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Inhination of Phospholipase A2-mediated Arachidonic Acid Release by Cyclic AMP Defines a Negative Feedback Loop for P2Y Receptor Activation in Madin-Darby Canine Kidney D1 Cells*

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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 (AA) 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 P2Y (previously termed P2U) purinergic receptor-promoted AA release is mediated through cPLA2 activation by MAP kinase (2). Other data have shown that stimulation of P2Y purinergic receptors in these cells also stimulates arachidonic acid stimulation of PLA2 and AA release initiated by receptor agonists. We have recently demonstrated that in MDCK-D1 cells P2Y2 (previously termed P2U) purinergic receptor-promoted AA release is mediated through cPLA2 activation by MAP kinase (2). We found that activation of the AC system can inhibit cPLA2-mediated AA release by P2Y2 purinergic receptors through the inhibition of MAP kinase in MDCK-D1 cells. Therefore, we define a negative feedback mechanism involving prostaglandins (3, 4). In the present study we sought to assess the relationship of these two signaling pathways activated by P2Y2 purinergic receptors. We found that the AC system can inhibit cPLA2-mediated AA release by P2Y2 purinergic receptors through the inhibition of MAP kinase in MDCK-D1 cells. Therefore, we define a negative feedback mechanism involving prostaglandins and leukotrienes (5) and 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 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).

[^1H]AA Release in Intact Cells—Cells were labeled with [^1H]AA by incubation with 0.5 μCi of [^1H]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 [^1H]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).

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leupeptin, and 1 mM dithiothreitol), 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 (supernatants after centrifugation at 4 °C for 10 min at 500 × g) were assayed for cPLA2 using 1-steinoyl-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-steinoyl-2[14C]arachidonyl-L-3-phosphatidyl choline, 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 phosphatidylyethanol was resolved by thin layer chromatography, as described previously (5). Phosphatidylyethanol 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 SDSPolyacrylamide gel electrophoresis loading buffer and boiled for 5 min, and samples were electrophoresed on SDSPolyacrylamide 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 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 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 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. 3, activation of cPLA2 activity 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 P2Y2 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.
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 Pₓᵧ₂ receptors utilize MAP kinase and protein kinase Cα 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 Pₓᵧ₂ Receptor agonists (2). In the present study, we found that activation of the AC/PKA system can inhibit Pₓᵧ₂ 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 Pₓᵧ₂ receptors, but not activated by α₁-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 α₁-adrenergic receptor-promoted AA release was unexpected, because MAP kinase activation is responsible for AA release/cPLA₂ activation by α₁-adrenergic receptors in MDCK-D₁ cells (7). The ability of the AC/PKA system to inhibit Pₓᵧ₂ receptor-mediated AA release was unexpected, because MAP kinase activation is responsible for AA release/cPLA₂ activation by α₁-adrenergic receptors in MDCK-D₁ cells (7). The ability of the AC/PKA system on α₁-adrenergic receptor-promoted AA release was unexpected, because MAP kinase activation is responsible for AA release/cPLA₂ activation by α₁-adrenergic receptors in MDCK-D₁ cells (7). The ability of the AC/PKA system on α₁-adrenergic receptor-promoted AA release was unexpected, because MAP kinase activation is responsible for AA release/cPLA₂ activation by α₁-adrenergic receptors in MDCK-D₁ cells (7).

In conclusion, the present study demonstrates that the AC/PKA system plays a negative role in the regulation of AA release/cPLA₂ activation by Pₓᵧ₂ receptors through inhibition of MAP kinase activation. This negative regulation occurs for Pₓᵧ₂ 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 Pₓᵧ₂ 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 Pₓᵧ₂ 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 Pₓᵧ₂ purinergic receptor pathway. The feedback cycle described herein may contribute to both physiologic and pharmacologic regulation of the Pₓᵧ₂ purinergic receptor signaling. Overall, these results, together with evidence that Pₓᵧ₂ 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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