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Distinct Roles for Protein Kinase C Isoforms in Regulating Platelet Purinergic Receptor Function

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ABSTRACT

ADP is a critical regulator of platelet activation, mediating its actions through two G protein-coupled receptors (GPCRs), $P2Y_1$ and $P2Y_{12}$. We have shown previously that the receptors are functionally desensitized, in a homologous manner, by distinct kinase-dependent mechanisms in which $P2Y_1$ is regulated by protein kinase C (PKC) and $P2Y_{12}$ by G protein-coupled receptor kinases. In this study, we addressed whether different PKC isoforms play different roles in regulating the trafficking and activity of these two GPCRs. Expression of PKCα and PKCδ dominant-negative mutants in 1321N1 cells revealed that both isoforms regulated $P2Y_1$ receptor signaling and trafficking, although only PKCδ was capable of regulating $P2Y_{12}$, in experiments in which PKC was directly activated by the phorbol ester phorbol 12-myristate 13-acetate (PMA). These results were paralleled in human platelets, in which PMA reduced subsequent ADP-induced $P2Y_1$ and $P2Y_{12}$ receptor signaling. PKC isoform-selective inhibitors revealed that novel, but not conventional, isoforms of PKC regulate $P2Y_{12}$ function, whereas both novel and classic isoforms regulate $P2Y_1$ activity. It is also noteworthy that we studied receptor internalization in platelets by a radioligand binding approach showing that both receptors internalize rapidly in these cells. ADP-induced $P2Y_1$ receptor internalization is attenuated by PKC inhibitors, whereas that of the $P2Y_{12}$ receptor is unaffected. Both $P2Y_1$ and $P2Y_{12}$ receptors can also undergo PMA-stimulated internalization, and here again, novel but not classic PKCs regulate $P2Y_{12}$, whereas both novel and classic isoforms regulate $P2Y_1$ internalization. This study therefore is the first to reveal distinct roles for PKC isoforms in the regulation of platelet P2Y receptor function and trafficking.

Activation of platelets occurs through a complex series of reactions in response to vessel injury and plays an essential role in thrombosis. One agonist, ADP, plays a central role in platelet activation by acting as a cofactor in the platelet responses to physiological agonists, including thromboxane A2, collagen, and thrombin. ADP activates two surface-expressed GPCRs, $P2Y_1$ and $P2Y_{12}$ (Kunapuli et al., 2003; Gachet, 2005). The combined stimulation of $P2Y_1$ receptor (coupled to G_q and phospholipase Cβ) and $P2Y_{12}$ receptor (negatively coupled to adenylyl cyclase through G_i) is necessary for the full platelet aggregation response to ADP, with platelet activation initiated by the $P2Y_1$ receptor and amplified by $P2Y_{12}$ (Gachet, 2005).

The attenuation of receptor-stimulated signal output upon sustained or recurrent agonist stimulation, a process known as desensitization, is a crucial physiological mechanism of adaptation observed for many GPCRs. Because ADP plays a crucial role in platelet activation, it is likely that the responsiveness of $P2Y_1$ and $P2Y_{12}$ receptors is tightly regulated. We have shown that both $P2Y_1$ and $P2Y_{12}$ receptor responses desensitize in human platelets (Hardy et al., 2005), which may underlie the observed desensitization of platelet responses after prolonged exposure to ADP (Poole et al., 1993;

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ABBREVIATIONS: GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; DNM, dominant-negative mutant; PMA, phorbol 12-myristate 13-acetate; ECL, enhanced chemiluminescence; A3P5P, adenosine-3′,5′-diphosphate; 2MeSADP, 2-methylthioadenosine 5′-diphosphate; GF109203X, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide); Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; PKC, protein kinase C; AM, Ro201724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; AR-C69931MX, 5′-adenylic acid, *N*-[2-(methylthio)-ethyl]-2-[(3,3,3-trifluoropropyl)thio]-, monoanhydride with (dichloromethylene)bis[phosphonic acid]; ELISA, enzyme-linked immunosorbent assay.

Baurand et al., 2000). Mechanisms underlying desensitization are complex and can involve phosphorylation of the receptor, uncoupling from G proteins, internalization, and ultimately intracellular down-regulation (Ferguson, 2001; von Zastrow, 2003). We have discovered recently that ADP pretreatment promotes $P2Y_1$ and $P2Y_{12}$ receptor desensitization by different kinase-dependent mechanisms. $P2Y_1$, but not $P2Y_{12}$, desensitization is mediated by protein kinase C (PKC) (Hardy et al., 2005). In contrast, agonist-induced desensitization of the $P2Y_{12}$ receptor, but not $P2Y_1$, is largely dependent on G protein-coupled receptor kinase activity.

It is important to address several important questions that were raised by our previous work. Because platelets express multiple isoforms of PKC (Crosby and Poole, 2002; Murugappan et al., 2004; Buensuceso et al., 2005), which isoforms are responsible for mediating homologous desensitization of the $P2Y_1$ receptor? Given that platelets express multiple G_q-coupled receptors, each leading to the activation of PKC, can activation of PKC cause "heterologous" regulation of $P2Y_{12}$ and, if so, which isoforms of PKC are involved? Do the receptors themselves become phosphorylated by PKC? Although our previous work addressed only the roles of kinases in regulating functional desensitization of the receptors, it is now important to address their role in receptor internalization and trafficking. In this study, we address these questions and reveal distinct roles for PKC isoforms in the regulation of platelet P2Y receptor function and trafficking.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), Lipofectamine 2000, and fetal bovine serum were obtained from Invitrogen (Paisley, UK). Radiochemicals were from PerkinElmer Life Sciences (Boston, MA). Complete protease inhibitor tablets were from Roche (Indianapolis, IN). Anti-hemagglutinin (HA)-monoclonal antibody (HA-11), goat anti-mouse fluorescein-conjugated secondary antibody (1:200), rhodamine-conjugated transferrin, and lysotracker red were purchased from Invitrogen. The bisindolylmaleimide GF109203X, Gö6976, and rottlerin were from Calbiochem (Merck Biosciences Ltd., Nottingham, UK). All other reagents were from Sigma (St. Louis, MO).

Adenovirus Infection and Cell Culture. Expression vectors for dominant-negative mutant (DNM) PKCα (rabbit) and PKCδ (mouse) in adenoviruses were kind gifts from Professors Motoi Ohba and Toshio Kuroki and have been described previously (Ohba et al., 1998; Mitsutake et al., 2001). Adenoviruses were amplified in human embryonic kidney 293 cells and harvested after 72 h, purified, and viral titers were estimated as described previously (Kanegae et al., 1994). 1321N1 human astrocytoma cells stably expressing either HA-tagged human $P2Y_1$ or $P2Y_{12}$ receptor were generated as described previously (Hardy et al., 2005). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 400 μg/ml G418 (Geneticin) at 37°C in a humidified atmosphere of 95% air/5% CO_2. For adenoviral infection, 1321N1 cells were infected between 50 and 200 plaque-forming units/cell in serum-free DMEM for 2 h. Infection with empty vector β-gal adenovirus was used as control.

Receptor Phosphorylation. Stably transfected cells in six-well dishes were washed twice with phosphate-free DMEM and incubated for 90 min at 37°C in the same media supplemented with 0.2 mCi/ml [^{32}P]orthophosphate and 0.2 U/ml apyrase. After incubation with the PKC inhibitor GF109203X (1 μM, 15 min) or vehicle alone, cells were treated with either ADP (10 μM, 5 min) or the protein kinase C activator phorbol 12-myristate 13-acetate (PMA; 1 μM; 15 min). After drug treatment, reactions were terminated by placing the cells on ice and washing twice with ice-cold phosphate-buffered saline. All subsequent procedures were performed at 4°C unless otherwise stated. Cells were subsequently lysed, and HA-tagged receptor was immunoprecipitated using a monoclonal anti-HA antibody (HA-11) as described previously (Mundell et al., 2004). Immune complexes were isolated by brief centrifugation, washed three times with immunoprecipitation buffer, and eluted from beads by the addition of 20 μl of electrophoresis sample buffer. After fractionation by SDS-PAGE and transfer to a nitrocellulose membrane, phosphoproteins were visualized by autoradiography for 24 to 72 h at −80°C. Receptor immunoprecipitation was determined by reprobing membranes with a polyclonal anti-HA antibody/horseradish peroxidase-conjugated antirabbit IgG and visualization by enhanced chemiluminescence (ECL). The extent of receptor phosphorylation was quantified by densitometric analysis of resulting autoradiographs.

Western Blotting. Protein expression in 1321N1 cells and platelets was determined by Western blotting. In brief, cells were lysed into ice-cold lysis buffer (20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM EDTA, and 1% Triton X-100, supplemented with Complete protease inhibitors), insoluble material was discarded by centrifugation (13,000*g*, 5 min), and SDS loading buffer (63 mM Tris, pH 6.5, 100 mM dithiothreitol, 1% SDS, 11.6% glycerol, and 0.02% bromphenol blue) was added to the cell lysates. Proteins were separated by SDS-PAGE. Gels were transferred to nitrocellulose membranes and blotted with PKC isoform-specific antibodies for PKCα, -β (Zhang et al., 2001), -δ, and -θ (Blass et al., 2002). Proteins were detected by ECL.

Measurement of Cytosolic $[Ca^{2+}]_i$ in 1321N1 Astrocytoma Cells. The cytosolic free Ca^{2+} concentration was determined using the fluorescent Ca^{2+} indicator fura-2-acetoxymethyl ester (fura-2/AM) as reported previously (Hardy et al., 2005). In brief, transfected cells were grown on poly(L-lysine)-coated glass coverslips and used at ~60% confluence. Cells were washed twice with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose, pH 7.4) and incubated with fura-2/AM (3 μM) at 37°C for 60 min. Glass coverslips were mounted into a quartz cuvette and placed into a thermostatically controlled cell holder at 37°C. Cells were continuously perfused with Locke's solution. Fluorescence was measured at 340 and 380 nM excitation and 510 nM emission. ADP (0.1–1 μM) was perfused onto cell monolayers as required. $[Ca^{2+}]_i$ was determined from ratiometric data as described previously (Grynkiewicz et al., 1985).

Measurement of cAMP Accumulation in 1321N1 Astrocytoma Cells. Cells infected with PKC-DNM adenoviruses or β-gal alone controls as described above were grown to 80% confluence and exposed to a desensitizing dose of ADP (1 nM, 15 min) or PMA (1 μM, 15 min) in the presence of the phosphodiesterase inhibitor Ro201724 (250 μM). Apyrase (0.2 U/ml) was then added directly to each well and incubated for 1 min at 37°C to remove the desensitizing ADP. Cells were then washed, forskolin (1 μM) was added in the absence or presence of ADP, and plates were incubated at 37°C for 10 min. Endogenous β_2 adrenoceptor responses were also examined in 1321N1 cells by measuring isoproterenol (1 μM)-stimulated cAMP accumulation. cAMP accumulation was terminated by the addition of ice-cold 100% trichloroacetic acid, and supernatant was neutralized with 1 M NaOH and 50 mM Tris-HCl and 4 mM EDTA, pH 7.4. cAMP levels were determined as described previously (Mundell et al., 1997). Data are expressed as cAMP production (picomoles of cAMP per well) or as the percentage of inhibition of forskolin-stimulated adenylyl cyclase.

Internalization of HA-$P2Y_1$ and HA-$P2Y_{12}$ in 1321N1 Cells. HA-tagged surface-receptor loss was assessed by ELISA as described previously (Daunt et al., 1997; Mundell et al., 2000). Cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly(L-lysine). Twenty-four hours later, cells were incubated with DMEM containing apyrase (0.1 U/ml) for 1 h at 37°C, washed, and then pretreated with the PKC inhibitor GF109203X (1 μM, 15 min). Cells were then challenged with DMEM containing ADP (10 μM, 15 min)

or PMA (1 μM, 15 min) at 37°C. Changes in surface-receptor expression were subsequently determined by an immunosorbent assay (ELISA) taking advantage of the HA-epitope tag (Daunt et al., 1997; Mundell et al., 2000), and expressed as either the percentage of surface receptor or as the percentage loss of surface receptor with the background signal from controls subtracted.

Preparation of Human Platelets. Human blood was drawn from healthy, drug-free volunteers on the day of the experiment. Acid citrate dextrose (120 mM sodium citrate, 110 mM glucose, and 80 mM citric acid, used at 1:7 v/v) was used as anticoagulant. Platelet-rich plasma was prepared by centrifugation at 200*g* for 17 min, and platelets were then isolated by centrifugation for 10 min at 1000*g*, in the presence of 0.02 U/ml apyrase and prostaglandin E_1 (140 nM) for all assays other than the measurement of intracellular cAMP, in which prostaglandin E_{1f} was omitted. The pellet was resuspended to a density of 4×10^8 platelets/ml in a modified Tyrode's-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, and 5 mM glucose, pH 7.3). To this platelet suspension, 10 μM indomethacin and 0.02 U/ml apyrase were added, and a 30-min resting period was allowed before stimulation.

Measurement of Cytosolic $[Ca^{2+}]_i$ in Platelets. Measurement of cytosolic calcium was performed as described previously (Poole et al., 1995). In brief, 3 μM fura-2/AM was added to platelet-rich plasma and incubated at 37°C for 45 min in the presence of 10 μM indomethacin. Platelets were centrifuged and resuspended in modified Tyrode's solution. Platelets were treated for 15 min with the PKC inhibitors GF109203X (2 μM), Gö6976 (1 μM), rottlerin (10 μM), or vehicle alone. ADP (10 μM)-induced calcium responses were subsequently measured at 37°C in PMA (1 μM, 15 min), and non–PMA-treated platelets were measured using a Hitachi F-4500 spectrofluorimeter with fluorescence excitation made at 340 and 380 nm and emission at 510 nm.

Measurement of cAMP Levels in Platelets. Platelets were treated for 15 min with the PKC inhibitors GF109203X (2 μM), Gö6976 (1 μM), rottlerin (10 μM), or vehicle alone. PMA (1 μM, 15 min) and non–PMA-treated platelets were stimulated in the presence of the phosphodiesterase inhibitor isobutyl methylxanthine (100 μM) ± forskolin (1 μM) in the absence or presence of ADP (10 μM) for 5 min at 37°C. cAMP accumulation was terminated by the addition of ice-cold 100% trichloroacetic acid, and samples were left to lyse on ice for 1 to 2 h. The resulting samples were spun at 4000*g* for 5 min, and the cAMP-containing supernatant was neutralized with 1 M NaOH and 50 mM Tris-HCl and 4 mM EDTA, pH 7.4. cAMP levels were subsequently determined in each sample using a binding assay as described previously (Mundell et al., 1997). Data are presented as either the percentage of inhibition of forskolin-stimulated adenylyl cyclase.

Radioligand Binding in Human Platelets. In experiments assessing receptor internalization, platelets were pretreated with the PKC inhibitors with GF109203X (2 μM), Gö6976 (1 μM), rottlerin (10 μM), or vehicle alone. Platelets were subsequently stimulated with ADP (10 μM, 0–30 min), PMA (1 μM, 15 min), or vehicle alone. ADP was then removed by the addition of 0.2 U/ml apyrase for 3 min before fixing platelets. Platelets were fixed by continuous rotation for 25 min in the presence of 4% formaldehyde. Platelets were then isolated by centrifugation at 1000*g* (10 min) and then resuspended in binding buffer (20 mM HEPES and 1 mM $MgCl_2$) to a density of 4×10^8 platelets/ml. Aliquots of platelet suspension were incubated with [^{3}H]2MeSADP (3 Ci/mmol, 0.01 nM to 1 μM) and specific binding was determined in the presence of either unlabeled ligand (10 μM), the $P2Y_1$ receptor antagonist A3P5P (1 μM to 3.33 mM) or the $P2Y_{12}$ receptor antagonist AR-C69931MX (1 nM to 10 μM). After incubation for 20 min at room temperature, reactions were terminated by the addition of ice-cold binding buffer and rapid filtration through Whatman GF/C glass fiber filters under vacuum. Radioactivity bound to the filters was measured by scintillation counting.

Experimental Design and Statistics. Data were analyzed by the iterative fitting program Prism (GraphPad Software Inc., San Diego, CA). Log concentration-effect curves were fitted to logistic expressions for single-site analysis, whereas $t_{1/2}$ values for agonist-induced internalization were obtained by fitting data to single exponential curves. Where appropriate, statistical significance was assessed by Mann-Whitney *U* test or by two-way analysis of variance.

Results

PKC-Dependent $P2Y_1$ and $P2Y_{12}$ Receptor Phosphorylation. No studies to date have demonstrated the direct phosphorylation of either the $P2Y_1$ or $P2Y_{12}$ purinergic receptor, although both contain multiple potential phosphorylation sites with their C-terminal domain. At this time, the lack of specific $P2Y_1$ and $P2Y_{12}$ receptor antibodies able to consistently identify and immunoprecipitate receptor from platelets precluded studies of endogenous purinergic receptor phosphorylation. Therefore, we examined the phosphorylation of heterologously expressed HA epitope-tagged $P2Y_1$ and $P2Y_{12}$ receptors in P2Y-null 1321N1 cells. A polyclonal anti-HA antibody recognized specific immunoreactive bands in membranes from both $P2Y_1$ and $P2Y_{12}$ receptor-expressing cells (Fig. 1). These bands at 45 ($P2Y_1$) or 70 kDa ($P2Y_{12}$) were not present in vector alone pcNEO-transfected controls and run at apparent molecular masses consistent with other reports (Moran-Jimenez and Matute, 2000; Zhong et al., 2004). $P2Y_{12}$ receptor runs at a considerably higher apparent molecular mass than may be predicted from its primary amino acid sequence due to extensive *N*-linked glycosylation (Zhong et al., 2004). $P2Y_1$ receptor has been shown by electrophoretic mobility to have an apparent molecular mass of between 40 and 50 kDa (Moran-Jimenez and Matute, 2000). Phosphorylation studies revealed that both $P2Y_1$ and $P2Y_{12}$ receptors exist as phosphoproteins under basal conditions and that the addition of either ADP (10 μM, 5 min) or the PKC activator PMA (1 μM, 15 min) significantly increased receptor phosphorylation. Pretreatment with the PKC inhibitor GF109203X (1 μM, 15 min) significantly attenuated ADP, and PMA promoted $P2Y_1$ receptor phosphorylation. Therefore, PKC can directly phosphorylate either the agonist-occupied or -unoccupied $P2Y_1$ receptor. In contrast, GF109203X only attenuated PMA-promoted $P2Y_{12}$ receptor phosphorylation. Therefore, agonist-induced $P2Y_{12}$ receptor phosphorylation is not regulated by PKC, although this kinase can directly phosphorylate the agonist-unoccupied receptor.

PKC-Dependent Desensitization of $P2Y_1$ and $P2Y_{12}$ Receptor Activity in 1321N1 Cells. To identify more accurately the PKC isoforms that may regulate $P2Y_1$ and $P2Y_{12}$ receptor function, we examined their expression in both 1321N1 cells and human platelets. Western blotting revealed that although platelets expressed PKCs α, β, δ, and θ, 1321N1 cells expressed only PKCα and PKCδ (Fig. 2A). We therefore subsequently overexpressed dominant-negative catalytically inactive mutants of both of these isoforms in 1321N1 cells by an adenoviral infection approach (Fig. 2B, where immunoblotting for both PKCα and PKCδ is shown) and examined effects of expression upon receptor signaling and internalization. $P2Y_1$ receptor desensitization was examined by monitoring the cytosolic calcium response to ADP, as we have shown previously (Hardy et al., 2005). Overexpression of either isoform-specific DNM did not significantly at-

tenuate agonist-induced desensitization (Fig. 3A). A reduction in $P2Y_1$ receptor desensitization was only apparent in cells coexpressing both PKCα- and PKCδ-DNM (Fig. 3A). Pretreatment with PMA (1 μM, 15 min) significantly attenuated subsequent agonist-induced $P2Y_1$ receptor activity. As with the homologous desensitization of $P2Y_1$ receptor activity, heterologous PMA-induced desensitization was only attenuated when both PKCα- and PKCδ-DNM were coexpressed. Studies examining the G_i-coupled $P2Y_{12}$ purinergic receptor revealed that ADP-induced inhibition of forskolin-stimulated adenylyl cyclase [ADP induced an 80% inhibition of response, consistent with previous studies (Hardy et al., 2005)] was reduced after PMA (1 μM, 15 min) pretreatment to a similar extent to that found in ADP (10 nM, 15 min) desensitized cells (Fig. 3B). Actual numbers are shown in Fig. 3B(i), and normalized data are shown in Fig. 3B(ii). Data from Fig. 3B(i) also show that PMA is not able to induce the activation of adenylyl cyclase or inhibit forskolin-induced activity of the cyclase. Therefore, PKC can regulate the agonist-unoccupied $P2Y_{12}$ purinergic receptor in a heterologous manner. Overexpression of DNM-PKCδ and not DNM-PKCα selectively attenuated PMA-induced $P2Y_{12}$ purinergic receptor desensitization, whereas ADP-induced desensitization was unaffected by either DNM-PKC.

PKC-Dependent Internalization of $P2Y_1$ and $P2Y_{12}$ Receptors in 1321N1 Cells. Using 1321N1 cells stably expressing N-terminal HA-epitope-tagged versions of either receptor, we were able to quantify agonist-induced surface-receptor loss by ELISA (Mundell et al., 2004). Stimulation with either ADP (10 μM, 5 min) or PMA (1 μM, 15 min) induced internalization of both $P2Y_1$ and $P2Y_{12}$ receptors (Fig. 4). The rate of internalization is shown in Fig. 4, A and B, for $P2Y_1$ and $P2Y_{12}$ receptors, respectively. It is also important to note that we show that carbachol (1 mM), operating through endogenously expressed muscarinic M_3 receptors, is able heterologously to induce internalization of both $P2Y_1$ and $P2Y_{12}$ receptors. Pretreatment with GF109203X (1 μM, 15 min) selectively attenuated ADP-induced $P2Y_1$ receptor internalization, whereas that of the $P2Y_{12}$ receptor was unaffected (Fig. 4C). As expected, inhibition of PKC with GF109203X reversed internalization of both $P2Y_1$ and $P2Y_{12}$ receptors induced by PMA. ADP and PMA-induced $P2Y_1$ receptor internalization was partially inhibited by the expression of either PKCα- or PKCδ-DNM, with coexpression of both DNMs producing a more robust inhibition of receptor internalization (Fig. 4D). As with GF109203X pretreatment, expression of PKCα- or PKCδ-DNM did not attenuate ADP-induced $P2Y_{12}$ receptor internalization (Fig. 4D). It is interesting that, consistent with their effects on functional desensitization, expression of DNM-PKCδ but not DNM-PKCα attenuated PMA-induced $P2Y_{12}$ receptor internalization (Fig. 4D).

Regulation of $P2Y_1$ and $P2Y_{12}$ Receptor Desensitization by PKC in Human Platelets. Because our studies in 1321N1 cells revealed that PKC could regulate $P2Y_1$ and $P2Y_{12}$ receptor function, we sought to determine whether this was also the case in human platelets. We have reported that in response to ADP, PKC can regulate the desensitization of $P2Y_1$ purinergic receptor responses in human platelets (Hardy et al., 2005). Therefore, as expected, pretreatment with PMA resulted in a significant reduction in subsequent ADP-promoted $P2Y_1$ receptor-mediated calcium response (Fig. 5A). Inhibition of PKC with GF109203X (2 μM), a nonselective PKC inhibitor, significantly attenuated $P2Y_1$ receptor desensitization. Pretreatment with Gö6976 (1 μM), which inhibits calcium-dependent classic PKC isoforms (Martiny-Baron et al., 1993), including PKCα, and rottlerin (10 μM), a PKCδ isoform selective inhibitor (Gschwendt et

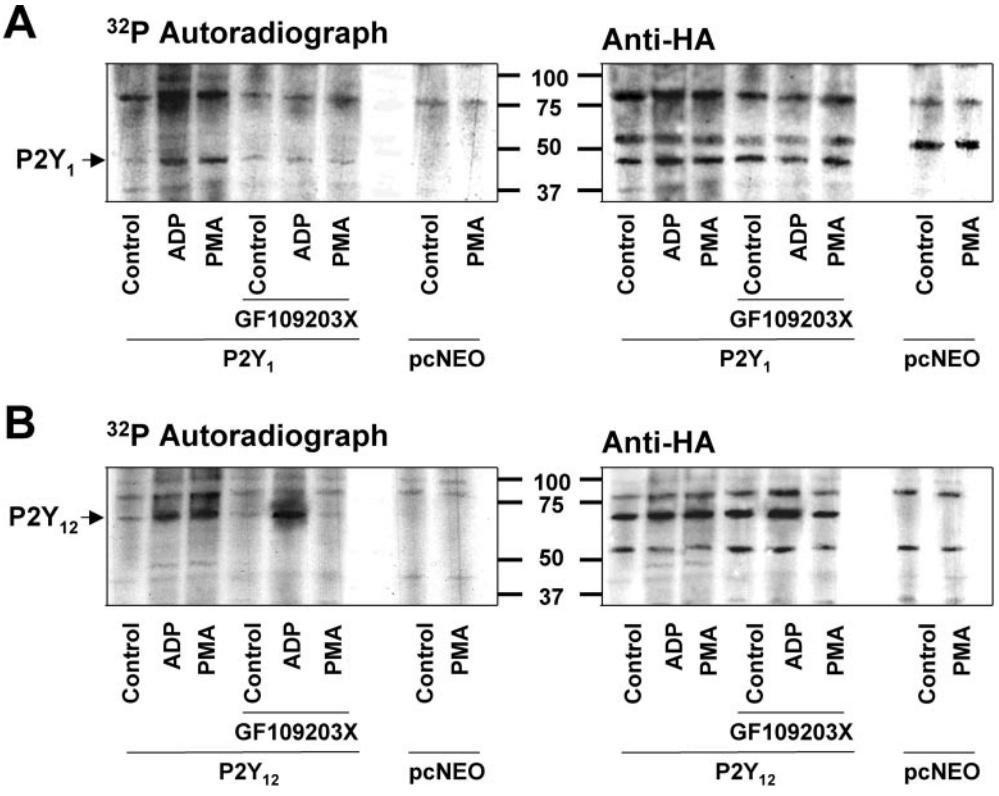

Fig. 1. Protein kinase C-mediated phosphorylation of HA-$P2Y_1$ and HA-$P2Y_{12}$. ^{32}P-labeled 1321N1 human astrocytoma cells stably expressing either HA-$P2Y_1$ (A), HA-$P2Y_{12}$ (B), or vector alone (pcNEO) were pretreated for 10 min with vehicle or 1 μM GF109203X before the addition of the PKC activator PMA (1 μM, 15 min), ADP (10 μM, 5 min), or vehicle alone. Phospho-HA-purinergic receptors were immunoprecipitated from membrane lysates and run on SDS-PAGE before transfer to nitrocellulose membranes. Specific phosphorylated bands at 45 kDa ($P2Y_1$, A) or 70 kDa ($P2Y_{12}$, B) not present in vector-alone pcNEO-transfected controls were subsequently identified by autoradiography. Similar amounts of receptor immunoprecipitation were confirmed (A and B, right) by reprobing membranes with a polyclonal anti-HA antibody/horseradish peroxidase-conjugated anti-rabbit IgG and visualization by ECL.

al., 1994), were unable to attenuate PMA-promoted $P2Y_1$-receptor desensitization (Fig. 5A). It is important to note, however, that pretreatment with both Gö6976 and rottlerin was able to partially attenuate the desensitization of $P2Y_1$ receptor activity. Stimulation of PKC with PMA also decreased ADP-induced $P2Y_{12}$ receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase [Fig. 5B(i) shows the actual numbers and B(ii) shows normalized data] to a level comparable with that induced by ADP pretreatment (Hardy et al., 2005). Pretreatment with either GF109203X or rottlerin attenuated PMA-induced $P2Y_{12}$ receptor desensitization, whereas Gö6976 had no effect on desensitization of the $P2Y_{12}$ response (Fig. 5B). Therefore, stimulation of PKC activity can promote the heterologous desensitization of both $P2Y_1$ and $P2Y_{12}$ receptor responses in human platelets. Finally, to demonstrate that, in the platelet system, the G_q-coupled $P2Y_1$ receptor may regulate $P2Y_{12}$, we chose to investigate the effect of $P2Y_1$ receptor blockade on ADP-induced desensitization of the $P2Y_{12}$ receptor response. Figure 6 shows that pretreatment of platelets with the $P2Y_1$ receptor antagonist A3P5P (1 mM) does not attenuate $P2Y_{12}$-mediated inhibition of adenylyl cyclase, but that in the presence of A3P5P, the ADP-induced desensitization of the $P2Y_{12}$ receptor is reduced, indicating partial heterologous regulation of $P2Y_{12}$ by the $P2Y_1$ receptor.

Internalization of $P2Y_1$ and $P2Y_{12}$ Receptors in Human Platelets. To study the internalization of purinergic receptors in human platelets, we made use of the nonspecific P2 ligand 2MeSADP (Takasaki et al., 2001). It is important to note that we chose to use formaldehyde-fixed platelets for our study. The reason for doing so was to be able to avoid complications in the binding assay produced by released ADP and ATP, which would compete with radioligand for binding sites. This has been a problem in other studies, and the use of fixed platelets to overcome this problem has been validated previously (Jefferson et al., 1988; Agarwal et al., 1989). Sat-

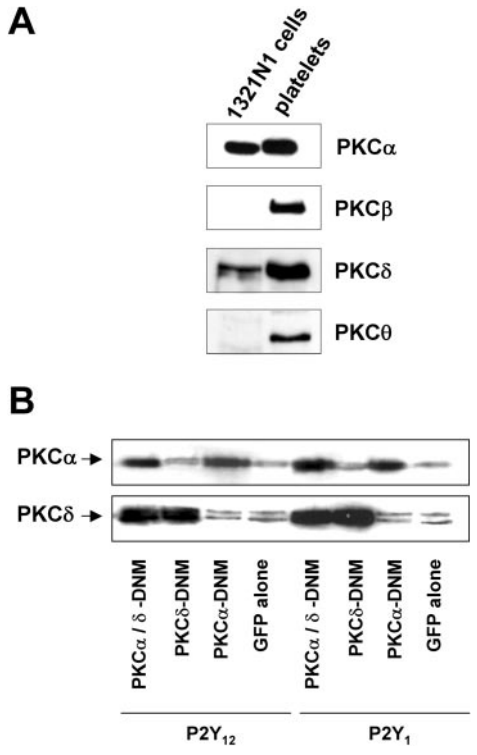

Fig. 2. PKC isoform expression in 1321N1 cells and human platelets. A, whole-cell lysates from 1321N1 cells and human platelets were subjected to SDS-PAGE, followed by immunoblotting with PKC isoform-specific antibodies as detailed in *Materials and Methods*. Data shown are representative of three experiments. B, 1321N1 cells were infected with βGal-PKCα-DNM, βGal -PKCδ-DNM, or βGal adenovirus alone as control. Whole-cell lysates from these cells were subjected to SDS-PAGE, followed by immunoblotting with PKC isoform-specific antibodies as detailed in *Materials and Methods*. Bands therefore represent the sum of native wild-type and heterologously expressed DNM-PKC. Data shown are representative of three experiments.

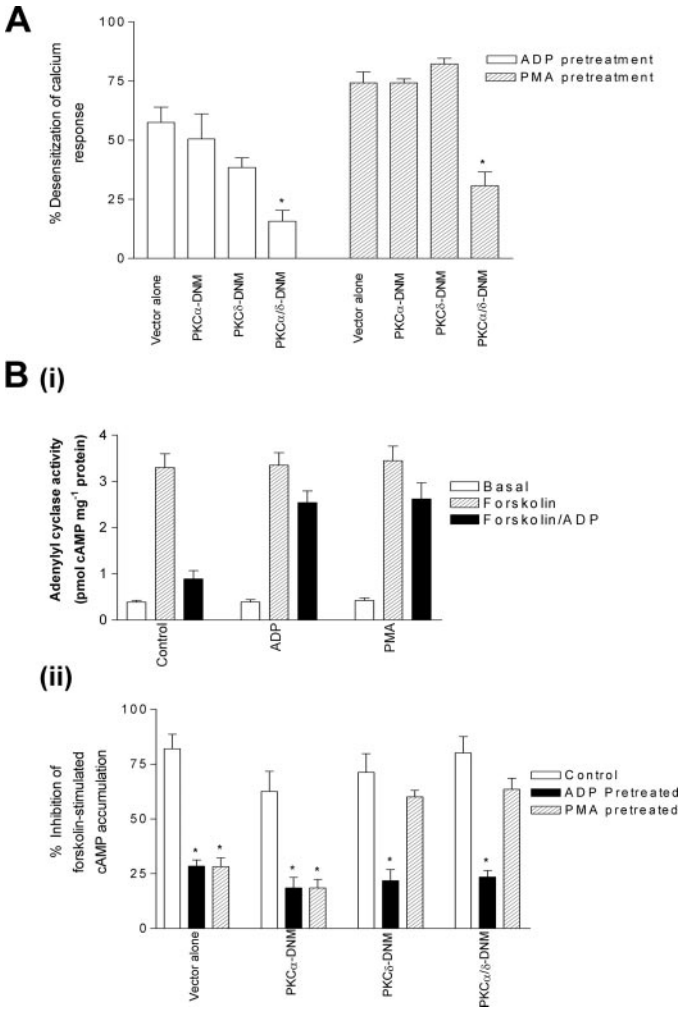

Fig. 3. PKC-mediated desensitization of $P2Y_1$ and $P2Y_{12}$ purinergic receptor responses in 1321N1 cells stably expressing each receptor. 1321N1 astrocytoma cells were infected with βGal-PKCα-DNM, βGal-PKCδ-DNM, both DNM constructs, or βGal adenovirus alone (vector alone) as control. A, desensitization of $P2Y_1$ receptors was assessed by comparing calcium responses to ADP (0.1 μM) before and after pretreatment addition of ADP (1 μM, 2 min) or PMA (1 μM, 15 min) as detailed in *Materials and Methods*. Results are expressed as a percentage of desensitization of response, and data are mean ± S.E.M. of at least three independent experiments. ∗, statistical significance at $p < 0.05$ for data compared with respective vector-alone control (Mann-Whitney *U* test). B, agonist (ADP, 10 nM)-dependent inhibition of forskolin (1 μM, 10 min)-stimulated adenylyl cyclase activity by $P2Y_{12}$ purinergic receptor activation after pretreatment with ADP (10 nM, 5 min), PMA (1 μM, 15 min) or vehicle alone was subsequently determined. B(i), for vector-alone control condition, data are presented as mean picomoles of cAMP per milligram of protein ± S.E.M. ($n \geq 4$). B(ii), for all conditions, including vector-alone control, normalized data are presented as mean ± S.E.M of at least four independent experiments, expressed as the percentage of inhibition of forskolin-stimulated adenylyl cyclase. ∗, statistical significance at $p < 0.05$ for data compared with respective nonpretreated agonist-induced inhibition of forskolin-stimulated controls (Mann-Whitney *U* test).

uration binding experiments measuring [^{3}H]2MeSADP binding to fixed platelets in the presence and absence of unlabeled radioligand (10 μM) indicated that there were 901 ± 41 [^{3}H]2MeSADP binding sites per platelet with an affinity of 4.9 ± 0.3 nM. Further saturation experiments using the $P2Y_1$ receptor antagonist A3P5P (1 mM) or the $P2Y_{12}$ receptor antagonist AR-C69931MX (1 μM) revealed two distinct binding populations of 184 ± 27 and 644 ± 11 [^{3}H]2MeSADP binding sites per platelet, which represent the $P2Y_1$ and $P2Y_{12}$ receptors, respectively. It is interesting that experiments using combined $P2Y_1$ and $P2Y_{12}$ receptor antagonists estimated the number of binding sites to be 844 ± 57, a number not significantly different from that obtained with unlabeled 2MeSADP. Further experiments using a fixed concentration of [^{3}H]2MeSADP (100 nM) revealed that the concentrations of A3P5P and AR-C69931MX used in the saturation analysis studies were indeed maximal (data not shown) and that the respective IC_{50} values for these antagonists were determined to be 19.9 ± 1.2 mM and 45.0 ± 2.4 nM, values which correlate well with those reported by Takasaki et al. (2001).

Therefore, in subsequent experiments (Fig. 7) examining receptor internalization, platelets were incubated with [^{3}H]2MeSADP (100 nM) in the presence of A3P5P (1 mM) or AR-C69931MX (1 μM) to give an estimate of either the $P2Y_1$ or $P2Y_{12}$ surface binding sites. After pretreatment with ADP (10 μM, 10 min), its subsequent removal with apyrase (0.2 U/ml, 3 min), and platelet fixation, there was a clear reduction in [^{3}H]2MeSADP binding to both $P2Y_1$ and $P2Y_{12}$ receptors compared with nonpretreated or apyrase alone-treated controls (Fig. 7A). Further studies showed that the $P2Y_{12}$ receptor internalized much more rapidly than the $P2Y_1$ receptor, although by 30 min, the relative surface expression of each receptor was comparable (Fig. 7B). Therefore, as in 1321N1 cells, both the $P2Y_1$ and $P2Y_{12}$ purinergic receptors internalize in a rapid agonist-dependent manner in human platelets.

PKC-Dependent Regulation of $P2Y_1$ and $P2Y_{12}$ Receptor Surface-Receptor Expression and Internalization in Human Platelets. To address the role PKC may play in this process, we used the PKC inhibitors GF109203X, Gö6976, and rottlerin described above. It is interesting that pretreatment of platelets with any of these inhibitors significantly increased the number of $P2Y_1$ binding sites, with GF109203X being the most potent (Fig. 7C). Pretreatment with GF109203X or rottlerin also significantly increased the

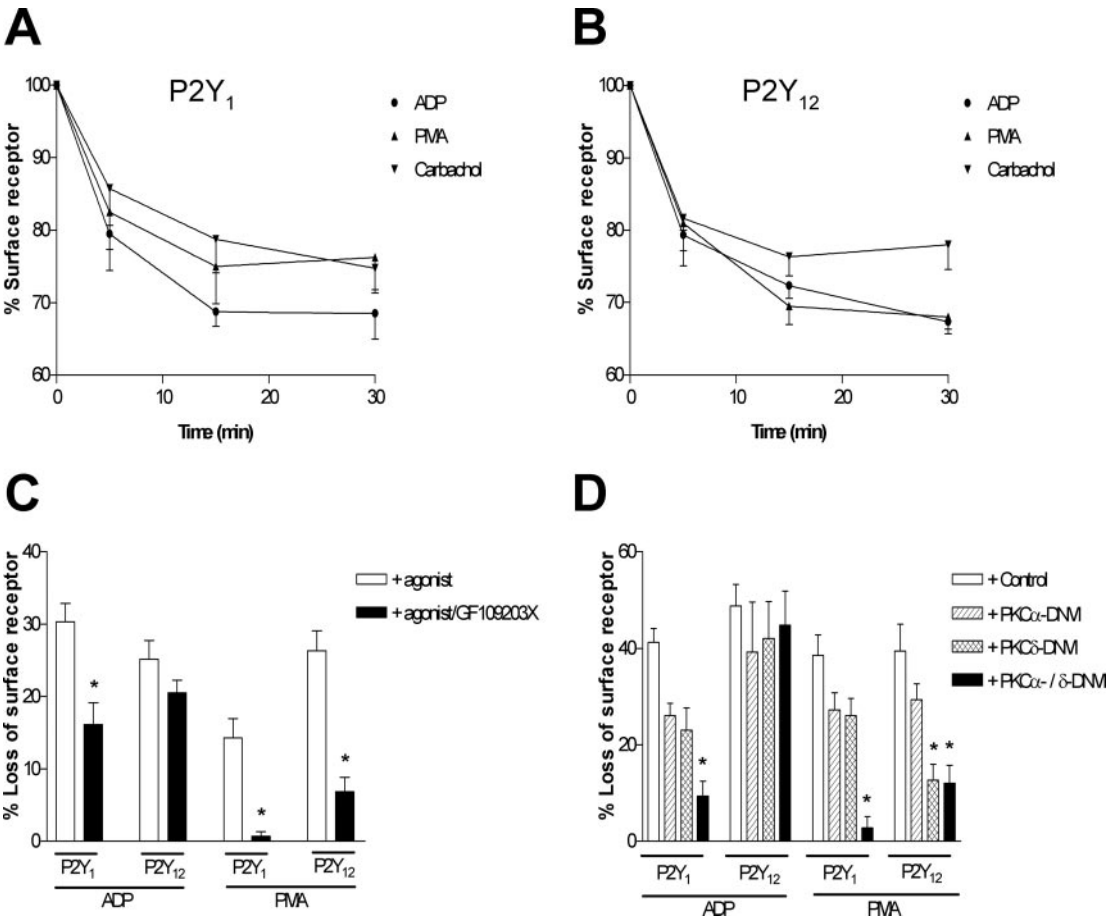

Fig. 4. PKC-mediated internalization of $P2Y_1$ and $P2Y_{12}$ purinergic receptor responses in 1321N1 cells stably expressing each receptor. $P2Y_1$ receptor- (A) or $P2Y_{12}$ receptor-expressing (B) 1321N1 cells were challenged with ADP (10 μM), the protein kinase C activator PMA (1 μM) or the muscarinic agonist carbachol (1 mM). Surface-receptor loss was assessed by ELISA at various time points after the addition of agonist. Data represent mean ± S.E.M. of five independent experiments. C, $P2Y_1$ or $P2Y_{12}$ receptor-expressing 1321N1 cells were pretreated with the PKC inhibitor GF109203X (1 μM, 15 min) and subsequently challenged with ADP (10 μM, 30 min) or the protein kinase C activator PMA (1 μM, 30 min). D, 1321N1 cells infected with βGal-PKCα-DNM, βGal-PKCδ-DNM, both DNM constructs, or βGal adenovirus alone as control were subsequently challenged with ADP (10 μM, 30 min) or PMA (1 μM, 30 min). Surface-receptor loss was assessed by ELISA. The data represent mean ± S.E.M. of five independent experiments. *, $p < 0.05$ compared with respective controls (Mann-Whitney U test).

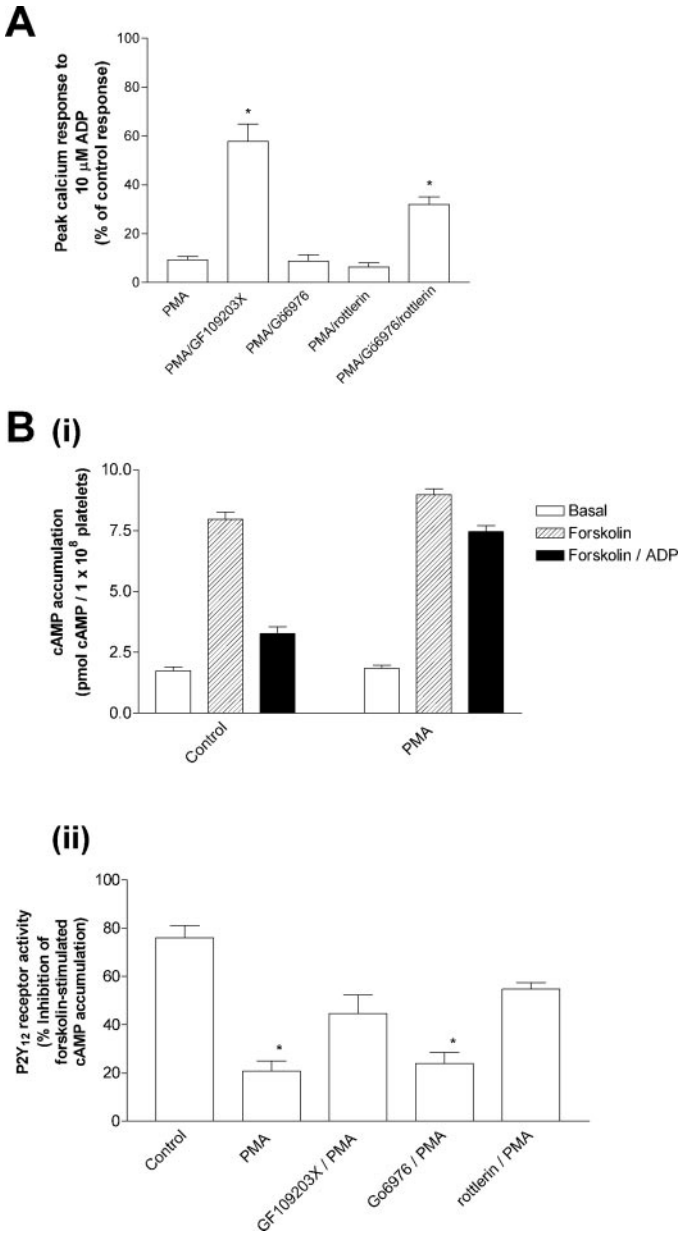

Fig. 5. PKC-dependent desensitization of purinergic receptor responses in human platelets. Platelets were pretreated for 15 min with either the nonspecific PKC inhibitor GF109203X (2 μM); Gö6976 (1 μM), which inhibits calcium-dependent PKC isoforms, including PKCα; rottlerin (10 μM), a PKCδ isoform selective inhibitor; and subsequently challenged with PMA (1 μM, 15 min) or vehicle alone. A, desensitization of $P2Y_1$ purinergic receptor responses was assessed by comparing peak calcium responses with 10 μM ADP in platelets treated with PMA versus vehicle alone. Values are mean ± S.E.M. of three independent experiments, and results are expressed as the ADP response after a desensitizing dose of PMA as a percentage of the control response. *, $p < 0.05$ for data compared with PMA treatment without PKC inhibitor (control; Mann-Whitney U test). B, platelets were either pretreated with PKC inhibitors (as described above) or with vehicle alone (control), as indicated. Agonist (ADP, 10 μM)-dependent inhibition of forskolin (1 μM, 5 min)-stimulated adenylyl cyclase activity by $P2Y_{12}$ purinergic receptor activation after pretreatment with vehicle alone (control) or PMA (1 μM, 15 min) was determined. B(i), data for control and PMA alone pretreatment conditions are presented as mean picomoles of cAMP per milligram of protein ± S.E.M. ($n = 3$). B(ii), values are shown as normalized data for all conditions, including control and PMA pretreatments, and represent mean ± S.E.M of three independent experiments expressed as the percentage of inhibition of forskolin-stimulated adenylyl cyclase. *, statistical significance at $p < 0.05$ for data compared with respective nonpretreated agonist-induced inhibition of forskolin-stimulated controls (Mann-Whitney U test).

number of $P2Y_{12}$ receptor binding sites, whereas Gö6976 had no significant effect (Fig. 7C). Inhibition of PKC activity with GF109203X, rottlerin, or Gö6976 selectively attenuated ADP (10 μM, 5 min)-induced $P2Y_1$ receptor internalization, whereas that of the $P2Y_{12}$ was unaffected (Fig. 7D), which is in agreement with our studies in 1321N1 cells (Fig. 4C). Stimulation of platelets with PMA (1 μM, 15 min) significantly enhanced the internalization of both the $P2Y_1$ and $P2Y_{12}$ receptor (Fig. 7D). Pretreatment with each of the PKC inhibitors reduced PMA-promoted $P2Y_1$ receptor internalization. Again, only GF109203X or rottlerin significantly reduced PMA-promoted $P2Y_{12}$ surface-receptor loss. Therefore,

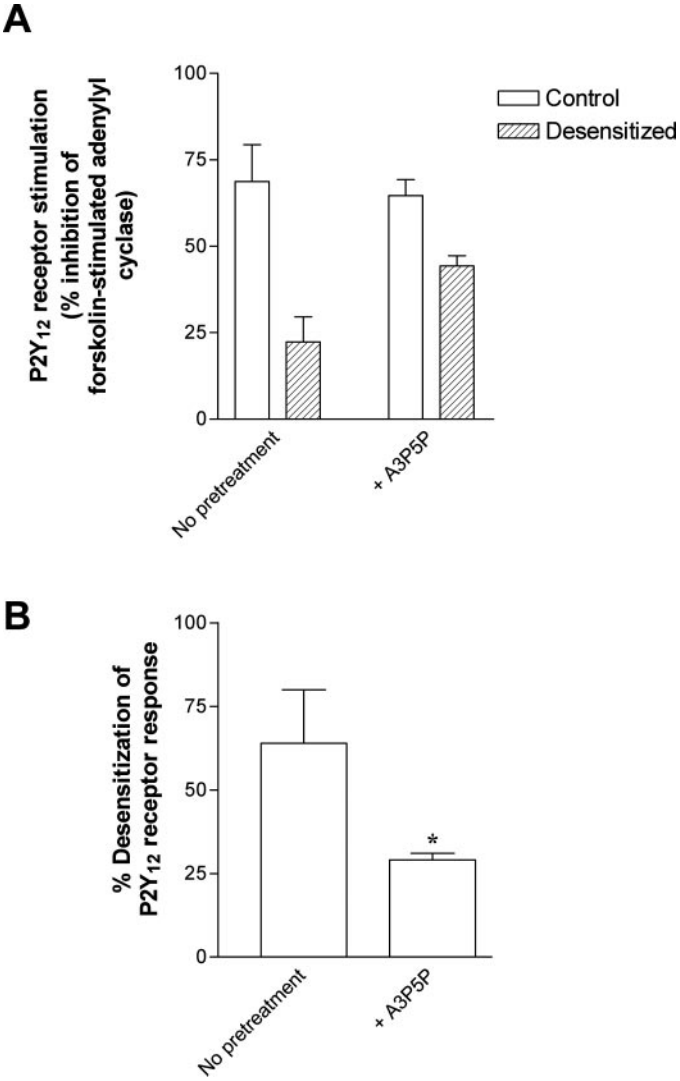

Fig. 6. Heterologous regulation of $P2Y_{12}$ receptor responses by $P2Y_1$ in platelets activated by ADP. Platelets were pretreated with the $P2Y_1$-selective antagonist A3P5P (1 mM) or with vehicle alone (no pretreatment) as control. Agonist (ADP, 10 μM)-dependent inhibition of forskolin (1 μM, 5 min)-stimulated adenylyl cyclase activity by $P2Y_{12}$ purinergic receptor activation was determined after pretreatment with vehicle alone (control) or a desensitizing addition of ADP (10 μM, 5 min). A, data are presented as the percentage of inhibition of adenylyl cyclase activity induced by the addition of ADP. B, data are presented as the percentage of desensitization of the $P2Y_{12}$ receptor response seen after pretreatment with ADP. Data are mean ± S.E.M of three independent experiments. *, statistical significance at $p < 0.05$ for data compared with respective nonpretreated agonist-induced inhibition of forskolin-stimulated controls (Mann-Whitney U test).

PKC can regulate the surface expression and internalization of both $P2Y_1$ and $P2Y_{12}$ receptors in human platelets.

Discussion

The activation of $P2Y_1$ and $P2Y_{12}$ purinergic receptors by ADP is critical for normal platelet function. To avoid inappropriate thrombosis, the sensitivity of these receptors to agonist needs to be continuously regulated. To date, the molecular mechanisms regulating platelet $P2Y_1$ and $P2Y_{12}$ purinergic receptor signaling and surface-receptor expression are relatively poorly understood. In this study investigating the regulation of these two clinically important GPCRs, we find both are phosphorylated after activation of PKC and demonstrate that their surface expression and activity are tightly regulated by this family of kinases. We show that specific PKC isoforms can differentially regulate $P2Y_1$ and $P2Y_{12}$ receptor function, demonstrating some redundancy of PKC isoforms for the regulation of $P2Y_1$ but lack of redundancy of PKC isoforms for regulation of $P2Y_{12}$, which is heterologously controlled by PKCδ.

Although $P2Y_1$ and $P2Y_{12}$ purinergic receptors play an essential role in ADP-induced platelet activation and are important pharmacological targets in the treatment of arterial thrombotic disease (Foster et al., 2001; Kunapuli et al., 2003; Gachet, 2005), minimal studies have investigated the regulation of function of these two GPCRs. In a recent study (Hardy et al., 2005), we demonstrated for the first time that both $P2Y_1$ and $P2Y_{12}$ receptors desensitize in platelets and show that these receptors desensitize by different kinase-dependent mechanisms, in which GRKs regulate the $P2Y_{12}$ receptor and PKC regulates agonist-induced desensitization of the $P2Y_1$ receptor in human platelets. Protein kinase C has been shown also to phosphorylate and regulate agonist-unoccupied receptors (Hipkin et al., 2000; Xiang et al., 2001; Mundell et al., 2002). At first, we demonstrated that both $P2Y_1$ and $P2Y_{12}$ receptors underwent ADP-induced phosphorylation and that activation of PKC by PMA also promoted the phosphorylation of both receptor subtypes. To our knowledge, this is the first demonstration that either of these GPCRs can be phosphorylated in an agonist-dependent or independent manner. The nonselective PKC inhibitor GF109203X attenuated ADP-stimulated $P2Y_1$ receptor phosphorylation, consistent with our recent demonstration that PKC regulates agonist-induced $P2Y_1$ receptor activity

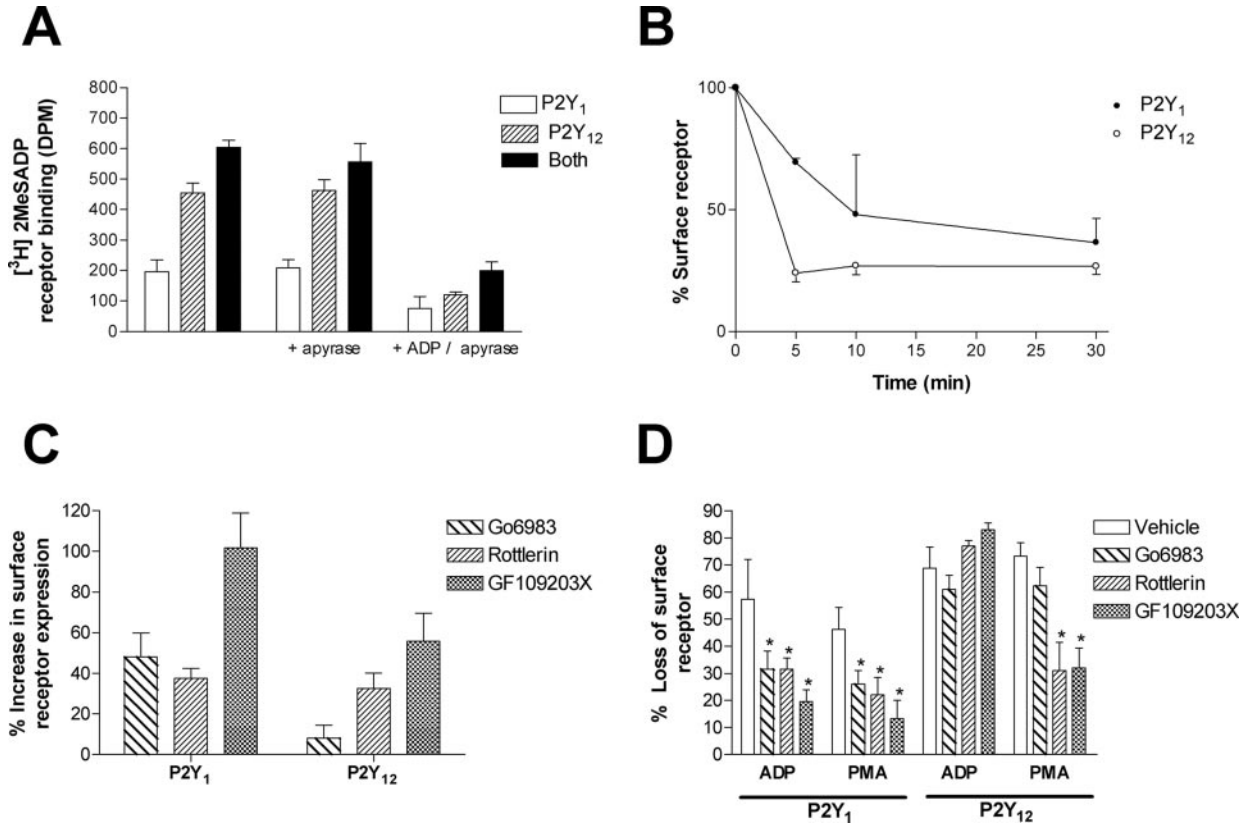

Fig. 7. Surface $P2Y_1$ and $P2Y_{12}$ receptor expression in human platelets is regulated by PKC. In experiments assessing receptor internalization, platelets were stimulated with ADP (10 μM) or vehicle alone for 5, 10, or 30 min. Platelets were incubated with [^{3}H]2MeSADP (100 nM), and specific receptor binding was determined in the presence of either the $P2Y_1$ receptor antagonist A3P5P (1 mM; $P2Y_1$), the $P2Y_{12}$ receptor antagonist AR-C69931MX (1 μM; $P2Y_{12}$), or a combination of both antagonists (Both). A, platelets were treated with ADP (10 μM) for 10 min. Data are expressed as receptor-specific [^{3}H]2MeSADP binding [[^{3}H]2MeSADP binding (absence of displacing ligand $-$ presence of displacing ligand)DPM] and represent means $\pm$ S.E.M. of three independent experiments. B, platelets were treated with ADP (10 μM) for 0 to 30 min. Data are expressed as the percentage of surface receptor and represent means $\pm$ S.E.M. of three independent experiments. C and D, platelets were pretreated for 15 min with either the nonspecific PKC inhibitor GF109203X (2 μM); Gö6976 (1 μM), which inhibits calcium-dependent PKC isoforms, including PKCα; or rottlerin (10 μM), a PKCδ isoform-selective inhibitor. C, $P2Y_1$ and $P2Y_{12}$ surface-receptor expression was compared in platelets pretreated with PKC inhibitors versus nonpretreated controls. Data are expressed as the percentage of increase in surface expression represent means $\pm$ S.E.M. of three independent experiments. D, platelets were subsequently challenged for 15 min with either ADP (10 μM), PMA (1 μM), or vehicle alone. Data are expressed as the percentage of loss of surface receptor and represent means $\pm$ S.E.M. of three independent experiments. $*$, $p < 0.05$ for data compared with ADP or PMA treatment without PKC inhibitor (vehicle; Mann-Whitney U test).

(Hardy et al., 2005). Although PKC can activate GRK2 and three isoforms, promoting their translocation to the cell membrane (Winstel et al., 1996), these kinases are unlikely to mediate agonist-independent PMA-dependent phosphorylation because GRKs only phosphorylate agonist-occupied receptors (Pitcher et al., 1998; Penn et al., 2000). We therefore propose, from the present data, that PKC isoforms lie directly upstream of phosphorylation of $P2Y_1$ and $P2Y_{12}$ receptors, at least in 1321N1 cells. These experiments were undertaken in P2Y receptor-null 1321N1 cells stably expressing epitope-tagged versions of both receptors. The lack of specific and high-affinity P2Y receptor antibodies with which to isolate either receptor from platelet cell membranes prevents similar studies in human platelets at this time. There are, however, a number of putative PKC phosphorylation sites located within the C terminus of both receptors, including Thr^{339} in the $P2Y_1$ receptor, which regulates PKC-dependent desensitization (Fam et al., 2003), and may be important as such in platelets.

Because phosphorylation of agonist-unoccupied receptors has been implicated in the desensitization and internalization of many GPCRs, we sought to determine its functional significance and to identify the specific PKC isoforms responsible. It is interesting to note that PMA pretreatment attenuated subsequent ADP-stimulated $P2Y_1$ and $P2Y_{12}$ receptor activity and promoted agonist-independent surface-receptor loss, the first demonstration of heterologous regulation of these GPCRs. Overexpression of DNM catalytically inactive forms of PKCα and PKCδ, the two PKC isoforms common to both platelets and 1321N1 cells, revealed that both isoforms regulated the agonist and PMA-induced desensitization and internalization of $P2Y_1$ receptors, because an attenuation of receptor desensitization and surface-receptor loss was only evident on coexpression of both DNMs. ADP-induced $P2Y_{12}$ receptor desensitization is regulated by GRKs, and for this receptor, we have ruled out any contribution from PKCs because expression of DNM PKC constructs did not have any effect (Hardy et al., 2005). It is interesting, however, that we found that heterologous PMA-promoted desensitization and internalization of $P2Y_{12}$ receptor function was regulated by PKCδ alone. In addition, we now show that not only receptor-independent activation of PKC but also activation of PKC by endogenous M_3 muscarinic receptors in 1321N1 cells is able to induce internalization of both $P2Y_1$ and $P2Y_{12}$ receptors (Fig. 4, A and B). Together, these novel findings in 1321N1 cells demonstrate that PKC-dependent phosphorylation of both $P2Y_1$ and $P2Y_{12}$ receptors can significantly decrease receptor function and promote a rapid loss of surface receptor. It is unclear at this time whether PMA-promoted loss of surface receptor plays a significant role in reduced receptor responsiveness or if PMA-stimulated receptor phosphorylation can lead to direct receptor/G protein-uncoupling. Detailed studies investigating which regions of these GPCRs regulate their internalization are planned. Such studies will allow us to make internalization-deficient receptor mutants and thereby determine whether surface-receptor loss plays a significant role in reduced signaling output.

Because PKC heterologously regulates $P2Y_1$ and $P2Y_{12}$ receptor function and trafficking in 1321N1 cells, we next examined these phenomena in human platelets. It is interesting that, as in 1321N1 cells, heterologous activation of PKC reduced subsequent $P2Y_1$ and $P2Y_{12}$ receptor responsiveness. In addition, we showed in Fig. 6 that in platelets, $P2Y_1$ receptors contribute partially to desensitization of $P2Y_{12}$ receptors in a heterologous manner. Because platelets lack a nucleus and significant protein synthetic machinery, approaches to disrupt or reduce endogenous protein function (e.g., expression of dominant-negative mutants) are not viable at present. Therefore, to determine the PKC isoforms that regulate purinergic receptor function, we used three PKC inhibitors: GF109203X (2 μM), a potent inhibitor of conventional and novel PKC isoforms (Toullec et al., 1991); Gö6976 (1 μM), an inhibitor with IC_{50} values in the nanomolar range for calcium-dependent PKC isoforms (Martiny-Baron et al., 1993), including PKCα; and rottlerin (10 μM), which selectively inhibits the calcium-independent PKC isoforms, inhibiting PKCδ with an IC_{50} value of approximately 5 μM; 10- to 30-fold higher concentrations are required to inhibit conventional PKC isoforms (Gschwendt et al., 1994). Using these selective inhibitors, we found that as in 1321N1 cells, classic and novel isoforms of PKC can regulate the heterologous desensitization of $P2Y_1$ receptor activity, whereas only PKCδ was capable of desensitizing $P2Y_{12}$ receptor activity in an agonist-independent manner.

To examine changes in purinergic receptor surface expression in human platelets, we used the P2Y receptor radioligand [^{3}H]2MeSADP in combination with the $P2Y_1$ receptor antagonist A3P5P and the $P2Y_{12}$ receptor antagonist AR-C69931MX. Our estimates of receptor number (see *Results*) are similar to those obtained by others (Baurand et al., 2000). This is important because we had chosen to use formaldehyde-fixed platelets for our study. In our study, after stimulation with ADP, the number of binding sites for both $P2Y_1$ and $P2Y_{12}$ receptors was significantly reduced. Recent investigations, in agreement with our own, indicate that agonist pretreatment with ADPβS also reduced the number of $P2Y_1$ receptor binding sites in stably transfected 1321N1 cells (Baurand et al., 2000, 2005). This was paralleled in the study of Baurand et al. (2005) by ADP-induced internalization of $P2Y_1$ receptors in platelets. In contrast, however, $P2Y_{12}$ receptor surface expression was reported by these authors not to change after pretreatment of 1321N1 cells with ADPβS and only to internalize transiently and very rapidly upon treatment of platelets with 5 μM ADP. The data relating to the $P2Y_{12}$ receptor are therefore in contrast with those of the present study, in which we show a more sustained internalization in platelets and 1321N1 cells. The reasons for the discrepancy between our data and those of Baurand et al. (2005) are not clear, although there are a number of methodological differences between the two studies. First, it should be noted that because ADPβS is less potent at $P2Y_{12}$ receptors than at $P2Y_1$ (Takasaki et al., 2001) and is a partial agonist at the $P2Y_{12}$ purinergic receptor (Cusack and Hourani, 1981), it may be unable to promote full internalization of this receptor (Clark et al., 1999). In addition, the study of Baurand et al. (2005) used green fluorescent protein-tagged receptor expressed in 1321N1 cells (Baurand et al., 2005), in contrast to the HA tag used in our study. The relatively bulky green fluorescent protein tag may unpredictably alter signaling and trafficking properties of the receptor. The platelet studies also differ in that Baurand et al. (2005) use an immunogold transmission electron microscopic approach, whereas the present study uses a radioligand binding approach. These methodological differences may explain the

different results obtained for $P2Y_{12}$ receptors. It is interesting that, in our study, pretreatment with PMA also reduced $P2Y_1$ and $P2Y_{12}$ receptor surface expression. This is the first demonstration that heterologous activation of PKC can promote the internalization of P2Y receptors in human platelets.

It is interesting that in the absence of agonist treatment, the surface expression of both receptors was increased after pretreatment with inhibitors of PKC (Fig. 7C), and it seems likely therefore that basal PKC activity is directly regulating surface-receptor number. In 1321N1 cells after agonist-induced internalization, both the $P2Y_1$ and $P2Y_{12}$ receptors can subsequently recycle to the cell surface (data not shown). It is therefore possible that an attenuation of agonist-independent receptor internalization by inhibition of PKC, coupled with recycling of receptor already present in endocytic compartments back to the cell surface, together lead to increased cell surface-receptor number.

As with heterologous receptor desensitization in platelets, PMA-dependent $P2Y_1$ receptor loss is regulated by both classic isoforms of PKC and PKCδ, whereas only PKCδ regulates agonist-independent $P2Y_{12}$ receptor internalization. It is unclear why there is functional redundancy between PKCα and PKCδ in their ability to regulate $P2Y_1$ receptor function in human platelets. These two isoforms play different roles in platelet function (Crosby and Poole, 2003; Murugappan et al., 2004, 2005; Pula et al., 2005), and their different modes of activation may in turn determine their ability to phosphorylate downstream targets. It is also unclear why PKCδ specifically attenuates $P2Y_{12}$ receptor signaling. The activity of PKC isoforms is tightly regulated by multiple molecular mechanisms, including interaction with binding-partner proteins (Poole et al., 2004). A greater understanding of the protein-protein interactions between particular PKC isoforms and membrane-associated anchoring protein, which serve to recruit the PKC isoforms to distinct subcellular compartments in close proximity to receptor target substrates, should prove useful in unraveling patterns of GPCR/PKC isoform specificity.

In conclusion, because ADP performs a pivotal role in the formation of stable platelet aggregates, the activity of purinergic receptors may maintain the delicate balance between rest and activation that underlies platelet sensitivity. Our results show for the first time that regulation of platelet purinergic receptor expression and activity by specific PKC isoforms may play a significant role in hemostatic function.

Acknowledgments

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