**P₂ Purinergic Receptor Agonists Enhance cAMP Production in Madin-Darby Canine Kidney Epithelial Cells via an Autocrine/Paracrine Mechanism**

(Received for publication, August 30, 1995, and in revised form, November 20, 1995)

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Mechanisms of cross-talk between different classes of signaling molecules are inadequately understood. We have used clonal Madin-Darby canine kidney (MDCK-D₁) epithelial cells as a model system to investigate the effects of extracellular nucleotides (e.g. ATP, UTP), which promote increase in activity of several phospholipases, on cAMP production. In contrast to observations in some other cell systems, ATP and UTP were not additive with the β-adrenergic receptor agonist isoproterenol, but were synergistic with forskolin in increasing cAMP production, indicating that Gₛₐ is activated by these nucleotides. Additionally, we found that (a) nucleotide-induced increases in cAMP were blocked by indomethacin, a cyclooxygenase inhibitor, (b) arachidonic acid increased cellular cAMP levels in an indomethacin-sensitive fashion, and (c) PGE₂, the major metabolite of arachidonic acid, stimulated cAMP formation. Overall, our results suggest a mechanism by which extracellular nucleotides stimulate release of arachidonic acid which is metabolized to PGE₂ which, in turn, acts in an autocrine/paracrine fashion via prostaglandin receptors to activate Gₛₐ and increase cAMP. Based on the ability of extracellular nucleotides to stimulate the formation and release of prostaglandins in MDCK-D₁ epithelial and other cells, we hypothesize that receptor-mediated prostaglandin release may be a general mechanism that regulates cAMP formation in many types of cells.

Extracellular ATP, an important extracellular signaling molecule, is stored and released from sympathetic neurotransmitter vesicles and from stressed/damaged cells. Receptors that interact specifically with ATP (classified as P₂ purinergic receptors) are present in many tissues and cell types (1–4). Two subclasses of G protein-coupled P₂ purinergic receptors include P₂ₐ and P₂ₐ receptor subtypes. In particular, P₂ₐ receptors bind ATP, while P₂ₐ receptors respond to both ATP and UTP (2, 5). Additionally, ATP can be metabolized to adenosine, the agonist for P₁ purinergic receptors.

Interaction of extracellular nucleotides with P₂ purinergic receptors elicits a range of intracellular signaling responses. The most common response elicited by ATP is the activation of phospholipase C with subsequent increases in 1,4,5-inositol trisphosphate formation, diacylglycerol production, increased intracellular Ca²⁺, and activation of protein kinase C (PKC) (5–8). Activation of phospholipase A₂ and phospholipase D has also been reported to occur in response to nucleotides (9–11). In addition, in rat hepatocytes, FRTL-5 thyroid cells, mouse ventricular myocytes, and C6-gloma cells, extracellular ATP inhibits cAMP production by both pertussis toxin-sensitive and insensitive pathways (8, 12–17).

MDCK cells are a well differentiated epithelial cell line and have been widely used as a model system for studying the regulation of epithelial cell function (18–20). In these cells, cAMP regulates Cl⁻ ion secretion, polarized vesicle transport, and Na⁺/K⁺ ATPase activity (21–24). MDCK-D₁ cells, a clonal line derived from parental cells (25, 26), appear to express both P₂ₐ and P₂ₐ purinergic receptors (10, 27). Because of the importance of cAMP in regulating epithelial cell function and the potential interaction between P₂ purinergic receptor response pathways and the adenylyl cyclase pathway, we examined the effect of extracellular nucleotides on cAMP generation in MDCK-D₁ cells. In contrast to observations in other cell lines, we found that both ATP and UTP stimulated cAMP production in MDCK-D₁ cells. Several mechanisms regulating phospholipase activation to modulation of cAMP accumulation could explain this result. In particular, increases in Ca²⁺ or PKC activity following phospholipase C activation can alter adenylyl cyclase activity.

In this study, however, we demonstrated that nucleotide-induced phospholipase activation leads to increased levels of cAMP by the release of arachidonic acid, and in turn, cyclooxygenase-derived products. We propose that utilization of this autocrine/paracrine pathway may be a general mechanism for regulating cAMP production in epithelial cells and other cell types.

**MATERIALS AND METHODS**

**Cell Culture—** MDCK-D₁ cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% serum as described previously (25, 26). Cells were used in assays at 60–80% confluence. At cell densities greater than this, basal cAMP levels were increased.

**Measurement of cAMP Accumulation—** Prior to treatment of cells, growth medium was removed 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 HEPES-buffered Dulbecco’s modified Eagle’s medium and various agents as described in the figure legends. Unless otherwise indicated, incubations with agonist were conducted for 5 min at 37 °C in the presence of 200 μM IBMX, a phosphodiesterase inhibitor, and terminated by aspiration of medium and addition of 7.5% trichloroacetic acid.

The abbreviations used are: PKC, protein kinase C; MDCK-D₁, clonal Madin-Darby canine kidney cells; ATP₆, adenosine 5’-O(thio-triphosphate); PGE₂, prostaglandin E₂.

*This work was supported by Grants GM 40781, GM 31987, HL 35018 from National Institutes of Health and Postdoctoral Fellowship GM 16172 from National Institutes of Health (to S. R. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ATP and UTP Increase cAMP Formation in Epithelial Cells

**RESULTS AND DISCUSSION**

We assessed the ability of various P2 receptor and P2 receptor agonists to affect cAMP accumulation in MDCK-D1 cells. As shown in Fig. 1a, both ATP and UTP stimulated cAMP formation. It is unlikely that this response to ATP resulted from breakdown to adenosine and activation of P1 purinergic receptors, since adenosine failed to alter cAMP production, whereas ATP-S, a nonhydrolyzable ATP analog, stimulated cAMP formation to the same extent as ATP. This suggested that extracellular nucleotides acting at P2 purinergic receptors were mediating cAMP formation. The ability of UTP, a P2U receptor agonist, and 2-methylthio-ATP, a P2Y receptor agonist, to increase cAMP indicate that both P2U and P2Y receptors are positively coupled to adenylyl cyclase activation in MDCK-D1 cells. P2 purinergic receptor-mediated stimulation of cAMP formation was concentration-dependent (Fig. 1b), exhibiting an average EC50 of 1.8 μM for ATP and 2.1 μM for UTP. The ability of nucleotides to stimulate cAMP production in MDCK-D1 cells occurred at concentrations previously shown to activate phospholipase-linked pathways in this and other cell systems (10, 12, 14, 28). At maximally effective concentrations, UTP-stimulated cAMP formation was typically not as great as that occurring with ATP; a result consistent with the interaction of ATP with multiple (presumably both P2U and P2Y) receptor populations.

In general, P2U and P2Y purinergic receptors mediate the activation of phospholipase C with subsequent increases cellular Ca2+ levels (5–8). Increased intracellular Ca2+ is known to stimulate adenylyl cyclase through a variety of mechanisms, including interaction with calmodulin and activation of PKC (29–32). Since ATP and UTP increase intracellular Ca2+ ion concentration in MDCK-D1 cells (27), it is possible that nucleotides stimulate cAMP production by a Ca2+/calmodulin-dependent process. Because Ca2+/calmodulin-stimulated adenylyl cyclase isozymes, types I and VIII, have not been detected in kidney (33, 34), it is unlikely that the nucleotide-stimulated increase in cAMP in MDCK cells results from a direct interaction of Ca2+/calmodulin with adenylyl cyclase. We therefore examined the extent to whichactivation of PKC could mimic the effect of nucleotides on cAMP production in these cells. As shown in Fig. 2, treatment of cells with the phorbol ester, phorbol 12-myristate 13-acetate, for 10 min had no effect on basal or forskolin-stimulated cAMP accumulation. Since protein kinase C-dependent modulation of the activity of certain isoforms of adenylyl cyclase is conditional upon Gαs activation (35, 36), we examined the effect of nucleotides on β-adrenergic receptor-stimulated cAMP production. As shown in Fig. 3, ATP and UTP were not additive with the β-adrenergic receptor agonist, isoproterenol, in increasing cAMP formation. It therefore appears that in MDCK-D1 cells, PKC activation is not directly responsible for the nucleotide-mediated increase in cAMP.

**Fig. 1.** P2 receptor agonists increase cAMP in MDCK cells.

MDCK-D1 cells were treated as described under “Materials and Methods.” a, cells were incubated in the presence or absence of the indicated nucleotide (100 μM), adenosine (Adn; 100 μM), or isoproterenol (Iso; 10 μM). b, cells were stimulated with the indicated concentrations of ATP and UTP. Incubations were for 5 min in the presence of 200 μM isobutylmethylxanthine. Cells were treated and cAMP assayed as described under “Materials and Methods.” Data represent the mean ± S.E. of triplicate determinations from a representative experiment with similar results obtained in at least three separate experiments.

**Fig. 2.** Activation of PKC does not mimic nucleotide effect on cAMP production. MDCK-D1 cells were preincubated in the presence or absence of 50 ng/ml phorbol 12-myristate, 13-acetate (PMA), a PKC activator, for 10 min at 37°C. Subsequently, isobutylmethylxanthine and either 100 μM nucleotide or 1 μM forskolin (Fsk) was added and incubations continued as described under “Materials and Methods.” The data represent the mean ± S.E. of triplicate determinations with similar results obtained in multiple experiments.
ATP and UTP were not additive with each other or with the response elicited by isoproterenol. This lack of additivity is consistent with the utilization of a common pathway by both P2 purinergic receptors and β-adrenergic receptors. In contrast, nucleotide- and isoproterenol-stimulated cAMP production were both synergistic with forskolin. As shown here in studies with isoproterenol and PGE2 (Fig. 3 and inset) and as demonstrated previously (37–39), synergistic effects of such agonists and forskolin in stimulating cAMP production is associated with activation of Gsα, probably by enhancing the ability of Gsα to interact with adenylyl cyclase (39, 40). Thus, it appears that the ATP and UTP-mediated increase in cAMP production involves Gsα activation.

To date, however, there is no evidence for the interaction of P2 purinergic receptors directly with Gsα. We hypothesized therefore, that the activation of Gsα might be secondary to purinergic receptor-mediated stimulation of arachidonic acid release and the subsequent formation of arachidonic acid metabolites, perhaps those derived from the action of cyclooxygenase. In this regard, we have found that activation of phospholipases, in particular phospholipase A2, in MDCK-D1 cells results in the release of arachidonic acid and, in turn, to the formation of PGE2 (41). Furthermore, PGE2 release is inhibited by indomethacin, a cyclooxygenase inhibitor (42). Since both ATP and UTP stimulate the release of arachidonic acid in these cells (10, 27), it seemed possible that P2 purinergic receptor-mediated cAMP production was secondary to the metabolism of arachidonic acid. Indeed, as shown in Fig. 4, arachidonic acid stimulated cAMP formation with a time course that parallels that of nucleotide-mediated activation. For both exogenous arachidonic acid and nucleotides, maximal cAMP levels were obtained by 5 min. The effect of arachidonic acid to increase cAMP formation occurred at concentrations between 1 and 10 μM, and importantly, the ability of arachidonic acid to stimulate cAMP accumulation was completely inhibited by 1 μM indomethacin (Fig. 5a). This result led us to predict that cyclooxygenase activity and presumably prostaglandin produced from arachidonic acid might be responsible for the effect of ATP and UTP to stimulate cAMP production. Results presented in Fig. 5b confirm this prediction in that nucleotide-stimulated cAMP production, like that elicited by arachidonic acid, was inhibited by indomethacin. Taken together, our results indicate that in MDCK-D1 cells cyclooxygenase products, most likely PGE2, produced following P2 purinergic receptor activation of
phospholipases mediate the cAMP response observed in response to extracellular nucleotides. Modulation of adenylyl cyclase activity by extracellular nucleotides has been previously noted, but, in general, the effect of nucleotides on cAMP accumulation is inhibitory. Roles for G_{i} activation, Ca^{2+} and PKC have been implicated in ATP-mediated inhibition of cAMP production (8, 14, 16, 17). Notwithstanding these inhibitory effects on adenylyl cyclase, there are reports of extracellular nucleotide-mediated increases in cAMP formation. For example, in bovine aortic endothelial cells, ATP results in a 3-fold increase in cAMP production and a greater than additive increase when added together with forskolin (43). The mechanism by which ATP elicited this response appears to involve both PKC activation and ATP interaction with methylxanthine-sensitive receptors. Similarly, in L cells and adrenal medullary endothelial cells, ATP sensitizes adenylyl cyclase to stimulation by hormones and forskolin (28, 44). Sensitization of adenylyl cyclase to activation by G_{s}-linked receptor agonists or forskolin have been described for PKC-dependent modulation of distinct adenylyl cyclase isoforms (35, 36, 45, 46). It therefore seems likely that in aortic endothelial cells, L cells, and adrenal medullary cells PKC mediates the ability of P_{2} purinergic receptor agonists to increase adenylyl cyclase activity. In Swiss 3T3 fibroblasts and human A431 epidermoid carcinoma cells, however, ATP-stimulated cAMP production is sensitive to cyclooxygenase inhibitors (47, 48). This effect is associated with the ability of ATP to stimulate mitogenesis in these cells (48, 49). In contrast to results presented here, in both 3T3 and A431 cells there is an initial delay of 15 min before cAMP increases are observed, and maximal cAMP accumulation is not achieved until 45 min after addition of ATP. Therefore, it seems less likely that nucleotide-mediated cAMP formation in 3T3 cells or A431 cells serves a role in rapid alterations of cellular responses. Our results in MDCK-D_{1} cells demonstrate that extracellular nucleotides rapidly stimulate cAMP formation by a mechanism involving the liberation of arachidonic acid, and production of prostaglandin, presumably PGE_{2}. PGE_{2} then acts in an autocrine/paracrine manner at a prostaglandin receptor to activate G_{s}, and subsequently adenylyl cyclase. The ability of P_{2} nucleotide receptors to stimulate cAMP by this mechanism, therefore, depends on several properties. First, phospholipases must be activated and arachidonic acid must be released in quantities sufficient for substantial prostaglandin formation. Second, cyclooxygenase must be present and active in the time frame during which arachidonic acid is available. Third, the cells must possess prostaglandin receptors, which are positively coupled to adenylyl cyclase. In MDCK-D_{1} cells, as well as in 3T3 and A431 cells, these criteria have been fulfilled. We predict that other cell types will show similar features. Thus, extracellular nucleotides released from granules or in other settings, such as cell damage, can regulate cAMP formation in a variety of cell types. In many cases this effect is inhibitory. However, depending on cell type, nucleotides can stimulate cAMP formation by at least two different mechanisms. In aortic endothelial cells, L cells, and adrenal medullary cells this stimulation appears to be mediated by PKC. In MDCK-D_{1} epithelial, fibroblast, and epidermoid carcinoma cells, nucleotide-stimulated cAMP formation results from the cyclooxygenase-dependent metabolism of arachidonic acid. In this way, P_{2} receptors can regulate both rapid (e.g. secretion) and long term (e.g. mitogenesis) cAMP-dependent cell functions.
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J. Biol. Chem. 1996, 271:2029-2032.
doi: 10.1074/jbc.271.4.2029

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