ATP Activates cAMP Production via Multiple Purinergic Receptors in MDCK-D1 Epithelial Cells

BLOCKADE OF AN AUTOCRINE/PARACRINE PATHWAY TO DEFINE RECEPTOR PREFERENCE OF AN AGONIST*

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Extracellular nucleotides regulate function in many cell types via activation of multiple P₂-purinergic receptor subtypes. However, it has been difficult to define which individual subtypes mediate responses to the physiological agonist ATP. We report a novel means to determine this by exploiting the differential activation of an autocrine/paracrine signaling pathway. We used Madin-Darby canine kidney epithelial cells (MDCK-D1) and assessed the regulation of cAMP formation by nucleotides. We found that ATP, 2-methylthio-ATP (MT-ATP) and UTP increase cAMP production. The cyclooxygenase inhibitor indomethacin completely inhibited ATP, 2-methylthio-ATP (MT-ATP), whereas P₂Y₂ receptors respond to both ATP and UTP (2, 5). Additionally, ATP can be metabolized by ecto-ATPases to adenosine, the agonist for P₁-purinergic receptors.

We have studied P₂ receptor-mediated signaling events in a well differentiated kidney epithelial cell line, Madin-Darby canine kidney (MDCK-D1), derived from distal tubule/collecting duct. P₂-purinergic receptors expressed on these cells respond to extracellular nucleotides by increasing the activity of various phospholipases (phospholipases C, D, and A₂), the activity of protein kinase C, the concentration of intracellular calcium, the production of cAMP, and transepithelial ion transport (6–12).

In several other cell types, stimulation of P₂ receptors decreases intracellular cAMP production via a pertussis toxin-sensitive mechanism (13–18). In contrast, ATP was shown to activate adenylyl cyclase in HL60 cells (19, 20). Using MDCK-D1 cells, we found that P₂ receptor agonists increase cAMP production (10) and tested hypotheses regarding several potential mediators for the increase in cAMP production, protein kinase C, Gₛ, adenosine, and arachidonic acid metabolites. Our results indicated that ATP and UTP, acting at P₂-purinergic receptors, increase cAMP via an indomethacin-sensitive pathway, implying that cyclooxygenase-derived products mediate this response.

In the current study, we sought to distinguish between the actions of ATP and other nucleotides that act at different P₂ receptor subtypes. Our data indicate that P₂Y₂ receptor-mediated cAMP formation occurs via an indomethacin-sensitive pathway. In contrast, MT-ATP (acting at other classes of receptors, perhaps P₂Y₁ and/or P₂Y₁₁ receptors) preferentially couples to indomethacin-insensitive pathways to increase cAMP production. The physiological agonist ATP increases cAMP via preferential interaction with P₂Y₂ receptors.

MATERIALS AND METHODS

Cell Culture—MDCK-D₁ cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% serum as described previously (8). Cells were used in assays at 60–80% confluency. Basal cAMP levels were increased at cell densities greater than this.

Measurement of cAMP Accumulation—Before treatment of cells, growth medium was removed and cells were equilibrated for 30 min at

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1 The abbreviations used are: MDCK-D₁, clonal Madin-Darby canine kidney cells; PGₑₑ₆₆, prostaglandin E₆₆; MT-ATP, 2-methylthioadenosine 5’-triphosphate; RT, reverse transcriptase; PCR, polymerase chain reaction.
ATP Interaction with Purinergic Receptors in Epithelial Cells

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 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 isobutylmethylxanthine, a phosphodiesterase inhibitor, and terminated by aspiration of medium and addition of 7.5% trichloroacetic acid. Trichloroacetic acid extracts were frozen (−20°C) until assay. Intracellular cAMP levels were determined by radioimmunoassay (Calbiochem) of trichloroacetic acid extracts following acetylation according to the manufacturer’s protocol. Production of cAMP was normalized to the amount of acid-insoluble protein assayed by the Bio-Rad protein assay.

Measurement of PGE$_2$ Release—Cells were treated with indicated concentrations of nucleotide for 5 min in a total volume of 0.5 ml. Incubation medium was removed and assayed for PGE$_2$ content using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The amount of PGE$_2$ present in the medium in the absence of nucleotide was subtracted from each point.

RNA and DNA Isolation—Total RNA was isolated from MDCK-D1 using Trizol. MDCK-D1 DNA was isolated from cells grown in 150-mm culture dishes. Cells were scraped into ice-cold phosphate-buffered saline, collected by centrifugation (5 min at 500 x g) and resuspended in 5 volumes of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, 1 mg/ml proteinase K, pH 8.0). Following overnight incubation at 37°C, samples were extracted three times with phenol/chloroform (Ambion) and precipitated with ethanol (2 volumes) and 7.5 M ammonium acetate (0.5 volume).

Reverse Transcriptase (RT) Reaction—RNA (10 µg) was reverse transcribed in 25 µl of RT buffer (Life Technologies, Inc.) together with 0.002 OD units of random hexamers, 10 mM dithiothreitol, 800 µM dNTP, and 200 units Moloney murine leukemia virus RT. After a 1-h incubation at 37°C, reactions were stopped by boiling for 4 min and diluted to 100 µl in RNase-free water. To control for possible DNA contamination of the samples, reactions were also performed in the absence of RT enzyme.

PCR and Sequencing—PCR conditions were identical for all primers (primers: P$_2$Y$_1$, forward 5'-ACCCCCAGGACCTTCTCTTCGACT-3', reverse 5'-AGGAGGAGSSAGGCAGAAC-3' expected product size 422 base pairs; P$_2$Y$_2$, forward 5'-GTCCTCCCTGTTCTCTTACT-3', reverse 5'-GTCACTGCTGCTGGACCTTG-3' expected product size 539 base pairs; P$_2$Y$_3$, forward 5'-CTGGTTGTTGGATTGTCCTGTG-3', reverse 5'-GGTCGAGGTGAAGAGAAGC-3' expected product size 234 base pairs): 10 µl of reverse transcribed RNA or 1 µg of genomic DNA was added to a solution of 20 µl each of forward and reverse primer, 2.5 mM MgCl$_2$ buffer (Perkin-Elmer), PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTPs (Amersham Pharmacia Biotech), 5 units of AmpliTaq Gold Polymerase (Perkin-Elmer) and dH$_2$O in a total volume of 50 µl. Temperature cycling proceeded as follows: 1 cycle at 95°C for 10 min to activate the enzyme, 95°C for 30 s, 60°C for 90 s and 72°C for 90 s, for 40 cycles, followed by 72°C for 10 min. PCR products were then subjected to gel electrophoresis on a 1% Seakem-agarose gel (FMC Bioproducts). The bands were extracted using a Qiaquick gel extraction kit (Qiagen). The DNA was resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and 1 volume of the gel-extracted PCR product was purified using a Centricron concentrator (Amicon). Purified fragments were sequenced (ABI automated DNA sequencer, model 377) using the same forward primers that were used to generate the PCR fragments.

Chemicals—Chemicals were purchased from the following sources. For cAMP and anti-cAMP antibody from Calbiochem, 125I-cAMP from NEN Life Science Products, PGE$_2$ immunoassay kit from Cayman Chemical, all other reagents from Sigma. Trizol (Life Technologies, Inc.), Moloney murine leukemia virus reverse transcriptase/5X reaction buffer (Life Technologies, Inc.), DNA polymerization mix (Amersham Pharmacia Biotech), random hexamers (Amersham Pharmacia Biotech), AmpliTaq Gold polymerase (Perkin-Elmer), and dNTPs (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

In previous studies, we showed that extracellular nucleotides that act at P$_2$ purinergic receptors increase cAMP formation in MDCK-D1 kidney epithelial cells (10). The ability of ATP and UTP to stimulate cAMP was inhibited by the cyclooxygenase inhibitor indomethacin, indicating that this response was secondary to the release of arachidonic acid and most likely by its conversion to one or more prostaglandins. Because ATP is generally considered the endogenous ligand for P$_2$ purinergic receptors, our initial assumption was that P$_2$ receptors coupled to adenylyl cyclase activation secondary to the production of prostaglandins. Using indomethacin at a concentration that completely inhibits the ability of exogenous arachidonic acid to stimulate cAMP formation (Fig. 1A), we found that the increase in cAMP in response to UTP was also abolished (Fig. 1B). In contrast, the cyclooxygenase inhibitor did not significantly inhibit cAMP formation in response to the P$_2$Y$_1$ receptor agonist MT-ATP. In the presence of 1 µM indomethacin, the ability of ATP to increase cAMP was reduced, but only by ~80%. These results suggested the involvement of two signaling pathways in coupling P$_2$ receptors to adenylyl cyclase activation: P$_2$Y$_2$ receptors, whose ability to increase cAMP formation appears to be entirely dependent upon the formation of prostaglandin; and another P$_2$Y$_2$ receptor, whose activation of adenylyl cyclase is apparently independent of prostaglandin production.

We sought to determine the identity of the prostaglandins that coupled P$_2$ receptors with adenylyl cyclase activation. MDCK cells produce and release PGE$_2$, prostaglandin I$_2$ (prostacyclin), and prostaglandin F$_{2a}$ (21, 22). Of these, only PGE$_2$ and prostacyclin, tested using a stable prostacyclin analog (6a-PG1), were positively coupled to adenylyl cyclase activation (Fig. 2A) (10, 21). To correlate prostaglandin production with P$_2$ receptor activation, we measured the PGE$_2$ released following the addition of purinergic receptor agonists. At concentrations that increase cAMP formation, both ATP and UTP substantially increased PGE$_2$ release from MDCK cells (EC$_{50}$ ~ 30 µM, Fig. 2B). In contrast, receptor activation with MT-ATP did not increase PGE$_2$ release even at concentrations that maximally stimulated cAMP formation (Figs. 2B and 4A). The production of PGE$_2$ was completely blocked by indomethacin (1 µM) pretreatment (data not shown). These data correlate well with the sensitivity of cAMP production to indomethacin for P$_2$Y$_2$ receptor agonists (Fig. 1). Thus, by measuring cAMP formation in MDCK cells pretreated with indomethacin, we could specifically assess activation of indomethacin-insensitive P$_2$Y$_2$ receptors independent of the contribution of P$_2$Y$_2$ receptor activation.
Having defined conditions that allowed us to distinguish between indomethacin-insensitive and indomethacin-sensitive (P2Y2) receptor activation, we assessed the relative contributions of these two receptor pathways in cAMP accumulation stimulated by the physiological agonist ATP. We compared the ability of ATP to increase cAMP formation via both pathways (i.e. absence of indomethacin) with that of the indomethacin-insensitive pathway alone (i.e. following pretreatment with indomethacin). As shown in Fig. 3A, pretreatment of cells with indomethacin had no significant effect on the ability of MT-ATP to stimulate cAMP formation (EC50 ~ 25 μM in each case). In contrast, ATP displayed a substantial difference in its ability to activate adenylyl cyclase following pretreatment of MDCK cells with indomethacin (Fig. 3B). In untreated cells, ATP, acting via both pathways, displayed an apparent EC50 ~ 10 μM. However, following indomethacin treatment, ATP, acting selectively via indomethacin-insensitive receptors, demonstrated a greatly reduced potency to increase cAMP (EC50 ~ 100 μM). This result indicates that in untreated cells, the cAMP formed in response to ATP arises by the preferential activation of P2Y2 receptors.

The substantial inhibition of ATP-stimulated cAMP formation by indomethacin suggested that ATP was activating multiple P2 receptors, but with greater apparent potency at P2Y2 receptors. Given the previous evidence for expression of P2Y1 and P2Y2 receptors in MDCK-D1 cells (8, 23) (and the efficacy of MT-ATP in stimulating cAMP formation), we initially hypothesized that the indomethacin-insensitive response to MT-ATP resulted from activation of P2Y1 receptors. To test this assumption, we used suramin as a P2Y1 receptor antagonist. In cells preincubated with indomethacin, suramin competitively inhibited MT-ATP-stimulated cAMP formation (Fig. 4A) and displayed a pA2 ~ 5.2 (i.e. an antagonist concentration required to increase EC50 for agonist 2-fold), which closely matches that previously reported for competition at P2Y1 receptors (1). Suramin also completely inhibited the indomethacin-insensitive component of ATP-stimulated cAMP formation (Fig. 4B). These data are consistent with the notion that ATP acts at P2Y1 receptors to increase cAMP by a prostaglandin-independent pathway.

Recently, a new P2Y receptor subtype, P2Y11, which responds to ATP and MT-ATP, was identified and shown to couple to adenylyl cyclase activation (24). However, ATP activated the cloned P2Y11 receptor with much greater potency (EC50 ~ 30 μM) than we observed for the indomethacin-insensitive cAMP response in MDCK cells. Nevertheless, we assessed whether MDCK-D1 cells express this receptor subtype. Indeed, RT-PCR analysis indicated that P2Y1, P2Y2, and P2Y11 receptors are all expressed in MDCK-D1 cells (Fig. 5). PCR of DNA and RT-PCR of RNA yielded bands of the anticipated size for each of the three receptors. Omission of reverse transcriptase from the RT-PCR reaction failed to yield any product indicating the absence of contaminating DNA. The identity of the products (in the case of the P2Y1, the larger of the two products) was confirmed by direct sequencing using the forward primer. Thus, although the pharmacology of the indomethacin-insensitive cAMP response is consistent with that of a P2Y1 receptor, we cannot exclude the possible contribution of P2Y11 receptors in mediating the indomethacin-insensitive response to ATP.

Previous studies have demonstrated the ability of P2 receptor agonists to regulate transepithelial ion transport in MDCK cells (11, 12). PGE2 and other agents that increase cAMP are known to couple receptors to ion transport in epithelial cells. In
demonstrate the indomethacin-sensitive cAMP production elicited by P2Y2 agonists provide the most likely molecular basis for the ability of these receptors to couple to ion transport. Moreover, our finding that MDCK cells also express P2Y11 receptors raises the possibility that this receptor subtype may regulate ion transport via activation of adenylyl cyclase.

In MDCK-D1 cells, cAMP is a messenger that is considerably “downstream” from the initial occupancy by agonist of the P2Y2 receptors. Nucleotide-mediated stimulation of cAMP formation requires receptor occupancy, activation of cyclic phospholipase A2 (via apparent involvement of Ca2+ and multiple protein kinase C isozymes (see Ref. 9), cyclooxygenase-mediated formation of PGE2, PGE2 release from cells and autocrine/paracrine activation of PGE2 receptors, Gαs, and adenylyl cyclase). Thus, indomethacin, an inhibitor of an intermediate step in this scheme, can be used to define the relative ability of ATP to act at P2Y2 receptors in MDCK-D1 cells. Inhibitors that act on other components of the signaling pathways, which are not shared by the different P2-purinergic receptors, might also be used in this manner. We predict that selective inhibitors of the receptors that couple prostaglandin binding to activation of adenylyl cyclase (presumably EP2 receptors) (25) should also distinguish the indomethacin-sensitive (P2Y2) and indomethacin-insensitive components of ATP action.

The existence of multiple subtypes appears to be very common for G-protein-coupled receptors. Pharmacological approaches that involve use of receptor-selective agonists and antagonists have not kept pace with the discovery of receptor subtypes by molecular cloning strategies. In the case of P2-purinergic receptors, the absence of high affinity antagonists and the limited specificity of agonists have made it difficult to define precisely the cellular function of different receptor subtypes (4). In many cases receptor subtypes that recognize the same physiologic agonist preferentially activate different signaling pathways. In addition to the P2-purinergic receptors and the physiologic agonist ATP, other examples include receptors for adenosine, norepinephrine/epinephrine, dopamine, acetylcholine (muscarinic receptors), histamine, prostaglandins, and serotonin as well as receptor for certain peptides and peptide hormones (e.g. angiotensin and vasopressin) (26). Most work to date has emphasized linkage to different classes of G-proteins as the explanation for differences in signaling. The current studies show that one can use blockade of downstream signals that result from differences in signaling cascades to define contribution of different receptor subtypes that recognize the same physiologic agonist. Such downstream differences may prove useful for the analysis of other receptor systems, in particular those for which response has been attributed, at least in part, to generation of cyclooxygenase-derived products (e.g. see Refs. 27–30).

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ATP Interaction with Purinergic Receptors in Epithelial Cells

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