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Comments

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Inhibition of Phospholipase A₂-mediated Arachidonic Acid Release by Cyclic AMP Defines a Negative Feedback Loop for P_{2Y} Receptor Activation in Madin-Darby Canine Kidney D₁ Cells*

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In Madin-Darby canine kidney D₁ cells extracellular nucleotides activate P_{2Y} receptors that couple to several signal transduction pathways, including stimulation of multiple phospholipases and adenylyl cyclase. For one class of P_{2Y} receptors, P_{2Y2} receptors, this stimulation of adenylyl cyclase and increase in cAMP occurs via the conversion of phospholipase A₂ (PLA₂)-generated arachidonic acid (AA) to prostaglandins (e.g. PGE₂). These prostaglandins then stimulate adenylyl cyclase activity, presumably via activation of prostanoid receptors. In the current study we show that agents that increase cellular cAMP levels (including PGE₂, forskolin, and the β -adrenergic agonist isoproterenol) can inhibit P_{2Y} receptor-promoted AA release. The protein kinase A (PKA) inhibitor H89 blocks this effect, suggesting that this feedback inhibition occurs via activation of PKA. Studies with PGE₂ indicate that inhibition of AA release is attributable to inhibition of mitogen-activated protein kinase activity and in turn of P_{2Y} receptor stimulated PLA₂ activity. Although cAMP/PKA-mediated inhibition occurs for P_{2Y} receptor-promoted AA release, we did not find such inhibition for epinephrine (α_1 -adrenergic) or bradykinin-mediated AA release. Taken together, these results indicate that negative feedback regulation via cAMP/PKA-mediated inhibition of mitogen-activated protein kinase occurs for some, but not all, classes of receptors that promote PLA₂ activation and AA release. We speculate that receptor-selective feedback inhibition occurs because PLA₂ activation by different receptors in Madin-Darby canine kidney D₁ cells involves the utilization of different signaling components that are differentially sensitive to increases in cAMP or, alternatively, because of compartmentation of signaling components.

Arachidonic acid (AA)¹ and its eicosanoid metabolites (e.g. prostaglandins and leukotrienes) play critical roles in the initiation or modulation of a broad spectrum of biological responses, including many inflammatory processes. In mammalian cells AA is normally stored in membrane phospholipids

and released primarily by phospholipase A₂ (PLA₂). Among several types of mammalian PLA₂s, the 85-kDa cytosolic form (cPLA₂) appears to specifically catalyze receptor-promoted AA release. Considerable efforts have been made in recent years to study the mechanism for the activation of cPLA₂ and the subsequent release of AA. In a variety of cell types, cPLA₂ activation occurs as a result of phosphorylation by mitogen-activated protein (MAP) kinase (1).

We have recently demonstrated that in MDCK-D₁ cells P_{2Y2} (previously termed P_{2U}) purinergic receptor-promoted AA release is mediated through cPLA₂ activation by MAP kinase (2). Other data have shown that stimulation of P_{2Y2} purinergic receptors in these cells also stimulates adenylyl cyclase (AC) activity, increasing cellular cAMP levels through an autocrine/paracrine mechanism involving prostaglandin E₂ (PGE₂) production subsequent to cPLA₂-mediated AA release (3, 4). In the present study we sought to assess the relationship of these two signaling pathways activated by P_{2Y2} purinergic receptors. We found that activation of the AC system can inhibit cPLA₂-mediated AA release by P_{2Y2} purinergic receptors through the inhibition of MAP kinase in MDCK-D₁ cells. Therefore, we define a negative feedback mechanism via an autocrine/paracrine cycle in which P_{2Y2} receptors can attenuate the activation of PLA₂ and AA release initiated by receptor agonists.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK-D₁ cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% serum and passaged every 3–4 days by trypsinization using trypsin/EDTA. Cells were used for experiments when grown to approximately 70% confluence.

Assay of cAMP—Growth medium was removed from cells, and cells were equilibrated for 30 min at 37 °C in serum-free Dulbecco's modified Eagle's medium containing 20 mM Hepes buffer (DMEH, pH 7.4). Subsequently cells were incubated in fresh DMEH with PGE₂ for 5 min at 37 °C in the presence of 200 μ M isobutylmethylxanthine or 100 μ M Ro20-1724, two different phosphodiesterase inhibitors. Reactions were terminated by aspiration of medium and addition of 7.5% trichloroacetic acid. Intracellular cAMP levels were determined by radioimmunoassay (Calbiochem, CA) of trichloroacetic acid extracts following acetylation, as described previously (3).

[³H]AA Release in Intact Cells—Cells were labeled with [³H]AA by incubation with 0.5 μ Ci of [³H]AA (specific activity, 100 Ci/mmol; NEN Life Science Products) per ml for approximately 20 h in 24-well plates. Cells were washed four times with DMEH supplemented with 5 mg/ml bovine serum albumin and then incubated in the same medium at 37 °C for 15–20 min to equilibrate the temperature. Agents of interest were then added in 1 ml of 37 °C medium after removing equilibration medium. Release of [³H]AA was assayed and normalized to the percentage of incorporated radioactivity, as described previously (2).

cPLA₂ Activity Assay of Cell Lysates—cPLA₂ activity was assayed in lysates prepared from cells incubated with various agents, as described previously (2). Briefly, cells were incubated with indicated agonists in DMEH for 10 min at 37 °C, washed with ice-cold buffer containing 250 mM sucrose, 50 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM EDTA, phosphatase inhibitors (200 μ M Na₃VO₄, 1 mM levamisole) and protease inhibitors (500 μ M phenylmethylsulfonyl fluoride, 8 μ M pepstatin, 16 μ M

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¹ The abbreviations used are: AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, 85-kDa cytosolic form of PLA₂; MAP, mitogen-activated protein; MDCK, Madin-Darby canine kidney; AC, adenylyl cyclase; PGE₂, prostaglandin E₂; PKA, protein kinase A.

leupeptin, and 1 mM diisopropyl fluorophosphate), and then scraped into ice-cold buffer identical to the washing buffer except that sucrose was omitted but 100 nM okadaic acid and 1 mM dithiothreitol were added. Scraped cells were sonicated, and cell lysates (supernatants after centrifugation at 4 °C for 10 min at 500 × g) were assayed for cPLA₂ using 1-stearoyl-2-[¹⁴C]arachidonoyl-L-3-phosphatidyl choline as substrate in the assay buffer described above containing 10 mg/ml bovine serum albumin and 10 mM CaCl₂. Cell lysates (100 μl) were added to an equal volume of substrate in a shaking 37 °C water bath so that final concentrations were, in addition to phosphatase and protease inhibitors, 20 μM 1-stearoyl-2-[¹⁴C]arachidonoyl-L-3-phosphatidyl choline, 5 mM CaCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 50 mM Hepes, pH 7.4, and 10–30 μg of protein (determined by a Bradford protein assay kit (Bio-Rad) with a bovine serum albumin standard). Reactions were stopped by adding 0.75 ml of 1:2 (v/v) chloroform/methanol. Samples were processed and assayed for [¹⁴C]AA by thin layer chromatography, as described previously (2).

Assay of Phospholipase D Activation—MDCK-D₁ cells were labeled by an overnight incubation with [³H] palmitate. Washed cells were then incubated for 1 h with 20 μM indomethacin (to block PGE₂ formation), with 0.5 μM isobutylmethylxanthine for 20 min, with 20 μM PGE₂ or 50 μM isoproterenol for 20 min, and then with either 300 μM ATP or 300 μM UTP for 10 min. Cells were lysed and phosphatidylethanol was resolved by thin layer chromatography, as described previously (5). Phosphatidylethanol was expressed as the percentage of total cellular radioactivity.

Phosphorylation-induced Mobility Shift, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblotting of MAP Kinase—Cells were washed five times with DMEH supplemented with 2 mg/ml bovine serum albumin, incubated in this medium at 37 °C for 1 h, and then with specified agonists for 3 min. Reactions were stopped by aspiration of medium and washing of cells four times with ice-cold buffer (62.5 mM Tris HCl, pH 6.8, plus 10% glycerol), and protease and phosphatase inhibitors were used for PLA₂ activity assays. Scraped cells were lysed in SDS-polyacrylamide gel electrophoresis loading buffer and boiled for five min, and samples were electrophoresed on SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide. Following transfer to an Immobilon-P polyvinylidene fluoride membrane (Millipore) and blocking for 1 h with 5% nonfat dry milk dissolved in phosphate-buffered saline, membranes were incubated with 1:2000–3000 diluted anti-p42 MAP kinase rabbit serum for 90 min and then with 1:2000 diluted horseradish-peroxidase-linked donkey anti-rabbit immunoglobulin for 1 h (both in 5% nonfat dry milk dissolved in phosphate-buffered saline) and then washed four times with phosphate-buffered saline (5 min each wash). MAP kinase bands were visualized using ECL immunoblotting detection reagents (Amersham Pharmacia Biotech).

Data Presentation—Unless otherwise specified, the data shown in figures are the means ± S.D. of triplicate measurements and are representative of results obtained in two to four experiments. Results were analyzed for statistical significance by one-way analysis of variance (with Bonferroni's correction, where appropriate).

RESULTS

Increase in Cellular cAMP Inhibits P_{2Y2} Receptor-promoted AA Release and cPLA₂ Activation in MDCK-D₁ Cells—We have previously demonstrated in MDCK-D₁ cells that activation of P_{2Y2} purinergic receptors results in AA release via activation of cPLA₂ (2) and that stimulation of P_{2Y2} purinergic receptors in these cells can increase cellular cAMP levels via the action of the PGE₂ generated from AA (3, 4). To further investigate the relationship of these two signaling pathways activated by P_{2Y2} receptors, we examined the effects of cAMP-increasing agents on P_{2Y2} receptor-promoted AA release. As shown in Fig. 1, agents that increase cAMP in MDCK-D₁ cells (PGE₂, forskolin, and Ro20-1724) inhibited AA release stimulated by the P₂ receptor agonists ATP and UTP. Isoproterenol also inhibited the AA release stimulated by these purinergic agonists (data not shown).

PGE₂-mediated stimulation of cAMP and inhibition of P_{2Y2} receptor-promoted AA release displayed a similar concentration-response relationship, with a nearly maximal effect of PGE₂ achieved at 1–10 μM both for production of cAMP and inhibition of AA release (Fig. 2). Because P_{2Y2} receptor-promoted AA release in MDCK-D₁ cells is mediated by activation

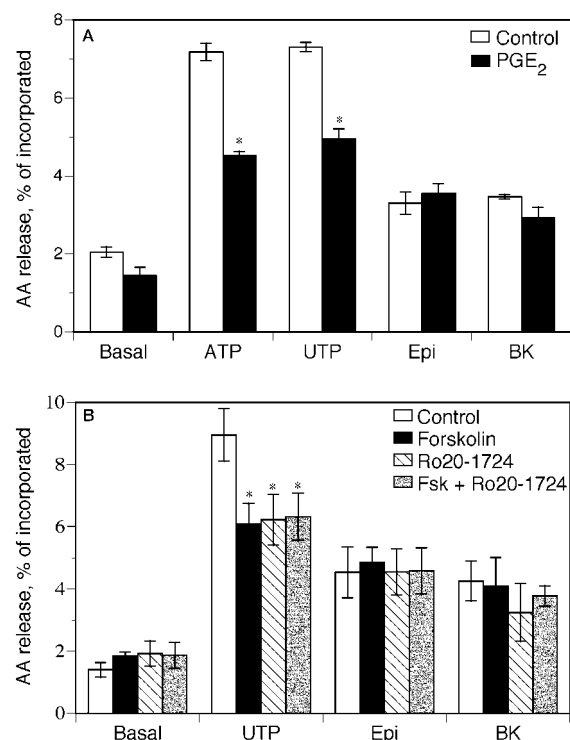


FIG. 1. Differential effects of PGE₂ and forskolin on AA release promoted by ATP, UTP, bradykinin, or epinephrine. MDCK-D₁ cells labeled with [³H]AA were incubated with 10 μM PGE₂ (A) or 30 μM forskolin and/or 100 μM Ro20-1724 (B) for 20 min and then with either medium (basal), 300 μM ATP (A only), 300 μM UTP, 50 μM epinephrine, or 1 μM bradykinin for 10 min to measure [³H]AA release as described under "Experimental Procedures." *, *p* < 0.05.

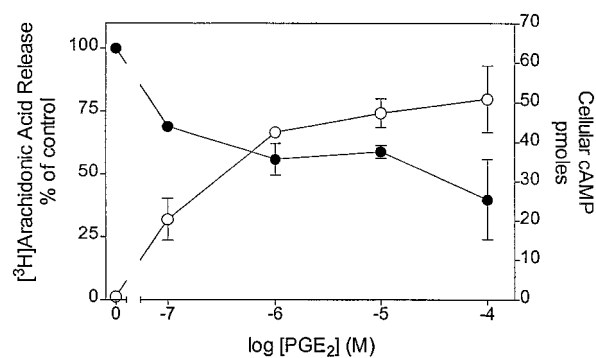


FIG. 2. Concentration response of effects of PGE₂ on cAMP levels and 300 μM UTP-promoted AA release of MDCK-D₁ cells. Cells were assayed for [³H]AA or in parallel for cAMP content as described under "Experimental Procedures" and in the legend to Fig. 1. ●, AA; ○, cAMP.

of cPLA₂ (2), we tested whether an increase in cAMP would blunt activation of this lipase. Indeed, as shown in Fig. 3, activation of PLA₂ activity in cell lysates by the specific P_{2Y2} agonist UTP was substantially inhibited by treatment of cells with PGE₂. Therefore, increases in cellular cAMP decrease AA release and cPLA₂ activation by P_{2Y2} receptors in MDCK-D₁ cells.

MDCK-D₁ cells possess α₁-adrenergic receptors and B₂-bradykinin receptors that promote AA release in response to stimulation by epinephrine and bradykinin, respectively (8–10). As do P_{2Y2} receptors, α₁-adrenergic receptors and bradykinin receptors promote AA release through activation of cPLA₂ in MDCK-D₁ cells (2, 3, 7). Therefore, we tested whether increases in cAMP would regulate AA release by these different receptors. Unlike the results obtained for P_{2Y2} receptors, AA release

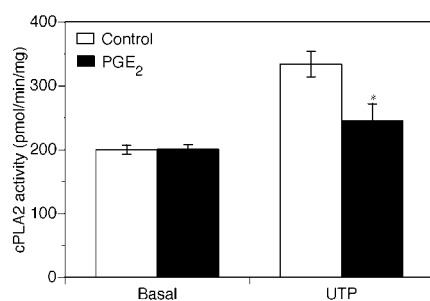


FIG. 3. Effect of PGE₂ on UTP-promoted activation of PLA₂ in MDCK-D₁ cells. MDCK-D₁ cells were incubated with or without 10 μ M PGE₂ for 20 min and then with 300 μ M UTP for 10 min. Cell lysates were prepared, and PLA₂ activity was assayed as described under "Experimental Procedures." The effect of PGE₂ on UTP-promoted PLA₂ activity was statistically significant. *, $p < 0.05$.

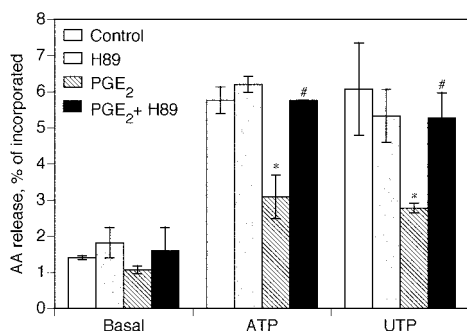


FIG. 4. Effect of H89 on PGE₂-mediated inhibition of AA release promoted by ATP and UTP. [³H]AA-labeled MDCK-D₁ cells were incubated with either 10 μ M PGE₂ (20 min), 1.33 μ M H89 (1 h prior to PGE₂ incubation), PGE₂ plus H89, or with neither agent, and then with 300 μ M ATP or UTP for 10 min to measure [³H]AA release, as described under "Experimental Procedures." *, $p < 0.05$ relative to control; #, $p < 0.05$ relative to PGE₂ alone.

elicited by α_1 -adrenergic receptors and bradykinin receptors was not inhibited by forskolin, Ro20-1724 (an inhibitor of phosphodiesterase), or both (Fig. 1B). Treatment with PGE₂ alone or with PGE₂ and Ro20-1724 yielded similar results (data not shown). Neither forskolin nor PGE₂ stimulation of cAMP accumulation was diminished by the presence of various concentrations of epinephrine or bradykinin, as compared with the presence of ATP or UTP (data not shown).

Activation of PKA and Inhibition of MAP Kinase Are Responsible for the cAMP-mediated Inhibition of P_{2Y2} Receptor-promoted AA Release—To test whether the inhibitory effect of the increase in cellular cAMP on P_{2Y2} receptor-promoted AA release and cPLA₂ activation in MDCK-D₁ cells is mediated by the activation of PKA, we examined the effect of the PKA inhibitor H89 on PGE₂-mediated inhibition of AA release. As shown in Fig. 4, PGE₂-mediated inhibition of ATP- or UTP-stimulated AA release was completely prevented by pretreatment of cells with H89, whereas H89 had no statistically significant effect on basal or P_{2Y2} receptor-promoted AA release. These data suggest that activation of PKA by cAMP is responsible for the inhibitory effects of increased cellular cAMP levels on P_{2Y2} receptor-promoted AA release.

Because MAP kinase plays a critical role in the activation of cPLA₂ and AA release in MDCK-D₁ cells (2), we next assessed whether activation of MAP kinase by P_{2Y2} receptors was inhibited by pretreatment of cells with PGE₂. Consistent with this idea were results with epinephrine, which also activates MAP kinase activity in MDCK-D₁ cells (7). PGE₂ blocked the MAP kinase activation by UTP but not that by epinephrine (Fig. 5). As shown in Fig. 6, the UTP-induced gel shift of MAP kinase was inhibited by incubation of cells with PGE₂. This PGE₂-

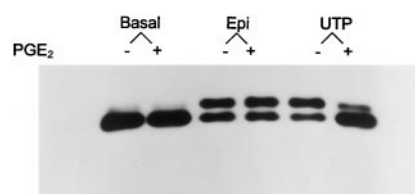


FIG. 5. Effect of PGE₂ on activation of MAP kinase by UTP and epinephrine. MDCK-D₁ cells were incubated in the absence or presence of 10 μ M PGE₂ for 20 min and then with 50 μ M epinephrine (*Epi*) or 300 μ M UTP for 3 min. MAP kinase activation was assessed as described under "Experimental Procedures."

	H89	-	+	-	+	+	-
PGE ₂	-	-	-	+	-	-	+
UTP	-	-	+	+	+	+	+

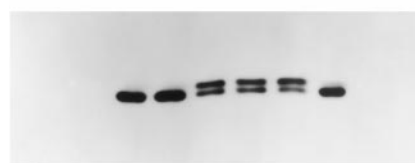


FIG. 6. Effects of PGE₂ and H89 on UTP-promoted activation of MAP kinase. MDCK-D₁ cells were incubated with 10 μ M PGE₂, 1.33 μ M H89, or both as stated in the legend to Fig. 4 and then with or without 300 μ M UTP for 3 min. MAP kinase activation was assessed as described under "Experimental Procedures."

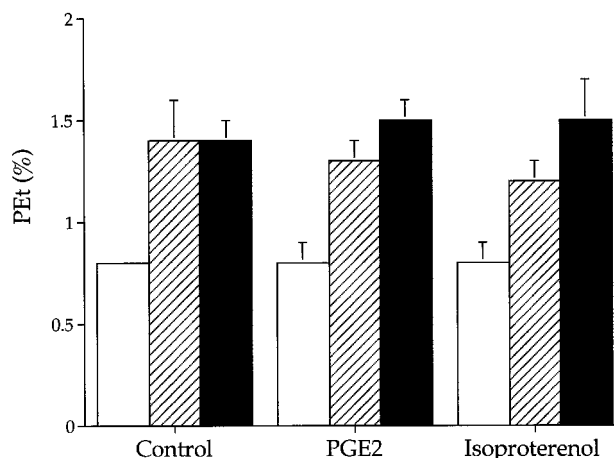


FIG. 7. Lack of effect of PGE₂ and isoproterenol on ATP- and UTP-promoted PLD activation. MDCK-D₁ cells were labeled with [³H]palmitate and then incubated with 20 μ M PGE₂ and 50 μ M isoproterenol prior to incubation with 300 μ M ATP or 300 μ M UTP and assayed for PLD activity, as described under "Experimental Procedures."

mediated inhibition of MAP kinase was reversed by treatment of cells with H89, suggesting that activation of PKA is responsible for both the cAMP-mediated inhibition of MAP kinase activation and inhibition of AA release and cPLA₂ activation. Based on these and previous data related to the role of MAP kinase on P_{2Y} receptor-promoted activation of cPLA₂ in MDCK-D₁ cells, we conclude that the cAMP/PKA system negatively regulates cPLA₂ activated by P_{2Y2} receptors through the inhibition of MAP kinase activation.

P_{2Y} Receptor Activation of Phospholipase D Activity Is Not Inhibited by Elevation of cAMP—In addition to activation of cPLA₂, P_{2Y} receptors can also increase phospholipase D activity in MDCK-D₁ cells (5). To determine whether the inhibitory effect of cAMP on cPLA₂ activation occurs at more upstream levels of the signaling cascade, such as at the level of receptor or G protein, we measured the effect of increasing cAMP on phospholipase D activity. The inability of increases in cAMP to

blunt ATP- and UTP-mediated phospholipase D activity (Fig. 7) argues that a more distal, nonshared component, such as MAP kinase (Figs. 5 and 6), is the site of negative regulation of cPLA₂/AA release by increases in cAMP.

DISCUSSION

Inhibitory effects of cAMP-increasing agents on AA release have been observed in several previous studies (*e.g.* Refs. 11–13). However, the molecular mechanism for this phenomenon was not defined in these earlier studies. We recently showed that P_{2Y2} receptors utilize MAP kinase and protein kinase C α as parallel pathways for the activation of cPLA₂ in MDCK-D₁ cells; blockade of either of these two pathways impairs the activation of cPLA₂ by P_{2Y2} receptor agonists (2). In the present study, we found that activation of the AC/PKA system can inhibit P_{2Y2} receptor-promoted cPLA₂ activation by inhibiting the MAP kinase signaling pathway. This finding is consistent with the reports that cAMP and cAMP-mediated activation of PKA can inhibit MAP kinase activation in other types of cells (14–16).

AA release activated by P_{2Y2} receptors, but not activated that by α_1 -adrenergic or B₂-bradykinin receptors, was inhibited by the AC/PKA system (Fig. 1). Because we have found that MAP kinase appears not to be involved in the regulation of AA release/cPLA₂ activation by bradykinin receptors in MDCK-D₁ cells (6), we were not surprised by the absence of inhibition of bradykinin receptor-promoted AA release by the AC/PKA system. Another group has used MDCK-D₁ cells and observed slight inhibition of bradykinin receptor-promoted AA release by cAMP (17). However, a similar inhibition also was noted of “basal” AA release in that study. The absence of inhibition by the AC/PKA system on α_1 -adrenergic receptor-promoted AA release was unexpected, because MAP kinase activation is responsible for AA release/cPLA₂ activation by α_1 -adrenergic receptors in MDCK-D₁ cells (7). The ability of the AC/PKA system to inhibit P_{2Y2} receptor-mediated, but not α_1 -adrenergic receptor-mediated activation of MAP kinase (Fig. 4), leads us to conclude that the differential inhibitory effects of the AC/PKA system on AA release lies in differences in MAP kinase activation by the two receptors. This conclusion is also supported by studies showing a lack of inhibition by cAMP of P₂ receptor-mediated activation of PLD activity (Fig. 7). Other data indicate that MAP kinase activation is not required for PLD activation by P₂ receptors or α_1 -adrenergic receptors in MDCK cells (18).² We speculate that P_{2Y2} purinergic and α_{1b} -adrenergic receptors are coupled to cPLA₂ and MAP kinase through different signaling pathways (perhaps via different G proteins) that are differentially sensitive to increases in cAMP. Alternatively, signaling components utilized by the two types of receptors are compartmentalized such that increases in cAMP selectively regulate components unique to the P_{2Y2} receptor pathway.

In conclusion, the present study demonstrates that the AC/PKA system plays a negative role in the regulation of AA release/cPLA₂ activation by P_{2Y2} receptors through inhibition of MAP kinase activation. This negative regulation occurs for P_{2Y2} receptors but not for two other classes of receptors coupled to cPLA₂/AA release and is apparently secondary to effects of cAMP/PKA to inhibit MAP kinase activation. Because the P_{2Y2} receptor can activate the AC/PKA system by promoting cPLA₂-mediated release of AA and its subsequent conversion to PGE₂ (3, 4), our results define a feedback cycle whereby P_{2Y2} receptors in MDCK-D₁ cells activate AA release and production of PGE₂. PGE₂, in turn, activates the AC/PKA system and then inhibits MAP kinase to decrease the AA signaling cascade. Such a cycle could serve to produce homologous desensitization of the purinergic receptor pathway in response to nucleotides and thus would blunt ongoing production of AA and AA metabolites. Moreover, the cross-talk that occurs between AC-stimulating pathways and the purinergic pathway also represents a mechanism for the heterologous desensitization of the P_{2Y} purinergic receptor pathway. The feedback cycle described herein may contribute to both physiologic and pharmacologic regulation of the P_{2Y} purinergic receptor signaling. Overall, these results, together with evidence that P_{2Y2} receptors in MDCK-D₁ cells are coupled to cAMP production via release of AA and its conversion to PGE₂, define a potentially important feedback loop for regulation of AA formation.

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² M. Balboa and P. A. Insel, unpublished observations.

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