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Inhibition of Phospholipase A_2-mediated Arachidonic Acid Release by Cyclic AMP Defines a Negative Feedback Loop for P_{2Y} Receptor Activation in Madin-Darby Canine Kidney D_1 Cells*

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In Madin-Darby canine kidney D_1 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 A_2 (PLA_2)-generated arachidonic acid (AA) to prostaglandins (*e.g.* PGE_2). 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 PGE_2, 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 PGE_2 indicate that inhibition of AA release is attributable to inhibition of mitogen-activated protein kinase activity and in turn of P_{2Y} receptor stimulated PLA_2 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 PLA_2 activation and AA release. We speculate that receptor-selective feedback inhibition occurs because PLA_2 activation by different receptors in Madin-Darby canine kidney D_1 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 A_2 (PLA_2). Among several types of mammalian PLA_2s, the 85-kDa cytosolic form ($cPLA_2$) appears to specifically catalyze receptor-promoted AA release. Considerable efforts have been made in recent years to study the mechanism for the activation of $cPLA_2$ and the subsequent release of AA. In a variety of cell types, $cPLA_2$ activation occurs as a result of phosphorylation by mitogen-activated protein (MAP) kinase (1).

We have recently demonstrated that in MDCK-D_1 cells P_{2Y2} (previously termed P_{2U}) purinergic receptor-promoted AA release is mediated through $cPLA_2$ 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 E_2 (PGE_2) production subsequent to $cPLA_2$-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 $cPLA_2$-mediated AA release by P_{2Y2} purinergic receptors through the inhibition of MAP kinase in MDCK-D_1 cells. Therefore, we define a negative feedback mechanism via an autocrine/paracrine cycle in which P_{2Y2} receptors can attenuate the activation of PLA_2 and AA release initiated by receptor agonists.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK-D_1 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 PGE_2 for 5 min at 37 °C in the presence of 200 μM isobutylmethylxanthine or 100 μM 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).

[^{3}H]AA Release in Intact Cells—Cells were labeled with [^{3}H]AA by incubation with 0.5 μCi of [^{3}H]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 [^{3}H]AA was assayed and normalized to the percentage of incorporated radioactivity, as described previously (2).

$cPLA_2$ Activity Assay of Cell Lysates—$cPLA_2$ 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 μM Na_3VO_4, 1 mM levamisole) and protease inhibitors (500 μM phenylmethylsulfonyl fluoride, 8 μM pepstatin, 16 μM

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[1] The abbreviations used are: AA, arachidonic acid; PLA_2, phospholipase A_2; $cPLA_2$, 85-kDa cytosolic form of PLA_2; MAP, mitogen-activated protein; MDCK, Madin-Darby canine kidney; AC, adenylyl cyclase; PGE_2, prostaglandin E_2; 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 dithiothrietol were added. Scraped cells were sonicated, and cell lysates (supernatants after centrifugation at 4 °C for 10 min at 500 × *g*) were assayed for $cPLA_2$ using 1-stearoyl-2[^{14}C]arachidonyl-L-3-phosphatidyl choline as substrate in the assay buffer described above containing 10 mg/ml bovine serum albumin and 10 mM $CaCl_2$. 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[^{14}C]arachidonyl-L-3-phosphatidyl choline, 5 mM $CaCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothrietol, 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 [^{14}C]AA by thin layer chromatography, as described previously (2).

Assay of Phospholipase D Activation—MDCK-D$_1$ cells were labeled by an overnight incubation with [^{3}H] palmitate. Washed cells were then incubated for 1 h with 20 μM indomethacin (to block PGE_2 formation), with 0.5 μM isobutylmethylxanthine for 20 min, with 20 μM PGE_2 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 PLA_2 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 $cPLA_2$ Activation in MDCK-D$_1$ Cells—We have previously demonstrated in MDCK-D$_1$ cells that activation of P_{2Y2} purinergic receptors results in AA release via activation of $cPLA_2$ (2) and that stimulation of P_{2Y2} purinergic receptors in these cells can increase cellular cAMP levels via the action of the PGE_2 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- D$_1$ cells (PGE_2, forskolin, and Ro20–1724) inhibited AA release stimulated by the P_2 receptor agonists ATP and UTP. Isoproterenol also inhibited the AA release stimulated by these purinergic agonists (data not shown).

PGE_2-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 PGE_2 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-D$_1$ cells is mediated by activation

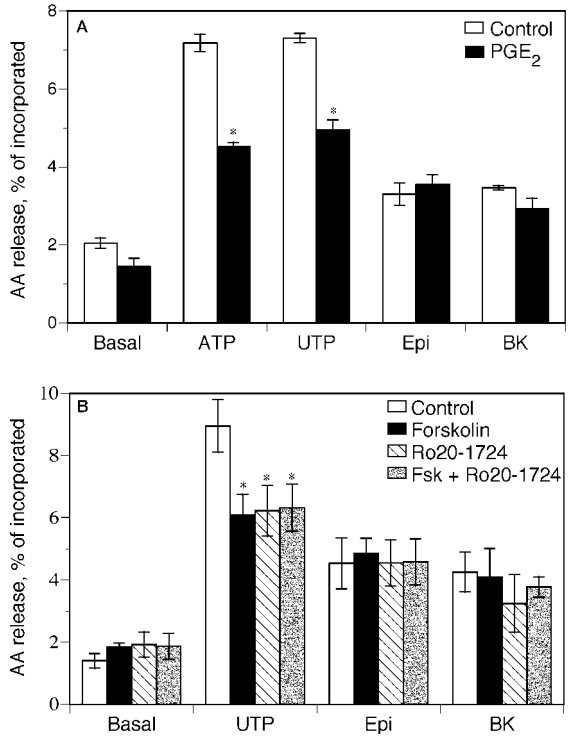

FIG. 1. **Differential effects of PGE_2 and forskolin on AA release promoted by ATP, UTP, bradykinin, or epinephrine.** MDCK-D$_1$ cells labeled with [^{3}H]AA were incubated with 10 μM PGE_2 (*A*) or 30 μM forskolin and/or 100 μM Ro20–1724 (*B*) for 20 min and then with either medium (basal), 300 μM ATP (*A* only), 300 μM UTP, 50 μM epinephrine, or 1 μM bradykinin for 10 min to measure [^{3}H]AA release as described under "Experimental Procedures." *, $p < 0.05$.

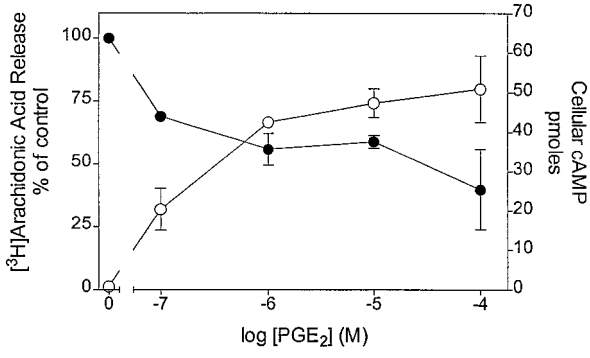

FIG. 2. **Concentration response of effects of PGE_2 on cAMP levels and 300 μM UTP-promoted AA release of MDCK-D$_1$ cells.** Cells were assayed for [^{3}H]AA or in parallel for cAMP content as described under "Experimental Procedures" and in the legend to Fig. 1. ●, AA; ○, cAMP.

of $cPLA_2$ (2), we tested whether an increase in cAMP would blunt activation of this lipase. Indeed, as shown in Fig. 3, activation of PLA_2 activity in cell lysates by the specific P_{2Y2} agonist UTP was substantially inhibited by treatment of cells with PGE_2. Therefore, increases in cellular cAMP decrease AA release and $cPLA_2$ activation by P_{2Y2} receptors in MDCK-D$_1$ cells.

MDCK-D$_1$ cells possess α_1-adrenergic receptors and B_2-bradykinin receptors that promote AA release in response to stimulation by epinephrine and bradykinin, respectively (8–10). As do P_{2Y2} receptors, α_1-adrenergic receptors and bradykinin receptors promote AA release through activation of $cPLA_2$ in MDCK-D$_1$ 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

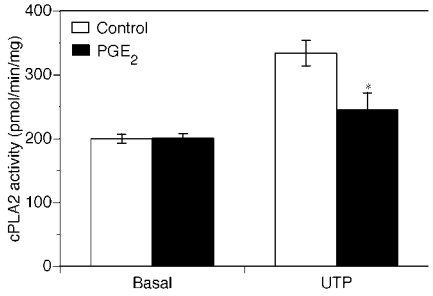

FIG. 3. **Effect of PGE$_2$ on UTP-promoted activation of PLA$_2$ in MDCK-D$_1$ cells.** MDCK-D$_1$ cells were incubated with or without 10 μM PGE$_2$ for 20 min and then with 300 μM UTP for 10 min. Cell lysates were prepared, and PLA$_2$ activity was assayed as described under "Experimental Procedures." The effect of PGE$_2$ on UTP-promoted PLA$_2$ activity was statistically significant. *, $p < 0.05$.

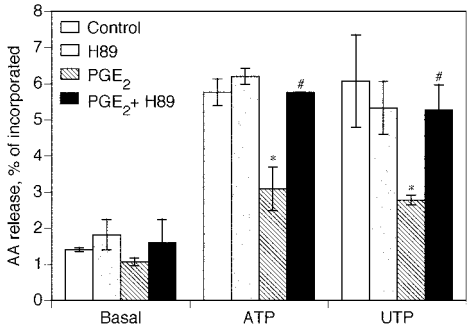

FIG. 4. **Effect of H89 on PGE$_2$-mediated inhibition of AA release promoted by ATP and UTP.** [^{3}H]AA-labeled MDCK-D$_1$ cells were incubated with either 10 μM PGE$_2$ (20 min), 1.33 μM H89 (1 h prior to PGE$_2$ incubation), PGE$_2$ plus H89, or with neither agent, and then with 300 μM ATP or UTP for 10 min to measure [^{3}H]AA release, as described under "Experimental Procedures." *, $p < 0.05$ relative to control; #, $p < 0.05$ relative to PGE$_2$ alone.

elicited by α_1-adrenergic receptors and bradykinin receptors was not inhibited by forskolin, Ro20–1724 (an inhibitor of phosphodiesterase), or both (Fig. 1*B*). Treatment with PGE$_2$ alone or with PGE$_2$ and Ro20–1724 yielded similar results (data not shown). Neither forskolin nor PGE$_2$ 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 cPLA$_2$ activation in MDCK-D$_1$ cells is mediated by the activation of PKA, we examined the effect of the PKA inhibitor H89 on PGE$_2$-mediated inhibition of AA release. As shown in Fig. 4, PGE$_2$-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 cPLA$_2$ and AA release in MDCK-D$_1$ cells (2), we next assessed whether activation of MAP kinase by P$_{2Y2}$ receptors was inhibited by pretreatment of cells with PGE$_2$. Consistent with this idea were results with epinephrine, which also activates MAP kinase activity in MDCK-D$_1$ cells (7). PGE$_2$ 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 PGE$_2$. This PGE$_2$-

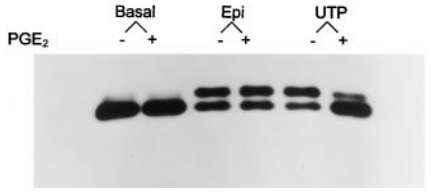

FIG. 5. **Effect of PGE$_2$ on activation of MAP kinase by UTP and epinephrine.** MDCK-D$_1$ cells were incubated in the absence or presence of 10 μM PGE$_2$ for 20 min and then with 50 μM epinephrine (*Epi*) or 300 μM UTP for 3 min. MAP kinase activation was assessed as described under "Experimental Procedures."

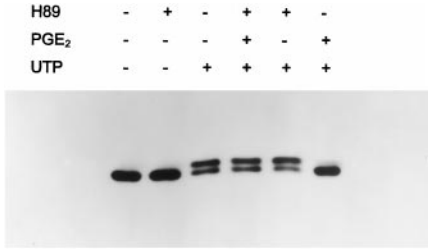

FIG. 6. **Effects of PGE$_2$ and H89 on UTP-promoted activation of MAP kinase.** MDCK-D$_1$ cells were incubated with 10 μM PGE$_2$, 1.33 μM H89, or both as stated in the legend to Fig. 4 and then with or without 300 μM UTP for 3 min. MAP kinase activation was assessed as described under "Experimental Procedures."

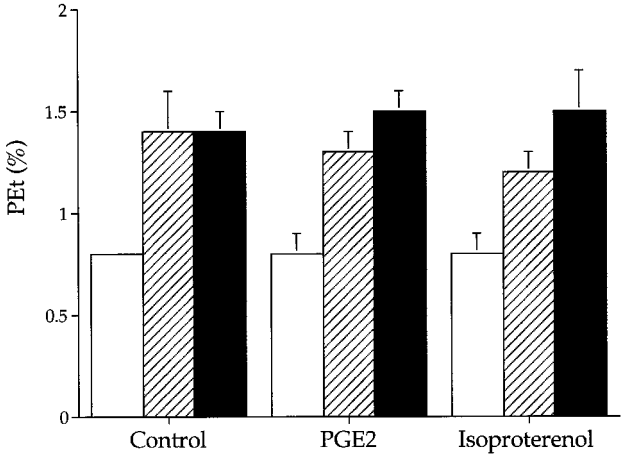

FIG. 7. **Lack of effect of PGE$_2$ and isoproterenol on ATP- and UTP-promoted PLD activation.** MDCK-D$_1$ cells were labeled with [^{3}H]palmitate and then incubated with 20 μM PGE$_2$ and 50 μM isoproterenol prior to incubation with 300 μM ATP or 300 μM UTP and assayed for PLD activity, as described 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 cPLA$_2$ activation. Based on these and previous data related to the role of MAP kinase on P$_{2Y}$ receptor-promoted activation of cPLA$_2$ in MDCK-D$_1$ cells, we conclude that the cAMP/PKA system negatively regulates cPLA$_2$ 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 cPLA$_2$, P$_{2Y}$ receptors can also increase phospholipase D activity in MDCK-D$_1$ cells (5). To determine whether the inhibitory effect of cAMP on cPLA$_2$ 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 $cPLA_2$/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 Cα as parallel pathways for the activation of $cPLA_2$ in MDCK-D_1 cells; blockade of either of these two pathways impairs the activation of $cPLA_2$ 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 $cPLA_2$ 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 B_2-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/$cPLA_2$ activation by bradykinin receptors in MDCK-D_1 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-D_1 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/$cPLA_2$ activation by α_1-adrenergic receptors in MDCK-D_1 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 P_2 receptor-mediated activation of PLD activity (Fig. 7). Other data indicate that MAP kinase activation is not required for PLD activation by P_2 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 $cPLA_2$ 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.

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

In conclusion, the present study demonstrates that the AC/PKA system plays a negative role in the regulation of AA release/$cPLA_2$ 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 $cPLA_2$/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 $cPLA_2$-mediated release of AA and its subsequent conversion to PGE_2 (3, 4), our results define a feedback cycle whereby P_{2Y2} receptors in MDCK-D_1 cells activate AA release and production of PGE_2. PGE_2, 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-D_1 cells are coupled to cAMP production via release of AA and its conversion to PGE_2, define a potentially important feedback loop for regulation of AA formation.

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