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, P2Y1 and P2Y12 (Kunapuli et al., 2003; Gachet, 2005). The combined stimulation of P2Y1 receptor (coupled to Gq and phospholipase Cβ) and P2Y12 receptor (negatively coupled to adenylyl cyclase through Gi) is necessary for the full platelet aggregation response to ADP, with platelet activation initiated by the P2Y1 receptor and amplified by P2Y12 (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 P2Y1 and P2Y12 receptors is tightly regulated. We have shown that both P2Y1 and P2Y12 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;
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 P2Y1 and P2Y12 receptor desensitization by different kinase-dependent mechanisms. P2Y1, but not P2Y12, desensitization is mediated by protein kinase C (PKC) (Hardy et al., 2005). In contrast, agonist-induced desensitization of the P2Y12 receptor, but not P2Y1, 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 platelet P2Y1 receptor? Given that platelets express multiple Gq-coupled receptors, each leading to the activation of PKC, can activation of PKC cause “heterologous” regulation of P2Y12 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 and lysotracker blue were purchased from Invitrogen. The bisindolylmaleimide GF109203X (1 μM), 15 min) or vehicle alone, cells were pretreated 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) (Zhang et al., 2001), -β (Kuroki et al., 2001), and -δ (Blass et al., 2002). Proteins were detected by ECL.

Measurement of Cytosolic [Ca2+] as a Function of PKC Activity in 1321N1 Astrocytoma Cells. The cytosolic free Ca2+ concentration was determined using the fluorescent Ca2+ 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 placed at ~60% confluence. Cells were washed twice with Locke’s solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl2, 2.2 mM CaCl2, 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. [Ca2+] was determined from ratiometric data as described previously (Gryniewicz et al., 1985).

Measurement of cAMP Accumulation in 1321N1 Astrocytoma Cells. Cells infected with PRC-DNM adenosinoviruses 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 Ro20-1724 (250 μM). Appyrase (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 β2 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 (pmol of cAMP per well) or as the percentage of inhibition of forskolin-stimulated adenylyl cyclase.

Internalization of HA-P2Y1 and HA-P2Y12 in 1321N1 Cells. HA-tagged surface-receptor loss was assayed 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 appyrase (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 200g for 17 min, and platelets were then isolated by centrifugation for 10 min at 1000g; in the presence of 0.02 U/ml apyrase and prostaglandin E1 (140 nM) for all assays other than the measurement of intracellular cAMP, in which prostaglandin E1 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 MgCl₂, 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 [Ca²⁺], 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), G66976 (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), G66976 (1 μM), rottlerin (10 μM), or vehicle alone. PKC (1 μM, 15 min) and non–PKC-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 4000g 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 adenyl cyclase.

Radioligand Binding in Human Platelets. In experiments assessing receptor internalization, platelets were pretreated with the PKC inhibitors with GF109203X (2 μM), G66976 (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 12 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 for 10 min at 1000g, rich plasma was prepared by centrifugation at 2000g at 1550 rpm for 25 min in the presence of 4% formaldehyde. Platelets were fixed by continuous rotation for 25 min in the presence of 4% formaldehyde. Platelets were then isolated by centrifugation (10 min) and then resuspended in binding buffer (20 mM HEPES and 1 mM MgCl₂) to a density of 4 × 10^8 platelets/ml. Aliquots of platelet suspension were incubated with [³²P]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 P2Y₁ receptor antagonist A3P5P (1 μM to 3.33 nM) or the P2Y₁₂ 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 t₁/₂ 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 P2Y₁ and P2Y₁₂ Receptor Phosphorylation. No studies to date have demonstrated the direct phosphorylation of either the P2Y₁ or P2Y₁₂ purinergic receptor, although both contain multiple potential phosphorylation sites with their C-terminal domain. At this time, the lack of specific P2Y₁ and P2Y₁₂ 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 P2Y₁ and P2Y₁₂ receptors in P2Y-null 1321N1 cells. A polyclonal anti-HA antibody recognized specific immunoreactive bands in membranes from both P2Y₁ and P2Y₁₂ receptor-expressing cells (Fig. 1). These bands at 45 (P2Y₁) or 70 kDa (P2Y₁₂) 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). P2Y₁ 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). P2Y₁ 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 P2Y₁ and P2Y₁₂ 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 P2Y₁ receptor phosphorylation. Therefore, PKC can directly phosphorylate either the agonist-occupied or -unoccupied P2Y₁ receptor. In contrast, GF109203X only attenuated PMA-promoted P2Y₁₂ receptor phosphorylation. Therefore, agonist-induced P2Y₁₂ receptor phosphorylation is not regulated by PKC, although this kinase can directly phosphorylate the agonist-unoccupied receptor.

PKC-Dependent Desensitization of P2Y₁ and P2Y₁₂ Receptor Activity in 1321N1 Cells. To identify more accurately the PKC isoforms that may regulate P2Y₁ and P2Y₁₂ 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. P2Y₁ 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<sub>1</sub> receptor desensitization was only apparent in cells coexpressing both PKC<sub>α</sub>- and PKC<sub>δ</sub>-DNM (Fig. 3A). Pretreatment with PMA (1 μM, 15 min) significantly attenuated subsequent agonist-induced P2Y<sub>1</sub> receptor activity. As with the homologous desensitization of P2Y<sub>1</sub> receptor activity, heterologous PMA-induced desensitization was only attenuated when both PKC<sub>α</sub>- and PKC<sub>δ</sub>-DNM were coexpressed. Studies examining the Gi-coupled P2Y<sub>12</sub> purinergic receptor function revealed that ADP-induced inhibition of forskolin-stimulated adenyl 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 adenyl cyclase or inhibit forskolin-induced activity of the cyclase. Therefore, PKC can regulate the agonist-unoccupied P2Y<sub>12</sub> purinergic receptor in a heterologous manner. Overexpression of DNM-PKC<sub>δ</sub> and not DNM-PKC<sub>α</sub> selectively attenuated PMA-induced P2Y<sub>12</sub> purinergic receptor desensitization, whereas ADP-induced desensitization was unaffected by either DNM-PKC<sub>α</sub>.

**PKC-Dependent Internalization of P2Y<sub>1</sub> and P2Y<sub>12</sub> 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<sub>1</sub> and P2Y<sub>12</sub> receptors (Fig. 4). The rate of internalization is shown in Fig. 4, A and B, for P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors, respectively. It is also important to note that we show that carbachol (1 mM), operating through endogenously expressed muscarinic M<sub>3</sub> receptors, is able heterologously to induce internalization of both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors. Pretreatment with GF109203X (1 μM, 15 min) selectively attenuated ADP-induced P2Y<sub>1</sub> receptor internalization, whereas that of the P2Y<sub>12</sub> receptor was unaffected (Fig. 4C). As expected, inhibition of PKC with GF109203X reversed internalization of both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors induced by PMA. ADP and PMA-induced P2Y<sub>1</sub> receptor internalization was partially inhibited by the expression of either PKC<sub>α</sub>- or PKC<sub>δ</sub>-DNM, with coexpression of both DNMs producing a more robust inhibition of receptor internalization (Fig. 4D). As with GF109203X pretreatment, expression of PKC<sub>α</sub>- or PKC<sub>δ</sub>-DNM did not attenuate ADP-induced P2Y<sub>12</sub> receptor internalization (Fig. 4D). It is interesting that, consistent with their effects on functional desensitization, expression of DNM-PKC<sub>δ</sub> but not DNM-PKC<sub>α</sub> attenuated PMA-induced P2Y<sub>12</sub> receptor internalization (Fig. 4D).

**Regulation of P2Y<sub>1</sub> and P2Y<sub>12</sub> Receptor Desensitization by PKC in Human Platelets.** Because our studies in 1321N1 cells revealed that PKC could regulate P2Y<sub>1</sub> and P2Y<sub>12</sub> 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<sub>1</sub> 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<sub>1</sub> receptor-mediated calcium response (Fig. 5A). Inhibition of PKC with GF109203X (2 μM), a nonselective PKC inhibitor, significantly attenuated P2Y<sub>1</sub> receptor desensitization. Pretreatment with Go6976 (1 μM), which inhibits calcium-dependent classic PKC isoforms (Martiny-Baron et al., 1993), including PKC<sub>α</sub>, and rottlerin (10 μM), a PKC<sub>δ</sub> isoform selective inhibitor (Gschwendt et al., 2005), was reduced after PMA (1 μM, 15 min) pretreatment. Pretreatment with GF109203X (1 μM, 15 min) significantly attenuated ADP-induced P2Y<sub>12</sub> receptor internalization (Fig. 4D). It is interesting that, consistent with their effects on functional desensitization, expression of DNM-PKC<sub>δ</sub> but not DNM-PKC<sub>α</sub> attenuated PMA-induced P2Y<sub>12</sub> receptor internalization (Fig. 4D).
al., 1994), were unable to attenuate PMA-promoted P2Y1-receptor desensitization (Fig. 5A). It is important to note, however, that pretreatment with both Go6976 and rotterlin was able to partially attenuate the desensitization of P2Y1 receptor activity. Stimulation of PKC with PMA also decreased ADP-induced P2Y12 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 rotterlin attenuated PMA-induced P2Y12 receptor desensitization, whereas Go6976 had no effect on desensitization of the P2Y12 response (Fig. 5B). Therefore, stimulation of PKC activity can promote the heterologous desensitization of both P2Y1 and P2Y12 receptor responses in human platelets. Finally, to demonstrate that, in the platelet system, the Gq-coupled P2Y1 receptor may regulate P2Y12, we chose to investigate the effect of P2Y1 receptor blockade on ADP-induced desensitization of the P2Y12 receptor response. Figure 6 shows that pretreatment of platelets with the P2Y1 receptor antagonist A3P5P (1 mM) does not attenuate P2Y12-mediated inhibition of adenylyl cyclase, but that in the presence of A3P5P, the ADP-induced desensitization of the P2Y12 receptor is reduced, indicating partial heterologous regulation of P2Y12 by the P2Y1 receptor.

**Internalization of P2Y1 and P2Y12 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-
uration binding experiments measuring \[^{3}H\]2MeSADP binding to fixed platelets in the presence and absence of unlabeled radioligand (10 \(\mu\)M) indicated that there were 901 \(\pm\) 41 \[^{3}H\]2MeSADP binding sites per platelet with an affinity of 4.9 \(\pm\) 0.3 nM. Further saturation experiments using the P2Y\(_{1}\) receptor antagonist A3P5P (1 mM) or the P2Y\(_{12}\) receptor antagonist AR-C69931MX (1 \(\mu\)M) revealed two distinct binding populations of 184 \(\pm\) 27 and 644 \(\pm\) 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 \(\pm\) 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 \(\pm\) 1.2 mM and 45.0 \(\pm\) 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 \(\mu\)M) to give an estimate of either the P2Y\(_{1}\) or P2Y\(_{12}\) surface binding sites. After pretreatment with ADP (10 \(\mu\)M, 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 pretreated 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, Go\(\delta\)6976, 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 \(\mu\)M), the protein kinase C activator PMA (1 \(\mu\)M) 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 \(\pm\) 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 \(\mu\)M, 15 min) and subsequently challenged with ADP (10 \(\mu\)M, 30 min) or ADP (10 \(\mu\)M, 30 min). Surface-receptor loss was assessed by ELISA at various time points after the addition of agonist. Data represent mean \(\pm\) S.E.M. of five independent experiments. D, 1321N1 cells infected with \(\beta\)Gal-PKCa-DNM, \(\beta\)Gal-PKCa-DNM, both DNM constructs, or \(\beta\)Gal adenovirus alone as control were subsequently challenged with ADP (10 \(\mu\)M, 30 min) or PMA (1 \(\mu\)M, 30 min). Surface-receptor loss was assessed by ELISA. The data represent mean \(\pm\) S.E.M. of five independent experiments. *, \(p < 0.05\) compared with respective controls (Mann-Whitney \(U\) test).
number of P2Y\textsubscript{12} receptor binding sites, whereas G\textsubscript{o}6976 had no significant effect (Fig. 7C). Inhibition of PKC activity with GF109203X, rottlerin, or G\textsubscript{o}6976 selectively attenuated ADP (10 \textmu M, 5 min)-induced P2Y\textsubscript{1} receptor internalization, whereas that of the P2Y\textsubscript{12} was unaffected (Fig. 7D), which is in agreement with our studies in 1321N1 cells (Fig. 4C). Stimulation of platelets with PMA (1 \textmu M, 15 min) significantly enhanced the internalization of both the P2Y\textsubscript{1} and P2Y\textsubscript{12} receptor (Fig. 7D). Pretreatment with each of the PKC inhibitors reduced PMA-promoted P2Y\textsubscript{1} receptor internalization. Again, only GF109203X or rottlerin significantly reduced PMA-promoted P2Y\textsubscript{12} surface-receptor loss. Therefore,

![Fig. 5. PKC-dependent desensitization of purinergic receptor responses in human platelets. Platelets were pretreated for 15 min with either the non-specific PKC inhibitor GF109203X (2 \textmu M); G\textsubscript{o}6976 (1 \mu M), which inhibits calcium-dependent PKC isoforms, including PKC\textalpha; rottlerin (10 \textmu M), a PKC\textbeta\textalpha} isoenzyme selective inhibitor; and subsequently challenged with PMA (1 \textmu M, 15 min) or vehicle alone. A, desensitization of P2Y\textsubscript{1} purinergic receptor responses was assessed by comparing peak calcium responses with 10 \textmu M ADP in platelets treated with PMA versus vehicle alone. Values are mean \pm 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 \textmu M)-dependent inhibition of forskolin (1 \textmu M, 5 min)-stimulated adenylyl cyclase activity by P2Y\textsubscript{12} purinergic receptor activation after pretreatment with vehicle alone (control) or PMA (1 \textmu M, 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 \pm S.E.M. (n = 3). B(ii), values are shown as normalized data for all conditions, including control and PMA pretreatments, and represent mean \pm 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).

![Fig. 6. Heterologous regulation of P2Y\textsubscript{12} receptor responses by P2Y\textsubscript{1} in platelets activated by ADP. Platelets were pretreated with the P2Y\textsubscript{1}-selective antagonist A23348P (1 mM) or with vehicle alone (no pretreatment) as control. Agonist (ADP, 10 \textmu M)-dependent inhibition of forskolin (1 \textmu M, 5 min)-stimulated adenylyl cyclase activity by P2Y\textsubscript{12} purinergic receptor activation was determined after pretreatment with vehicle alone (control) or a desensitizing addition of ADP (10 \textmu M, 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\textsubscript{12} receptor response seen after pretreatment with ADP. Data are mean \pm 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 P2Y₁ and P2Y₁₂ receptors in human platelets.

**Discussion**

The activation of P2Y₁ and P2Y₁₂ 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 P2Y₁ and P2Y₁₂ 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 P2Y₁ and P2Y₁₂ receptor function, demonstrating some redundancy of PKC isoforms for the regulation of P2Y₁, but lack of redundancy of PKC isoforms for regulation of P2Y₁₂, which is heterologously controlled by PKCδ.

Although P2Y₁ and P2Y₁₂ 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 P2Y₁ and P2Y₁₂ receptors desensitize in platelets and show that these receptors desensitize by different kinase-dependent mechanisms, in which GRKs regulate the P2Y₁₂ receptor and PKC regulates agonist-induced desensitization of the P2Y₁ 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 P2Y₁ and P2Y₁₂ 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 P2Y₁ receptor phosphorylation, consistent with our recent demonstration that PKC regulates agonist-induced P2Y₁ receptor activity.

**Fig. 7.** Surface P2Y₁ and P2Y₁₂ 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 [³H]2MeSADP (100 nM), and specific receptor binding was determined in the presence of either the P2Y₁ receptor antagonist A3P5P (1 mM; P2Y₁), the P2Y₁₂ receptor antagonist AR-C69931MX (1 μM; P2Y₁₂), or a combination of both antagonists (Both). A, platelets were treated with ADP (10 μM) for 10 min. Data are expressed as receptor-specific [³H]2MeSADP binding ([³H]2MeSADP binding (absence of displacing ligand) – presence of displacing ligand) DPM and represent means ± 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 ± 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); Go6983 (1 μM), which inhibits calcium-dependent PKC isoforms, including PKCε; or rottlerin (10 μM), a PKCδ isoform-selective inhibitor. C, P2Y₁ and P2Y₁₂ 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 ± 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 ± 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).
endogenous M3 muscarinic receptors in 1321N1 cells is able
independent activation of PKC but also activation of PKC by
lated subsequent ADP-stimulated P2Y1 and P2Y12 recep-
sible. It is interesting to note that PMA pretreatment atten-
significance and to identify the specific PKC isoforms respon-
some GRKs only phosphorylate agonist-occupied
receptors, at least in 1321N1 cells. These experiments were
performed on 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 phosphoryla-
sites located within the C terminus of both receptors,
including Thr339 in the P2Y1 receptor, which regulates PKC-
dependent desensitization (Fum et al., 2003), and may be
important as such in platelets.
Because phosphorylation of agonist-unoccupied receptors
has been implicated in the desensitization and internaliza-
tion of many GPCRs, we sought to determine its functional
significance and to identify the specific PKC isoforms respon-
sible. It is interesting to note that PMA pretreatment atten-
uated subsequent ADP-stimulated P2Y1 and P2Y12 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 P2Y1 receptors, because an attenuation of
receptor desensitization and surface-receptor loss was only
evident on coexpression of both DNMs. ADP-induced P2Y12
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 P2Y12 receptor function was regulated by
PKCδ alone. In addition, we now show that not only receptor-
dependent activation of PKC but also activation of PKC by
endogenous M3 muscarinic receptors in 1321N1 cells is able
to induce internalization of both P2Y1 and P2Y12 receptors
(Fig. 4, A and B). Together, these novel findings in 1321N1
cells demonstrate that PKC-dependent phosphorylation of
both P2Y1 and P2Y12 receptors can significantly decrease
receptor function and promote a rapid loss of surface recep-
tor. 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 phosphoryla-
tion can lead to direct receptor/G protein-uncoupling. De-
tailed 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 P2Y1 and P2Y12
receptor function and trafficking in 1321N1 cells, we next
examined these phenomena in human platelets. It is interest-
ing that, as in 1321N1 cells, heterologous activation of
PKC reduced subsequent P2Y1 and P2Y12 receptor respon-
siveness. In addition, we showed in Fig. 6 that in platelets,
P2Y1 receptors contribute partially to desensitization of
P2Y12 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 via-
able 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 conven-
tional and novel PKC isoforms (Toullec et al., 1991); Go6976
(1 μM), an inhibitor with IC50 values in the nanomolar range
for calcium-dependent PKC isoforms (Martiny-Baron et al.,
1993), including PKCα; and rottlerin (10 μM), which selec-
tively inhibits the calcium-independent PKC isoforms, inhib-
itng PKCδ with an IC50 value of approximately 5 μM; 10- to
30-fold higher concentrations are required to inhibit conven-
tional 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 P2Y1 receptor activity, whereas only PKCδ
was capable of desensitizing P2Y12 receptor activity in an
agonist-independent manner.
To examine changes in purinergic receptor surface expres-
sion in human platelets, we used the P2Y1 receptor radioli-
gand [3H]2MeSADP in combination with the P2Y1 receptor
antagonist A3P5P and the P2Y12 receptor antagonist AR-
69931MX. 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 formalde-
hyde-fixed platelets for our study. In our study, after stimu-
lation with ADP, the number of binding sites for both P2Y1
and P2Y12 receptors was significantly reduced. Recent inves-
tigations, in agreement with our own, indicate that agonist
pretreatment with ADPβS also reduced the number of P2Y1
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
P2Y1 receptors in platelets. In contrast, however, P2Y12
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 P2Y12 receptor are therefore in contrast with those of the
present study, in which we show a more sustained internal-
ization 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 meth-
odological differences between the two studies. First, it
should be noted that because ADPβS is less potent at P2Y12
receptors than at P2Y1 (Takasaki et al., 2001) and is a partial
agonist at the P2Y12 purinergic receptor (Cusack and Hou-
rani, 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 signal-
ing and trafficking properties of the receptor. The platelet
studies also differ in that Baurand et al. (2005) use an
munogold transmission electron microscopic approach,
whereas the present study uses a radioligand binding ap-
proach. 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 PKC$\delta$, whereas only PKC$\delta$ regulates agonist-independent P2Y$_{12}$ receptor internalization. It is unclear why there is functional redundancy between PKC$\alpha$ and PKC$\delta$ 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 PKC$\delta$ 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

We are grateful to Professors Motoi Ohba (Showa University, Shinagawa, Tokyo, Japan) and Toshio Kuroki (Gifu University, Gifu, Japan) for generation of PKC DNMs in adenosinergic and to Professor David Murphy (University of Bristol, Bristol, UK) for supplying these viruses. We also thank AstraZeneca for the generous supply of AR-C69931MX.

References


Address correspondence to: Dr. Alastair W. Poole, Department of Pharmacology, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK. E-mail: a.poole@bris.ac.uk