The G<sub>i</sub>-coupled P2Y12 Receptor Regulates Diacylglycerol-mediated Signaling in Human Platelets*  

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Stimulation of G<sub>i</sub>-coupled receptors activates phospholipase C and is supposed to promote both intracellular Ca<sup>2+</sup> mobilization and protein kinase C (PKC) activation. We found that ADP-induced phosphorylation of pleckstrin, the main platelet substrate for PKC, was completely inhibited not only by an antagonist of the G<sub>i</sub>-coupled P2Y1 receptor but also upon blockade of the G<sub>i</sub>-coupled P2Y12 receptor. The role of G<sub>i</sub> on PKC regulation required stimulation of phosphatidylinositol 3-kinase rather than inhibition of adenyl cyclase. P2Y12 antagonists also inhibited pleckstrin phosphorylation, Rap1b activation, and platelet aggregation induced upon G<sub>i</sub> stimulation by the thromboxane A<sub>2</sub> analogue U46619. Importantly, activation of phospholipase C and intracellular Ca<sup>2+</sup> mobilization occurred normally. Phorbol 12-myristate 13-acetate overcame the inhibitory effect of P2Y12 receptor blockade on PKC activation but not on Rap1b activation and platelet aggregation. By contrast, inhibition of diacylglycerol kinase restored both PKC and Rap1b activity and caused platelet aggregation. Stimulation of P2Y12 receptor or direct inhibition of diacylglycerol kinase potentiated the effect of membrane-permeable sn-1,2-dioctanoylglycerol on platelet aggregation and pleckstrin phosphorylation, in association with inhibition of its phosphorylation to phosphatatic acid. These results reveal a novel and unexpected role of the G<sub>i</sub>-coupled P2Y12 receptor in the regulation of diacylglycerol-mediated events in activated platelets.

It is generally accepted that, with a very few exceptions, platelet response to soluble agonists originates from the convergence of at least two different signal transduction pathways typically initiated by the heterotrimeric G-proteins G<sub>q</sub> and G<sub>i</sub>. Several receptors for platelet agonists are coupled to G<sub>q</sub>, including the P2Y1 receptor for ADP, the TPα receptor for the thromboxane A<sub>2</sub> (TxA<sub>2</sub>),<sup>3</sup> and the PAR1 and PAR4 receptors for thrombin (1, 2). Signaling downstream G<sub>q</sub> involves the stimulation of members of the β subfamily of phospholipase C (PLC), which generate the second messengers inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), responsible for Ca<sup>2+</sup> mobilization from intracellular stores and protein kinase C (PKC) activation, respectively (3). This pathway is necessary but not sufficient to elicit a full platelet response, and the concomitant activation of a G<sub>i</sub>-coupled receptor is absolutely mandatory (4).

To fulfill this requirement, some agonists, like ADP, directly stimulate multiple G-protein coupled receptors, one of which is associated to G<sub>i</sub> (5, 6), whereas others, such as TxA<sub>2</sub>, rely on the auxiliary action of secondary messengers released by activated platelets to stimulate a G<sub>i</sub>-coupled receptor (7). Despite their essential role, there are very few G<sub>i</sub>-coupled receptors on the platelet surface, and these include the α2-adrenergic receptor for epinephrine and the P2Y12 receptor for ADP (8, 9). Therefore, ADP, which is also one of the major component of the platelet releasate, is a crucial regulator of platelet function, and its receptors have gained increasing attention as potential target for anti-thrombotic agents, including thienopyridines (9–11).

The biochemical basis for the critical role of G<sub>i</sub> stimulation in platelet aggregation is not completely known. Inhibition of adenyl cyclase and reduction of intracellular cAMP levels do not appear sufficient to complement G<sub>q</sub>-dependent pathway for platelet aggregation (12). However, binding of ADP to the G<sub>i</sub>-coupled P2Y12 receptor regulates, through the G protein βγ dimers, additional intracellular effectors, including members of the class I phosphatidylinositol 3-kinase and the small GTPase Rap1b (13–17). Activation of phosphatidylinositol 3-kinase lies upstream stimulation of Rap1b (15–18), which in turn has emerged as a critical regulator of integrin αIIbβ3 and platelet aggregation (19–21). The phosphatidylinositol 3-kinase-Rap1b pathway has been proposed to be responsible for the G<sub>i</sub>-mediated contribution to platelet aggregation (15, 18, 22–26), but many aspects are not clearly defined.

In this work we report that whereas activation of PLC is exclusively controlled by G<sub>i</sub>-coupled receptors, the downstream stimulation of PKC requires the concomitant activation of the G<sub>i</sub>-coupled P2Y12 receptor. Moreover, we show that all the inhibitory effects of P2Y12 receptor antagonists on platelet function can be reversed by pharmacologic inhibition of the DAG metabolizing enzyme diacylglycerol kinase (DGK) but not by direct stimulation of PKC. These results indicate that DAG is...
FIGURE 1. Co-stimulation of P2Y1 and P2Y12 receptors is required for ADP-induced pleckstrin phosphorylation. A, 32P-labeled platelets were left untreated (bas) or were stimulated with 10 μM ADP in the absence of inhibitors or in the presence of the P2Y1 receptor antagonist MRS2179 (200 μM) or the P2Y12 receptor antagonist AR-C69931MX (1 μM) as indicated on the top for times ranging from 30 s to 3 min, as indicated on the bottom. As control, platelets were stimulated with 1 unit/ml thrombin (THR). Platelet proteins were separated by SDS-PAGE, and phosphorylated pleckstrin, indicated by the arrow on the right, was identified by autoradiography. B, autoradiographic analysis of pleckstrin phosphorylation in 32P-labeled platelets left untreated (bas) or stimulated with 100 nM PMA for the indicated times in the absence or in the presence of 1 μM AR-C69931MX. C, platelets were preincubated with buffer (none) or with the P2Y1 and P2Y12 receptors antagonists MRS2179 (200 μM), AR-C69931MX (1 μM), and 2MeSAMP (1 μM) as indicated on the bottom and then were left untreated (bas) or stimulated with 10 μM ADP for 1 min. PKC-directed protein phosphorylation was analyzed by immunoblotting with an anti-phospho(Ser) PKC substrates antibody. The position of the 47-kDa pleckstrin is indicated by the arrow on the right. D, platelets were incubated in the absence or presence of the P2Y12 receptor antagonist 2MeSAMP (1 μM) and then stimulated with the indicated doses of ADP for 1 min. Immunoblotting with an anti-phospho(Ser) PKC substrates antibody was used to detect phosphorylated pleckstrin, indicated by the arrow on the right. E, platelets were incubated with the P2Y12 receptor antagonists AR-C69931MX or 2MeSAMP in combination with the adenylyl cyclase inhibitor dideoxyadenosine (DDA, 100 μM) or were treated with the phosphatidylinositol 3-kinase inhibitors LY294002 (25 μM) or wortmannin (50 nM) as indicated on the bottom. Upon stimulation with 10 μM ADP, pleckstrin phosphorylation, indicated by the arrow, was analyzed by immunoblotting with the anti-phospho(Ser) PKC substrates antibody.
a critical messenger for platelet aggregation regulated through G<sub>i</sub>-dependent signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—ADP, U46619, thrombin, MRS2179, 2MeSAMP, phorbol 12-myristate 13-acetate (PMA), 1,2-dioleoylglycerol, prostaglandin E<sub>1</sub>, acetylsalicylic acid, sn-1,2 dioctanoylglycerol (DiC8), and apyrase were from Sigma. [32P]Orthophosphate, [3H(N)]-arachidonic acid (189 Ci/mmol) were from GE Healthcare. OCTOBER 24, 2008 • VOLUME 283 • NUMBER 43

**Platelet Isolation**—Human platelets were obtained from healthy volunteers in citric acid/citrate/dextrose (152 mM sodium citrate, 130 mM citric acid, 112 mM glucose). Whole blood was centrifuged at 120 x g for 10 min at room temperature. Apyrase (0.2 units/ml), prostaglandin E<sub>1</sub> (1 μM), and acetylsalicylic acid (1 mM) were then added to the platelet-rich plasma. Platelets were recovered by centrifugation at 720 x g for 15 min, washed with 10 ml of PBS buffer (20 mM PBS, 136 mM NaCl, pH 6.5), and finally gently resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, pH 7.4). The cell count was typically adjusted to 0.3 x 10<sup>9</sup> platelets/ml unless otherwise stated.

**Platelet Stimulation and Measurement of Aggregation**—All the experiments were performed with 0.1–0.4 ml samples of washed platelets placed at 37 °C in an aggregometer under constant stirring in the presence of 1 mM CaCl<sub>2</sub>. Platelets were stimulated with 10 μM ADP or 1 μM U46619 for 1 min unless otherwise stated in the figure legends. Preincubation with specific inhibitors, activators, or receptor antagonists was as follows: 1 or 2.5 nM PMA for 1 min, 0.1–3.5 μM DiC8 for 1 min, 200 μM MRS2179, 1 μM AR-C69931MX or 2MeSAMP for 2 min, 1 μM R59949 for 5 min, 50 nM wortmannin, 25 μM LY294002 for 15, 100 μM dideoxycadenosine for 30 min. When compounds were dissolved in DMSO, an equal volume of vehicle was added to control and samples. For aggregation measurement, stimulation was prolonged up to 7 min, and light transmission was continuously monitored. All the aggregation traces reported in the figures are representative of at least three different experiments.

**Measurement of Pleckstrin Phosphorylation and DiC8-PA Production Using 32P-Labeled Platelets**—Platelets, at the final concentration of 10<sup>9</sup> cells/ml in PBS buffer, were incubated with 0.1 mCi/ml [32P] for 90 min at 37 °C, centrifuged at 800 x g for 15 min, and finally resuspended in HEPES buffer containing 1 mM CaCl<sub>2</sub> and 5.5 mM glucose. Samples (0.2 ml) were preincubated with different inhibitors and then stimulated with ADP or U46619 as above indicated. The reaction was stopped by the addition 0.1 ml of SDS sample buffer 3X (37.5 mM Tris/HCl, pH 8.3, 288 mM glycine, 6% SDS, 1.5% dithiothreitol, 30% glycerol, 0.03% bronopol blue) and by heating at 95 °C for 3 min. Identical aliquots of total platelet proteins (20 μl) were sepa-
rated by SDS-PAGE on a 5–15% acrylamide gradient gel and stained with Coomassie Blue. Gels were then dried, and phosphorylation of pleckstrin was visualized upon autoradiography for about 18 h at −80 °C.

For measurement of DiC8 conversion to DiC8-PA, samples of 32P-labeled platelets (0.2 ml) were treated with 500 nM DiC8 in the presence or in the indicated concentrations of ADP plus 200 μM MRS2179, 1 μM AR-C69931MX, or 1 mM R59949 for 1 min. The reaction was stopped by the addition of 0.275 ml of chloroform and 0.55 ml of methanol. Phase partition was obtained through the addition of 0.275 ml of chloroform and 0.44 ml of 1 M HCl. Upon centrifugation at 800 g for 10 min, the lipids recovered in the lower phase were spotted on silica gel TLC plates and eluted in ethyl acetate/iso-octane/acetic acid/water (45:20:12:6). Spots on the TLC plates were visualized by autoradiography for about 18 h at −80 °C, and DiC8-derived PA production was quantified by analysis through ImageJ software.

Quantification of pleckstrin phosphorylation was performed by densitometric analysis. All the reported figures are representative of at least three different experiments.

**Evaluation of PKC-dependent Protein Phosphorylation Using an Anti-phospho(Ser) PKC Substrate Antibody**—Platelet stimulation on 0.1-ml samples (see above) was stopped by the addition of 0.05 ml of SDS sample buffer 3X. Proteins were separated by SDS-PAGE on a 5–15% acrylamide gradient gel, transferred to nitrocellulose, and probed by immunoblotting using an anti-phospho(Ser) PKC substrates antibody diluted 1:1000, as previously described (24). Immunoreactive bands were visualized by enhanced chemiluminescence reaction. Quantification of pleckstrin phosphorylation was performed by densitometric analysis. All the reported figures are representative of at least three different experiments.

**Measurement of Cytosolic Ca2+ Concentration**—Intracellular Ca2+ concentration was measured in Fura-2-AM-loaded platelets essentially as previously described (24). For these experiments, the final platelet concentration was 2 × 10⁹ cells/ml, and preincubation and stimulation was performed on 0.4-ml samples prewarmed at 37 °C under gentle stirring in a PerkinElmer Life Sciences LS3 spectrofluorometer in the presence of 1 mM EGTA. All determinations were repeated at least four times with platelets from different donors.

**Measurement of [3H]Inositol Phosphate Production**—Platelets in PIPES buffer (2 × 10⁹ cells/ml) were labeled with 0.125 mCi/ml myo-[2–3H]inositol for 3 h at 37 °C, centrifuged, and finally resuspended in HEPES buffer containing 5.5 mM glucose, 0.5 mM RGDS, 1 mM CaCl₂, and 10 mM LiCl at the concentration of 10⁹ cells/ml. Platelet samples (0.4 ml) were incubated in the presence or absence of 1 μM AR-C69931MX and stimulated under constant stirring with 1 μM U46619. The reaction was stopped by adding 0.55 ml of chloroform and 1.1 ml of methanol. Samples were placed on ice, and phase partition was obtained by the addition of 0.55 ml of 1 M HCl. The upper phase (2 ml), collected upon centrifugation at 800 g for 10 min, was neutralized with 1 ml of 2 M ammonium acetate, and [3H]inositol phosphates were separated by ion exchange chromatography on 1 ml of AG 1-X resin, as previously described (27) and quantified by scintillation counting in the gel phase.

**Measurement of DAG Accumulation**—Platelets in PIPES buffer (10⁹ cells/ml) were labeled with 2.5 μCi/ml [3H]arachidonic acid for 30 min at 37 °C and then resuspended in HEPES buffer containing 5.5 mM glucose, 0.5 mM RGDS, and 1 mM CaCl₂. Incubation and stimulation of platelet samples (0.2 ml) as well as phase partition was performed as described for the inositol phosphates measurement; however, the lower phases (0.4 ml) were collected and dried under N₂ flux. Lipids were resuspended with 20 μl of methanol/chloroform (1:1). Each sample was mixed with 1 μg of standard purified DAG and spotted on silica gel TLC plates. Elution was performed with diethyl ether:exane:acetic acid (70:30:1), and DAG spots were visualized with iodine vapors. The spots corresponding to DAG were scraped, and the radioactivity was measured by scintillation counting.

**Rap1b Activation Assay**—Activation of Rap1b in platelet samples (0.2 ml) was evaluated by a pulldown assay using the GST-tagged Rap binding domain of Rap1b (GST-Rap1b-RBD) essentially as previously reported (28). Quantification of...
Rap1b activation was performed by densitometric analysis of the immunoblots.

RESULTS

Stimulation of the G<sub>i</sub>-coupled P2Y<sub>12</sub> Receptor Is Necessary for ADP- or U46619-induced PKC Activation—The possible cross-talk between P2Y1 and P2Y12 receptors in the regulation of platelet PKC was evaluated by measuring the phosphorylation of the 47-kDa protein pleckstrin, the main PKC substrate, in 32P-labeled cells (29). Autoradiographic analysis allowed the detection of a clear, albeit faint, phosphorylation of pleckstrin in ADP-stimulated platelets which was rapid and transient (Fig. 1A). As expected, pleckstrin phosphorylation was totally suppressed upon blockade of the G<sub>q</sub>-coupled P2Y1 receptor by MRS2179. Surprisingly, however, we observed that ADP-induced pleckstrin phosphorylation was also completely abrogated upon platelet incubation with 1 or 2.5 nM PMA, a specific antagonist of the G<sub)i</sub>-coupled P2Y<sub>12</sub> receptor (Fig. 1A), which is not supposed to contribute to PLC regulation. By contrast, AR-C69931MX did not alter pleckstrin phosphorylation induced by the phorbol ester PMA (Fig. 1B) or by thrombin (data not shown).

Because our analysis with 32P-labeled platelets revealed no weaker metabolic labeling of the PKC substrates, we consolidated our observations with a different, immunological approach using an anti-phospho(Ser) PKC substrates antibody. This antibody detected a number of proteins in resting platelets whose reactivity was clearly increased upon stimulation with ADP (Fig. 1C). Such an increase was particularly evident for a 47-kDa band, which was identified as

FIGURE 4. Subthreshold concentrations of PMA restore agonist-induced pleckstrin phosphorylation but not platelet aggregation. A, analysis of pleckstrin phosphorylation evaluated by immunoblotting with the anti-phospho(Ser) PKC substrates antibody upon incubation with 1 or 2.5 nM PMA in the absence or presence of 1 μM AR-C69931MX as indicated. Stimulation was with 1 μM U46619 for 1 min, bas, untreated. B, analysis of platelet aggregation induced by 1 or 2.5 nM PMA. C, platelets were stimulated with 1 μM U46619 in the absence (none) or in the presence of 1 μM AR-C69931MX alone or in combination with 1 or 2.5 nM PMA as indicated on the right. The aggregation traces reported are representative of at least three different experiments.
pleckstrin based on immunoblotting analysis performed with $^{32}$P-labeled platelets (data not shown). None of the bands depicted in Fig. 1C was detected when platelets were preincubated with the PKC inhibitor Ro31-8220 (data not shown), confirming the specificity of the antibody. Using this alternative and more sensitive approach, we confirmed and extended our observations. ADP-induced pleckstrin phosphorylation as well as phosphorylation of other unidentified PKC substrates was completely suppressed not only by MRS2179 but also by AR-C69931MX (Fig. 1O). In addition, we found that a different P2Y12 antagonist, 2MeSAMP, was as efficient as AR-C69931MX in preventing ADP-induced activation of PKC (Fig. 1O). In a dose-dependent analysis, we found that even when platelets were stimulated with doses of ADP as high as 50 $\mu$M, pleckstrin phosphorylation was completely prevented by blockade of the P2Y12 receptor (Fig. 1D). These results demonstrate that co-stimulation of both P2Y1 and P2Y12 receptors by ADP is absolutely required for activation of PKC.

In platelets, the G<sub>T</sub>-coupled P2Y12 receptor is responsible for inhibition of adenyl cyclase and for activation of phosphatidylinositol 3-kinase (12–16). Fig. 1E shows that when P2Y12 receptor was blocked by AR-C69931MX or 2MeSAMP, direct inhibition of adenyl cyclase by dideoxyadenosine did not restore ADP-induced pleckstrin phosphorylation. By contrast, PKC-directed protein phosphorylation in ADP-stimulated platelets was prevented by the phosphatidylinositol 3-kinase inhibitors LY294002 and wortmannin (Fig. 1E). These results indicate that the P2Y12 receptor contributes to the regulation of PKC through the phosphatidylinositol 3-kinase-dependent pathway.

Similarly to the P2Y1 receptor for ADP, the platelet TXA<sub>2</sub> receptor, TPalpha, is coupled to G<sub>T</sub> and is able to stimulate PLC (1, 2). Nevertheless, platelet aggregation by TXA<sub>2</sub> or by its stable analogue U46619 requires concomitant stimulation of the G<sub>T</sub>-coupled P2Y12 receptor by secreted ADP (7, 22). We investigated whether the P2Y12 receptor participates in PKC regulation in U46619-activated platelets. In $^{32}$P-labeled cells, U46619 induced a stronger and more sustained phosphorylation of pleckstrin than ADP, which, however, was severely impaired when the P2Y12 receptor was blocked by AR-C69931MX (Fig. 2A). Moreover, inhibition of U46619-induced pleckstrin phosphorylation in AR-C69931MX- or 2MeSAMP-treated platelets was confirmed by the immunoblotting analysis with the anti-phospho(Ser) PKC substrates antibody (Fig. 2B). Incidentally, we noticed that the analysis of pleckstrin phosphorylation by immunoblotting with anti-phospho(Ser) PKC substrates antibody, which certainly generates stronger signals than the autoradiography approach, does not allow a reliable comparison of the strength of the response to different agonists. Although this observation has no impact on the present study, it certainly should be taken into consideration when choosing to adopt this technique. On stimulation with U46619, but not with ADP, washed platelets may form aggregates, and outside-in signaling through integrin alphaIIbbeta3 may contribute to PLC activation and PKC stimulation. To rule out the possibility that the inhibitory effect of P2Y12 antagonists on U46619-induced pleckstrin phosphorylation was a consequence of the inhibition of aggregation, comparative analysis were performed in the absence and presence of the integrin antagonist RGDS. Fig. 2C shows...
that even under non-aggregating conditions U46619-induced pleckstrin phosphorylation was inhibited by AR-C69931MX. A dose-dependent analysis revealed that inhibition of pleckstrin phosphorylation by blockade of the P2Y12 receptor was still evident when platelets were stimulated with higher doses of U46619 (Fig. 2D). In all the experiments performed, we noticed that whereas inhibition of pleckstrin phosphorylation was constantly complete upon stimulation with ADP, some relevant variability was observed on stimulation with U46619 (see for instance, Figs. 2, B and C). However, an accurate quantitative analysis of several different experiments revealed that the inhibitory effects of the antagonists of the P2Y12 receptor on U46619-induced pleckstrin phosphorylation was relevant, as it accounted for about 70–80% and was statistically significant (Fig. 2E).

The P2Y12 Receptor Does Not Influence Gq-mediated Activation of Phospholipase C Induced by U46619—

The effect of P2Y12 antagonists on the PKC activity could reflect a cross-talk between Gi and Gq at the level of PLC activation. Although ADP is clearly able to induce PLC activation under our experimental conditions, as revealed by measurement of PKC activation (Fig. 1) and intracellular Ca2+/H11001 mobilization (data not shown), we have been unable to reliably measure agonist-induced accumulation of inositol phosphates or DAG, probably because of the weakness of the response (data not shown), as suggested by early studies (30). Therefore, we took advantage from the evidence that the P2Y12 receptor is also implicated in PKC activation induced by U46619, which is a stronger activator of PLC. Using [3H]inositol- or [3H]arachidonic acid-labeled platelets, we found that neither accumulation of inositol phosphates nor DAG was affected by AR-C69931MX in U46619-treated platelets (Fig. 3A). Signals downstream of PLC branch into IP3-Ca2+/H11001 and DAG-PKC path-

**FIGURE 6.** Inhibition of U46619-induced Rap1b activation by the P2Y12 receptor antagonists is reversed by R59949 but not by PMA. Rap1b activation induced by stimulation of platelets with 1 μM U46619 for 1 min was analyzed by the pulldown assay with GST-RalGDS-RBD followed by immunoblotting with anti Rap1 antibody. In panel A platelets were preincubated with either 1 μM AR-C69931MX or 1 μM 2MeSAMP, and the effect of 1 μM R59949 was analyzed. TOT, total. In panel B some samples were incubated with AR-C69931MX, and the effect of 1 nM PMA is reported. The upper panels show the active form of Rap1b (GTP-Rap1b), whereas the lower panels report the level of total Rap1b present in the platelet lysates. The levels of Rap1b activation were quantified by densitomeric analysis of the immunoblots, and the results are summarized in panel C. The amount of active Rap1b in U46619-stimulated platelets in the absence of any inhibitor was taken as 100%. Data are the mean ± S.D. of eight different experiments. The recovery of Rap1b activity caused by inhibition of DGK with R59949, albeit partial, was found statistically significant.
ways. Although DAG-mediated activation of PKC was almost completely suppressed by the antagonists of P2Y12 receptor (Fig. 2), we found that, under the same conditions, IP$_3$-mediated mobilization of intracellular Ca$^{2+}$ was not affected (Fig. 3B). Altogether our results indicate that blockade of the P2Y12 receptor does not likely affect G$_i$-mediated PLC activation in U46619-stimulated platelets.

Restoration of PKC Activity Does Not Overcome the Inhibition of Platelet Aggregation Induced by P2Y12 Receptor Antagonists—It has been previously shown that both ADP- and U46619-induced platelet aggregation can be abrogated by blockade of the G$_i$-coupled P2Y12 receptor (5, 7). We addressed the possibility that this effect could be a consequence of the impaired PKC activation here described, and we analyzed whether restoration of PKC activity by subthreshold doses of PMA could overcome the inhibitory effect of AR-C69931MX. Using U46619-stimulated platelets as a model, we found that treatment with 1 nM PMA did not result in either pleckstrin phosphorylation or platelets aggregation, whereas 2.5 nM PMA induced a weak activation of PKC, associated to a very modest platelet aggregation (Fig. 4A and 4B). Fig. 4A shows that both 1 and 2.5 nM PMA were able to synergize with U46619 and restored normal PKC activation upon blockade of the P2Y12 receptor by AR-C69931MX. However, inhibition of U46619-induced platelet aggregation by AR-C69931MX was not overcome by either 1 or 2.5 nM PMA (Fig. 4C) despite normal PKC activation.

Inhibition of Diacylglycerol Kinase Bypasses the Need of P2Y12 Stimulation for Agonist-induced PKC Activation and Platelet Aggregation—The endogenous PKC activator DAG is typically metabolized and neutralized through phosphorylation by DGK, which, therefore, may indirectly regulate PKC function. To investigate whether DGK could be targeted by P2Y12, we analyzed the effect of a cell-permeable DGK inhibitor, R59949, on AR-C69931MX- and 2MeSAMP-mediated inhibition of platelet aggregation and pleckstrin phosphorylation induced by U46619. R59949 alone promoted only a small, but statistically significant increase of the levels of endogenous DAG, and caused a weak activation of PKC after 10 min (Fig. 5, A and B) but did not cause any detectable platelet aggregation (data not shown). Preincubation of platelets with R59949 for 5 min before stimulation with U46619 resulted in a very small increase of pleckstrin phosphorylation and in a modest potentiation of aggregation (Fig. 5, C and D). However, Fig. 5C shows that inhibition of PKC-directed protein phosphorylation by AR-C69931MX or 2MeSAMP in U46619-stimulated platelets was completely overcome by R59949. Similar results were also obtained when platelets were stimulated with ADP (data not shown). Interestingly, in the presence of R59949, U46619-induced evident platelet aggregation despite the inhibition of G$_i$ signaling by AR-C69931MX or 2MeSAMP (Fig. 5D). These results indicate that the need of P2Y12 stimulation for G$_i$-initiated platelet aggregation can be bypassed by the inhibition of DGK rather than by activation of PKC.

Beside activating PKC, PLC-generated DAG regulates other intracellular effectors, including CalDAG-GEFI, a nucleotide exchange factor for the small GTPase Rap1 (20, 21), which is important for integrin $\alpha$IIb$\beta$3 activation and platelet aggregation (19). As previously reported (13), we confirmed that U46619-induced activation of Rap1b was prevented by AR-C69931MX and 2MeSAMP (Fig. 6, A and B). However, we also found that the DGK inhibitor R59949, but not the PKC activator PMA, was able to partially overcome the effect of the P2Y12 receptor blockade on agonist-induced activation of Rap1 (Fig. 6, A and B). Because the recovery of Rap1b activity was not complete in the presence of R59949, we performed an accurate quantitative analysis of immunoblots from eight different experiments. The results are reported in Fig. 6C and show that in the presence of the P2Y12 receptor antagonists, U46619-induced activation of Rap1b was restored by about 50% by R59949 and that this effect resulted statistically significant.

Finally we investigated the interplay between G$_i$-dependent signaling and DAG-mediated effects on platelet aggregation using the cell-permeable DAG analogue, DiC8. A weak platelet aggregation was observed upon treatment of platelets with 3.5 μM but not with 2 μM DiC8 (Fig. 7A). Concomitant stimulation of P2Y12 receptor through the addition of ADP in the presence of MRS2179 significantly potentiated the effect of DiC8 on platelet aggregation (Fig. 7A). DiC8-induced PKC activation was dose-dependent and occurred in a range of concentrations much lower than those required for aggregation (Fig. 7B). We found that the faint pleckstrin phosphorylation detectable at 100 nM DiC8 was clearly potentiated by the DGK inhibitor R59949 (Fig. 7Bii). TLC analysis of the lipids extracted from $^{32}$P-labeled platelets revealed that exogenous DiC8 was actively phosphorylated to DiC8-PA by a DGK activity in intact cells and that this process was inhibited upon platelet incubation with R59949 (Fig. 7C). Similarly to R59949, we found that stimulation of the P2Y12 receptor by ADP in the presence of MRS2179 resulted in a potentiation of pleckstrin phosphorylation induced by exogenous DiC8 at all the doses analyzed (Fig. 7Bii). Concomitantly, an evident inhibition of DiC8 conversion to DiC8-PA in intact platelets was observed upon stimulation with ADP. This effect of ADP in $^{32}$P-labeled cells was dose-dependent and was prevented by AR-C69931MX but not by MRS2179, indicating that it was mediated by activation of the

**FIGURE 7.** Potentiation of DiC8-mediated platelet activation by stimulation of P2Y12 receptor. A, platelet aggregation was measured upon addition of 2 μM (i) or 3.5 μM (ii) DiC8 in the presence of buffer (none) or 10 μM ADP and 200 μM MRS2179, as indicated on the right. The reported traces are representative of at least three different experiments producing comparable results. B, platelets were stimulated for 1 min with 0–500 nM DiC8, as indicated at the bottom, in the absence or presence of 1 μM R59949 (i) or 10 μM ADP and 200 μM MRS2179 (ii). PKC-directed protein phosphorylation was analyzed by immunoblotting with an anti-phospho(Ser) PKC substrates antibody. The position of the 47-kDa pleckstrin is indicated by the arrow on the right. C, $^{32}$P-labeled platelets were treated with 500 nM DiC8 or with an equivalent volume of DMSO, as indicated on the top of the panel (i), and then left untreated (none) or stimulated with the indicated doses of ADP for 1 min in the absence or in the presence of 200 μM MRS2179, 1 μM AR-C69931MX, or 1 μM R59949, as indicated. Lipids were extracted, separated by TLC, and visualized by autoradiography. A representative image is reported in panel i. Accumulation of DiC8-derived PA was quantified by densitometric analysis of the autoradiographs, and the results are reported in panel (ii). The production of DiC8-PA in the presence of ADP or ADP plus antagonists is reported as percentage of that observed in samples treated with DiC8 alone. Results are the means ± S.D. of four different experiments. *p < 0.01 versus non-stimulated platelets.
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P2Y12 receptor (Fig. 7C). These results support the hypothesis that G\textsubscript{T}-dependent signaling potentiates the effect of endogenous DAG by limiting its conversion to PA.

**DISCUSSION**

In this work we have documented a novel interplay between G\textsubscript{q} and G\textsubscript{i} stimulation in human platelets as we have shown that activation of the G\textsubscript{T}-coupled P2Y12 receptor is essential for complete PKC activation in response to stimulation of G\textsubscript{T}-coupled receptors by ADP or U46619. The cross-talk between G\textsubscript{q} and G\textsubscript{i} is most likely to occur at the level of DAG metabolism, which plays a more crucial role than PKC itself in the regulation of platelet aggregation.

G\textsubscript{T}-coupled receptors are able to stimulate PLC\(\beta\) isoforms, which hydrolyze phosphatidylinositol 4,5-bisphosphate, and produce two intracellular messengers, IP\(3\) and DAG, which mediate the release of Ca\(^{2+}\) from internal stores and the activation of PKC, respectively (3). According to this paradigm, we have found that blockade of ADP binding to the G\textsubscript{T}-coupled P2Y1 receptor abolished PKC-mediated protein phosphorylation. Unexpectedly, however, we have found that antagonists of the G\textsubscript{T}-coupled P2Y12 receptor totally prevented PKC activation as well. Therefore, stimulation of the G\textsubscript{q} is necessary but not sufficient for ADP to induce PKC activation, and the concomitant stimulation of G\textsubscript{i} is also required. Importantly, G\textsubscript{T}-mediated regulation of PKC is not limited to ADP-stimulated platelets but is a common event when G\textsubscript{q} is stimulated. It has been previously shown that stimulation of the G\textsubscript{T}-coupled TP\(\alpha\) receptor by the TXA\(_2\) analogue U46619 activates PLC\(\beta\) but is not sufficient to trigger platelet aggregation unless activation of a G\textsubscript{T}-dependent pathway by binding of secreted ADP to the P2Y12 receptor occurs (2, 7, 22). In this context we have found that, similarly to what observed in ADP-stimulated platelets, the P2Y12 antagonists AR-C69931MX and 2MeSAMP prevented PKC-mediated protein phosphorylation induced by U46619. Therefore, even in this alternative experimental model, PKC activation is regulated by concomitant signals through G\textsubscript{q} and G\textsubscript{T}-coupled receptors. Direct measurement of inositol phosphate accumulation DAG production in U46619-stimulated platelets indicates that although G\textsubscript{i} regulates PKC activation, PLC is probably not affected by blockade of the P2Y12 receptor. Moreover, we also found that U46619-induced, IP\(3\)-dependent Ca\(^{2+}\) mobilization from intracellular stores was not affected by ADP scavengers or antagonists (Fig. 3B and Ref. 11) Previous works reported that ADP-induced Ca\(^{2+}\) mobilization is reduced by blockade of the P2Y12 receptor, suggesting a cross-talk between G\textsubscript{q} and G\textsubscript{i} at the level of PLC (31, 32). Using ADP as platelet agonist, we observed a comparable partial inhibition of Ca\(^{2+}\) release by AR-C69931MX (data not shown), but importantly, under the same conditions, pleckstrin phosphorylation was completely suppressed, indicating that G\textsubscript{i} signaling exerts an additional specific control on the DAG-PKC pathway. Moreover, it is of note that most of our study was performed with U46619-stimulated platelets under conditions in which PLC activation and Ca\(^{2+}\) mobilization were found to occur normally.

We have also addressed the fundamental question as to the functional implication of PKC regulation through P2Y12 recep-
Gi-mediated Regulation of DAG Signaling in Platelets

In conclusion, our results shed new light into the signaling pathway activated downstream of the P2Y12 receptor for ADP and required for integration of the Gq-dependent signals for platelet aggregation, reveal a novel role for Gi in the regulation of PKC activity, and point to the importance of DAG-metabolizing enzymes such as DGK in the regulation of platelet activation.

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