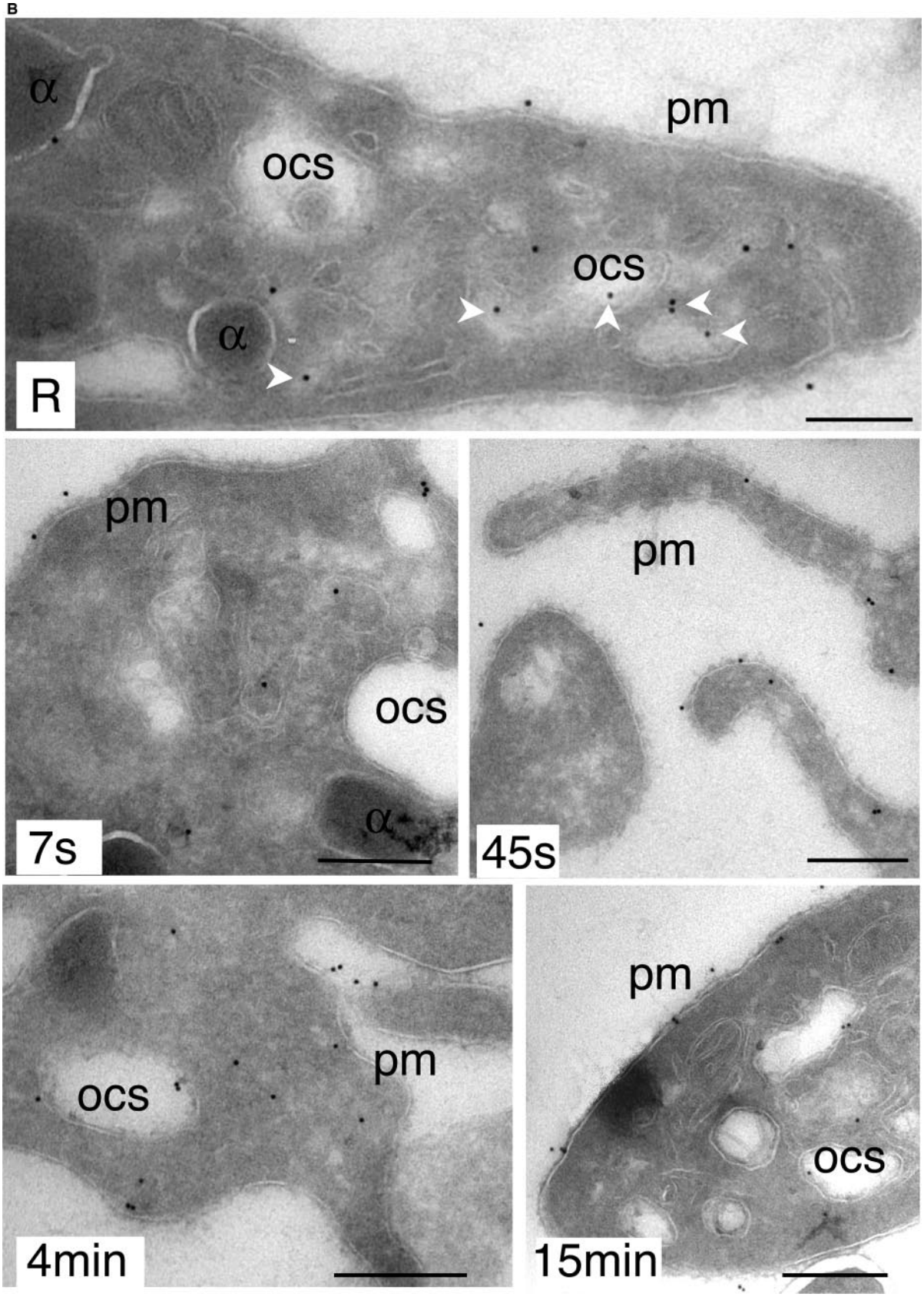

Fig. 6 cont. B, localization of the $P2Y_{12}$ receptors. $P2Y_{12}$ receptors were located on the external membranes in resting platelets (R). Some of the gold particles which seem apparently intracellular are in fact located on the OCS (arrowheads), which is an involution of, and continuous with, the outer platelet plasma membrane and not an inner compartment. After 7 s, some $P2Y_{12}$ receptors were associated with inner vesicles (arrows). At 45 s, and 4 and 15 min, the $P2Y_{12}$ receptors were mainly found on the external surface. Scale bars, 200 nm.

unaffected by sucrose. In addition, the internalized $P2Y_{12}$ receptors did not colocalize with transferrin, a marker of early endosomes, suggesting a different mechanism for internalization. Less is known about the mechanisms that link membrane proteins to non–clathrin-coated vesicles. It will be important to determine whether an adaptor protein for the $P2Y_{12}$ receptor directs the receptors to caveolae or other endocytic machinery.

Agonist-induced phosphorylation on putative serine, threonine, or tyrosine residues is a common feature of desensitization and internalization. Both the $P2Y_1$ and $P2Y_{12}$ receptors contain the DRYXXI/VXXP motif at the end of the third transmembrane domain that has been proposed as important for receptor internalization (Moro et al., 1993). In addition, $P2Y_1$ and $P2Y_{12}$ receptors also possess putative phosphorylated residues in the third intracellular loop and in the C-terminal part. The number of such residues is however higher in the C-terminal part of the $P2Y_1$ than the $P2Y_{12}$ receptor, and it has been suggested that the phosphorylation density could be more important for functional desensitization than for the internalization process (Hammes et al., 1999). The $P2Y_1$ receptor also possesses three putative protein kinase C phosphorylation sites, of which threonine 339 has been implicated in a protein kinase C-dependent negative feedback mechanism (Fam et al., 2003). Whether the different behavior of the $P2Y_{12}$ receptor is caused by structural motifs deserves further investigation. Like the $P2Y_1$ receptor, other platelet Gq-coupled receptors such as 5-hydroxytryptamine$_{2A}$, protease-activated receptor 1, or thromboxane A2 receptors are rapidly desensitized and internalized (Okwu et al., 1992; Roevens and de Chaffoy de Courcelles, 1995; Kawabata et al., 1999). Thus, in view of the central role of the $P2Y_{12}$ receptor in platelet activation and hemostasis, one may speculate that in vivo, in case of desensitization of Gq-coupled receptors, the Gi-coupled ADP pathway should still be able to potentiate the signals triggered by adhesion receptors such as GPIb, GPVI, or integrin $\alpha 2\beta 1$, which would represent a way for the cells to maintain their hemostatic properties (Gachet, 2001; Conley and Delaney, 2003; Hardy et al., 2004).

In summary, we have reported that the $P2Y_1$ and $P2Y_{12}$ receptors are differentially regulated after platelet activation. The $P2Y_1$ receptor is rapidly desensitized and internalized, whereas the $P2Y_{12}$ receptor remains functional and mainly persists at the plasma membrane. As a consequence, even in platelets refractory to stimulation by ADP, the $P2Y_{12}$ receptor is able to ensure platelet reactivity at sites of vessel injury, thus preventing loss of the hemostatic function.

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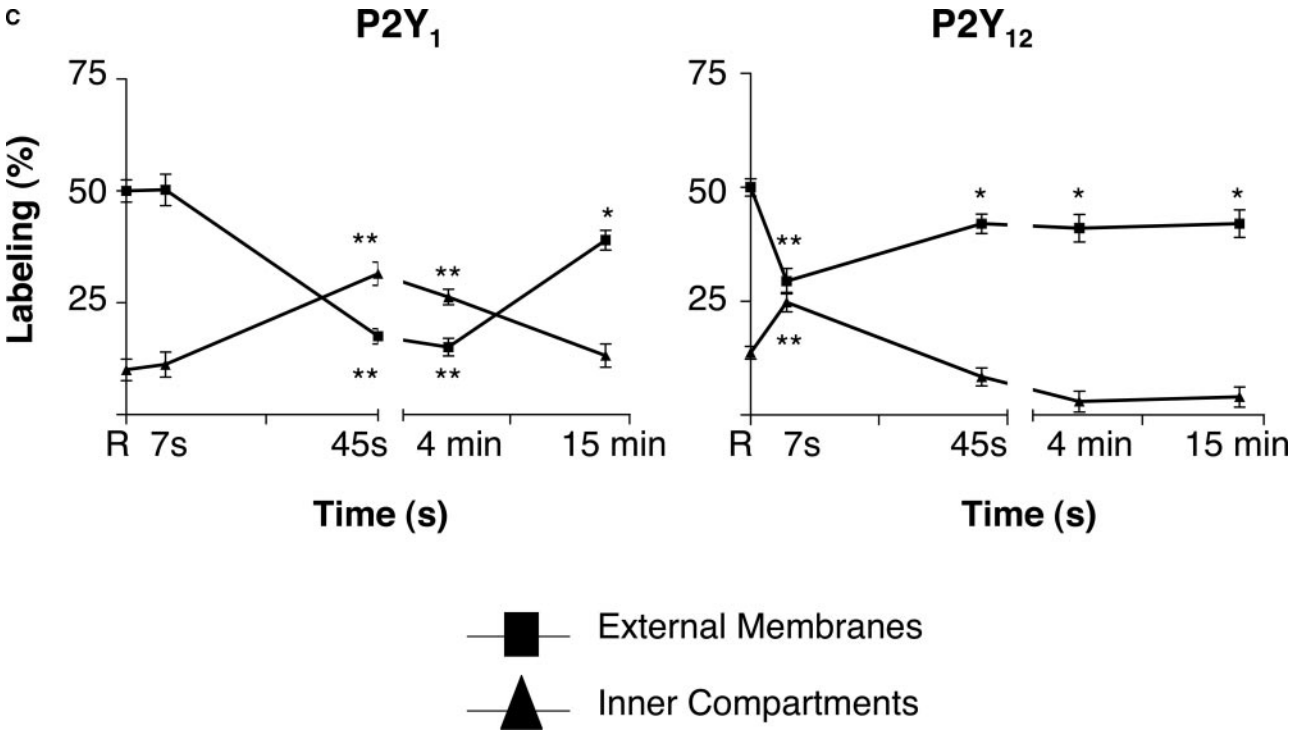

Fig. 6 cont. C, quantification of immunogold particles in resting and ADP-stimulated platelets. Immunogold particles on an average of 40 platelets were counted manually in randomly chosen platelets and assigned to various subcellular compartments: 1) the external membranes (EM) comprising the plasma membrane and open canalicular system, and 2) the inner compartments (IC), including the α-granules and platelet vesicles. The $P2Y_1$ or $P2Y_{12}$ and corresponding nonimmune labeling were determined for each compartment. The results are presented as specific $P2Y_1$ or $P2Y_{12}$ labeling expressed as percentage of the total labeling (corresponding to the specific plus the non immune labeling). Total labeling using the polyclonal antibodies against $P2Y_1$ and $P2Y_{12}$ was 22 ± 5.0 and 20 ± 3.6 particles per platelet, respectively, and the corresponding non immune labeling was 7.6 ± 0.9 and 7.14 ± 0.9 particles per platelet, respectively. *, $p < 0.05$; **, $p < 0.01$, versus resting, using one-way analysis of variance with Dunnett's post test.

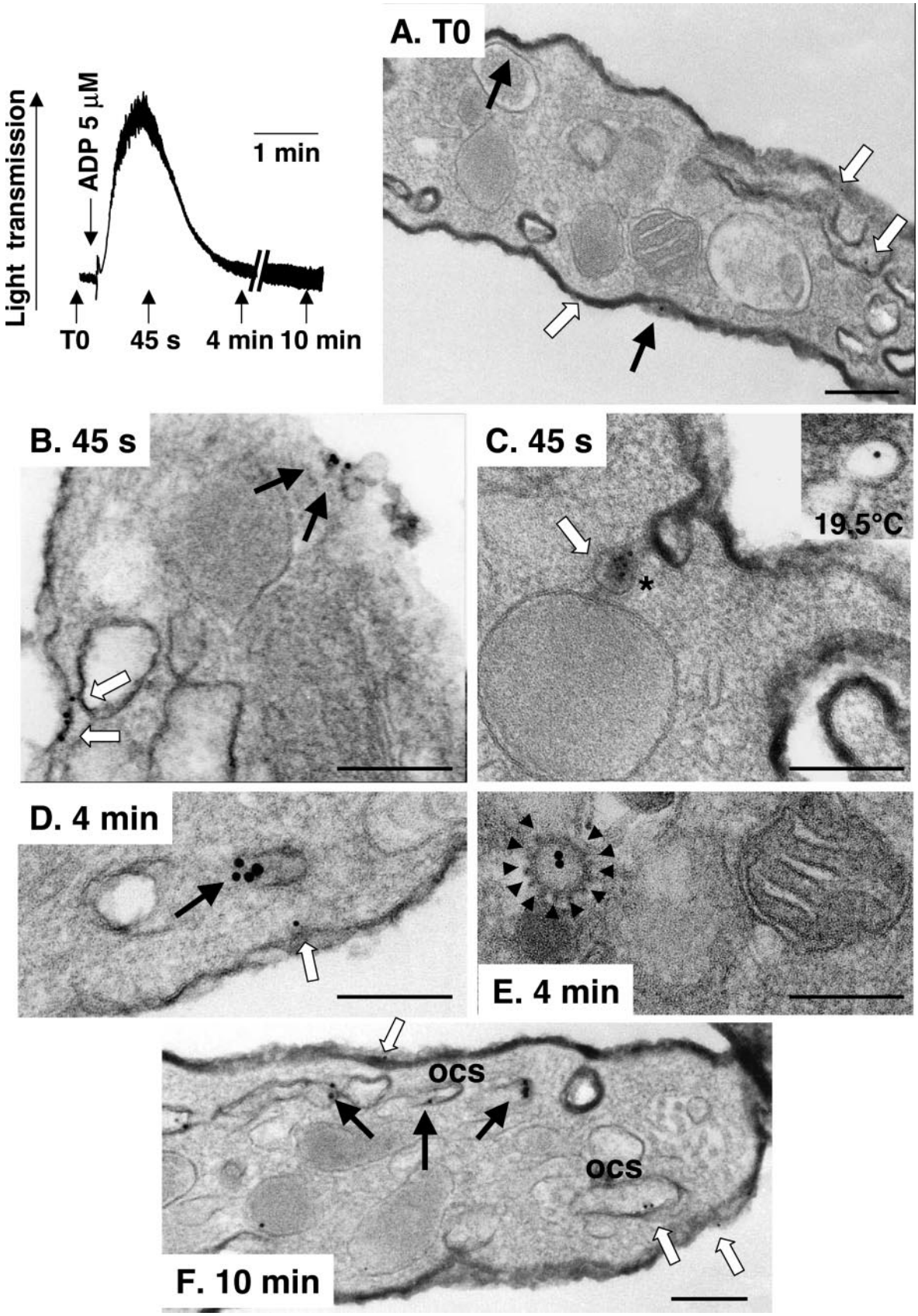

Fig. 7. Relocalization of the $P2Y_1$ and $P2Y_{12}$ receptors in human platelets treated with ADP. A, gold-labeled F(ab′)2 anti-$P2Y_1$ (15 nm), and anti-$P2Y_{12}$ (10-nm) antibodies were added to platelet suspensions before (T0) or after stimulation with 5 μM ADP. The platelets were then fixed with glutaraldehyde at different time points (B–F) and embedded in Epon. $P2Y_1$-15-nm gold particles are indicated with black arrows and $P2Y_{12}$-10-nm gold particles with white arrows. The plasma membrane and the OCS were stained with tannic acid, used to increase the contrast of external membranous systems (Gachet et al., 1993). Arrowheads indicated the presence of clathrin on $P2Y_1$-labeled vesicles. Scale bars, 200 nm.

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