In Madin-Darby canine kidney D₁ cells extracellular nucleotides activate P₂ₓ receptors that couple to several signal transduction pathways, including stimulation of multiple phospholipases and arachidonic acid. For one class of P₂ₓ receptors, P₂ᵧ receptors, this stimulation of arachidonic acid (AA) via prostaglandins (PGE₂) and adenylyl cyclase stimulation occurs via the phospholipase A₂ (PLA₂)-generated phospholipase A₂ activity. PLA₂ activation by different receptors that promote PLA2 activation and AA release. We speculate that receptor-selective feedback inhibition occurs via activation of PKA. We found that activation of the AC system can inhibit PLA₂-mediated AA release by P₂ᵧ 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 P₂ᵧ 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 (DMEM, pH 7.4). Subsequently, cells were incubated in fresh DMEM with PGE₂ 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. Intraacellular 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 μCi 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 DMEM 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 equilibrium 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 DMEM 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 NaVO₄, 1 mM levaninoside) and protease inhibitors (500 μM phenylmethylsulfonyl fluoride, 8 μM pepstatin, 16 μM Aprotinin). Cell lysates were prepared, and cPLA₂ activity was measured as described previously (2).
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 mM okadaic acid and 1 mM diithiothreitol were added. Scraped cells were sonicated, and cell lysates (supernatant after centrifugation at 4°C for 10 min at 5000 × g) were assayed for cPLA2 using 1-steroyl-2[14C]arachidonyl-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-steroyl-2[14C]arachidonoyl-3-phosphatidyl choline, 1 mM EDTA, 1 mM CaCl2, 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 DMEM 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 5 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-conjugated secondary antibody. 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 P2Y2 Receptor-promoted AA Release and cPLA2 Activation in MDCK-D1 Cells**—We have previously demonstrated in MDCK-D1 cells that activation of P2Y2 purinergic receptors results in AA release via activation of cPLA2 (2) and that stimulation of P2Y2 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 P2Y2 receptors, we examined the effects of cAMP-increasing agents on P2Y2 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 P2Y2 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 P2Y2 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 P2Y2 receptor-promoted AA release in MDCK-D1 cells is mediated by activation of cPLA2 (2), we tested whether an increase in cAMP would blunt activation of this lipase. Indeed, as shown in Fig. 2, activation of cPLA2 in cell lysates by the specific P2Y2 agonist UTP was substantially inhibited by treatment of cells with PGE2. Therefore, increases in cellular cAMP decrease AA release and cPLA2 activation by P2Y2 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 P2Y2 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 P2Y2 receptors, AA release...
elicited by α₁-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 P₂Y₂ Receptor-promoted AA Release—To test whether the inhibitory effect of the increase in cellular cAMP on P₂Y₂ 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 P₂Y₂ 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 P₂Y₂ 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 P₂Y₂ 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 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-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 P₂Y₅ receptor-promoted activation of cPLA₂ in MDCK-D₁ cells, we conclude that the cAMP/PKA system negatively regulates cPLA₂ activated by P₂Y₂ receptors through the inhibition of MAP kinase activation.

P₂Y₅ Receptor Activation of Phospholipase D Activity Is Not Inhibited by Elevation of cAMP—In addition to activation cPLA₂, P₂Y₅ 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
In conclusion, the present study demonstrates that the AC/PKA system plays a negative role in the regulation of AA release/cPLA2 activation by P2Y2 receptors through inhibition of MAP kinase activation. This negative regulation occurs for P2Y2 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 P2Y2 receptor can activate the AC/PKA system by promoting cPLA2-mediated release of AA and its subsequent conversion to PGE2, our results define a feedback cycle whereby P2Y2 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 P2Y2 purinergic receptor pathway. The feedback cycle described herein may contribute to both physiologic and pharmacologic regulation of the P2Y purinergic receptor signaling. Overall, these results, together with evidence that P2Y2 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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