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Cellular Release of and Response to ATP as Key Determinants of the Set-Point of Signal Transduction Pathways*

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The determinants of "basal" activity of signaling pathways regulating cellular responses are poorly defined. One possibility is that cells release factors to establish the set-point of such pathways. Here we show that treatment of Madin-Darby canine kidney cells with the nucleotidase apyrase decreases basal arachidonic acid release and cAMP production 30–40% and that inhibitors of P2Y receptor action also affect basal and forskolin-stimulated cAMP accumulation. Changing medium prominently increases extracellular levels of ATP in Madin-Darby canine kidney, COS-7, and HEK-293 cells. Mechanical stimulation of ATP release likely occurs in virtually every experimental protocol with cultured cells, implicating such release and P2Y receptor activation as critical in establishing the set-point for signal transduction pathways.

Signal transduction across the plasma membrane is a critical factor in the regulation of eukaryotic cells. A wealth of published studies have documented the role of exogenous hormones or neurotransmitters in the activation of such signaling pathways. By contrast, the importance of autocrine/paracrine signaling pathways in the regulation of cell function is often under appreciated because of the more dramatic effects of hormones and neuronal mediators. However, many cell types produce and/or release chemical mediators that can dramatically alter function of the same or nearby cells (1). Such local signaling events have the potential to sensitize or desensitize cells via cross-talk between signaling cascades or to propagate signals between cells based on release of mediators or direct cell to cell communication. Prostaglandins, derived from the conversion of arachidonic acid (AA)[1] by cyclooxygenase, are an example of such an autocrine/paracrine mechanism and are recognized to arise from physical perturbation of cell surfaces. Prostaglandins activate a diverse class of G protein-coupled receptors that increase cytoplasmic Ca^{2+} and regulate adenylyl cyclase activity (2).

Cells also can release nucleotides, such as ATP and UTP, in response to mechanical stress or biological activation (3–9). However, the role of released nucleotides (in contrast to exogenously added agents) in modulation of cell signaling pathways has not been defined. Specific targets for nucleotides include P2Y (G protein-coupled) and P2X (ion channel) receptors (10, 11). Although P2X and P2Y receptors have been identified in many cell types, their contribution to basal levels of ion conductance or activation of G protein-coupled effectors is not known. Release of ATP and activation of P2Y receptors has been implicated as a gap junction-independent mechanism for transducing waves of intracellular Ca^{2+} signals in various cell types (12, 13).

In the present studies, we have examined ATP release, P2Y receptors, and signal transduction pathways in Madin-Darby canine kidney (MDCK-D_1) cells, a well differentiated and widely utilized model system derived from distal tubule/collecting duct epithelium. $P2Y_1$ and $P2Y_2$ receptor subtypes are expressed in MDCK-D_1 cells and couple to phospholipase C, presumably via activation of $G_{q/11}$ family G proteins (14). $P2Y_{11}$ receptors are also expressed in these cells and have been suggested to couple to G_q as well as G_s in other cells (15). In MDCK-D_1 cells P2Y receptor agonists decrease membrane resistance, increase intracellular calcium, regulate ion transport, activate protein kinase C, and couple to the activation of cytosolic phospholipase A_2 ($cPLA_2$) via both mitogen-activated protein kinase-dependent and -independent pathways (13, 16–19). $cPLA_2$-mediated release of AA provides substrate for cyclooxygenase conversion into eicosanoids, in particular PGE_2, which then activates adenylyl cyclase activity through G_s-coupled prostanoid receptors (20). In the present experiments we tested whether nucleotides might be released by MDCK cells and thereby alter levels of second messengers via activation of P2Y receptors. We find that MDCK-D_1 cells release ATP in response to mild mechanical manipulation, thereby resulting in substantial basal release of arachidonic acid as well as an increase in the cellular levels of cAMP. We further find that HEK-293 and COS-7 cells also release ATP. Therefore, release of ATP by physical or chemical means contributes to the set-point of the cellular signaling pathways regulated by P2Y receptors. The results have important implications for released ATP as a modulator of signal transduction pathways in epithelial and other cell systems.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were obtained from Fisher. Radiolabeled chemicals were obtained from NEN Life Science Products. Forskolin was obtained from Calbiochem. ATP bioluminescence assay kit HS II was obtained from Roche Molecular Biochemicals. All other drugs and reagents were obtained from Sigma.

Cell Culture—MDCK-D_1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% fetal bovine serum and 7.5% horse serum. Cells were passaged every 3–4 days by trypsinization using trypsin/EDTA. Cells were used for experiments in 24-well plates (Costar) grown to approximately 60–70% confluence. In some experiments, cells were washed and cultured in serum-free DMEM for 24–48 h before assay.

Assay of cAMP—Cells were labeled with 1 μCi/well [^{3}H]adenine in growth medium for 90 min to allow incorporation of radiolabel into intracellular ATP pools. Growth medium was removed, and cells were washed extensively and equilibrated for 30 min at 37 °C in serum-free

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[1] The abbreviations used are: AA, arachidonic acid; MDCK cells, Madin-Darby canine kidney cells; $cPLA_2$, cytosolic phospholipase A_2; PGE, prostaglandin; DMEM, Dulbecco's modified Eagle's medium; DMEH, serum-free DMEM containing 20 mM HEPES buffer.

DMEM containing 20 mM HEPES buffer (DMEH). Cells were then incubated for 5 min in fresh DMEH with either 200 μM isobutylmethylxanthine or 100 μM Ro 20-1724 (to inhibit phosphodiesterases) along with various drugs of interest. Reactions were terminated by aspiration of medium and addition of 7.5% trichloroacetic acid. Approximately 1000 cpm of [^{32}P]cAMP internal standard was added to each sample, and the volume was brought to 1 ml with water. [^{3}H]cAMP and [^{3}H]ATP were separated from the supernatant fraction using a chromatography method modified from Salomon *et al.* (21) and as described previously (22).

Luciferin/Luciferase Detection of ATP—For luciferin/luciferase detection of ATP, cells were preincubated for 30 min in 0.5 ml of DMEH at 37 °C. Media were then gently aspirated without tilting the plate, and fresh media containing the indicated drugs were gently added to the side of the well. In time course experiments, 100 μl of medium was collected from the top of each well at the indicated time, making sure to avoid contact of the pipette tip with the cells. When drug effects were measured, cells were equilibrated for 60 min in media, a small volume of drug was gently applied, and then 100 μl of medium was collected 5 min later. All samples were centrifuged to eliminate possible cell contaminants. An ATP bioluminescence kit containing luciferin/luciferase reagent was used to detect ATP (ATP bioluminescence assay kit HS II, Roche Molecular Biochemicals), and luminescence was measured in a Monolight 2010 luminometer. Bioluminescence controls were performed with each drug solution to eliminate drug effect on luciferase activity as well as to control for ATP contamination.

[^{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) per ml for approximately 20 h in 24-well plates. Cells were washed three times with DMEH, pH 7.4, supplemented with 5 mg/ml bovine serum albumin and allowed to equilibrate at 37 °C for 15 min. This equilibration medium was aspirated, and drugs of interest were added to the wells and incubated with cells for 20 min. Assays were terminated by removal of medium and transferring this medium into tubes containing 50 μl of 55 mM EDTA, 55 mM EGTA. 250 μl of 0.5% Triton X-100 was added to each well to solubilize cellular membranes. Liquid scintillation counting was performed to quantitate released [^{3}H]AA in media. The results were normalized as a percentage of incorporated radioactivity measured from detergent-solubilized cells.

Data Presentation and Analysis—Data were obtained in triplicate, averaged for each condition in an experiment, and are presented as the mean ± S.E. of at least three experiments. Paired *t* test was used to determine statistical significance. For concentration-response relationships, the data were fit by nonlinear regression analysis (with variable slope) using Prism by GraphPad (San Diego, CA). EC_{50} and maximal response are reported as the mean ± S.E. of individual experiments.

RESULTS

Cyclic AMP Accumulation in MDCK-D$_1$ Cells

Effect of Cyclooxygenase Inhibition—We measured cAMP accumulation under basal and forskolin-stimulated conditions in MDCK-D_1 cells pretreated for 20 min in the absence or presence of indomethacin (1 μM). As shown in Fig. 1*A*, we found that basal levels of cAMP production were reduced 40% by incubation of the cells with indomethacin, whereas this treatment inhibited forskolin-stimulated (10 μM) response by almost 60%. A different cyclooxygenase inhibitor, aspirin (100 μM), inhibited basal and forskolin-stimulated cAMP accumulation in a similar fashion (data not shown). We analyzed cAMP production under unstimulated and forskolin-stimulated conditions as a measure of the basal activity state of adenylyl cyclase, taking advantage of the ability of forskolin to potentiate G_s activation of this enzyme by enhancing the coupling of G_s to adenylyl cyclase (23, 24). These results indicate that appreciable AA metabolites are generated by MDCK-D_1 cells in the basal state and that these metabolites substantially contribute to both basal and forskolin-stimulated cAMP generation. The latter finding implies that cellular response to forskolin, commonly assumed to measure catalytic activity of adenylyl cyclase (25), can include a substantial contribution of G protein-coupled receptor, in particular prostaglandin receptor, activation.

We examined whether cyclooxygenase-derived AA metabolites play a role in response to agonists that activate $cPLA_2$ in MDCK cells (19, 26, 27). ATP (100 μM), UTP (100 μM), phenylephrine (1 μM), and bradykinin (1 μM) each stimulated cAMP production, and these responses were substantially inhibited by indomethacin (Fig. 1*A*). When these agonists were combined with a low concentration of forskolin (0.1 μM), indomethacin eliminated responses to UTP, phenylephrine, and bradykinin (Fig. 1*A*, *inset*). The remaining levels of cAMP are the small amounts stimulated by forskolin in the absence of synergy from activated G_s (*i.e.* forskolin in the presence of indomethacin). The exception is the response to ATP, which activates a sizable indomethacin insensitive-cAMP response (20). Varying concentrations of phenylephrine and bradykinin were tested for their ability to stimulate cAMP production in either control cells or cells preincubated with indomethacin (1 μM). To control for the possibility that phenylephrine may activate β-adrenergic receptors, the β-blocker propranolol (1 μM) was included in these drug conditions. Phenylephrine stimulated cAMP accumulation with an EC_{50} of 23 nM and a maximal response about 50% over basal levels (Fig. 1*B*). Bradykinin activated cAMP production with similar potency (EC_{50} = 22 nM) but was more efficacious, stimulating almost 3-fold increases in cAMP levels. Indomethacin completely eliminated cAMP responses to both of these agonists, indicating that these effects are attributable to cyclooxygenase-derived AA metabolites. Thus, bradykinin and α_1-adrenergic receptors, as well as $P2Y_2$ receptors, increase cAMP formation in MDCK-D_1 cells in a cyclooxygenase-de-

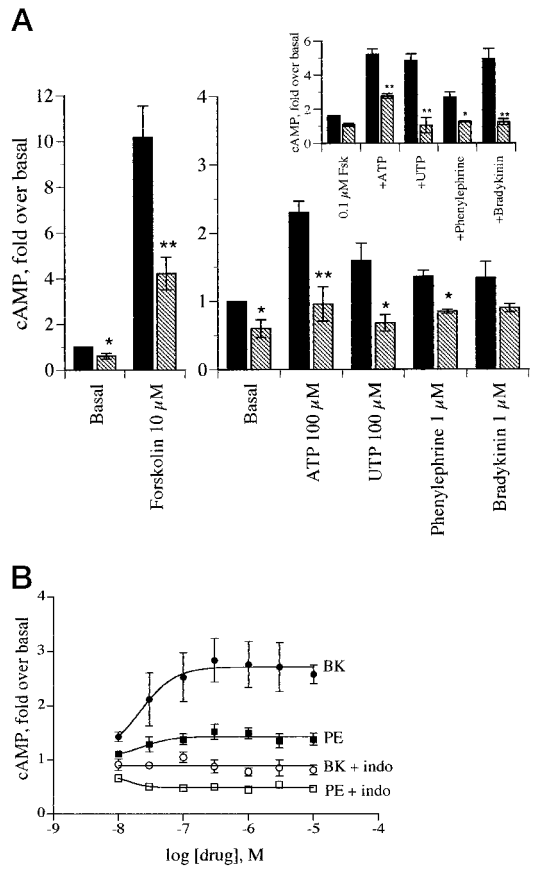

FIG. 1. **Sensitivity of cAMP formation to cyclooxygenase inhibition.** *A*, cAMP accumulation was measured in either control (*solid bars*) and indomethacin-treated (1 μM, *hatched bars*) MDCK-D_1 cells. *Inset*, cAMP responses were measured in the presence of a low, sensitizing concentration of forskolin (0.1 μM). *B*, cAMP accumulation concentration response curves to phenylephrine (*circles*) and bradykinin (*squares*) in control (*closed symbols*) or indomethacin-treated (*open symbols*) MDCK-D_1 cells. The lines represent fit of the data using nonlinear regression analysis. Each point is mean ± S.E. of 3–5 experiments.

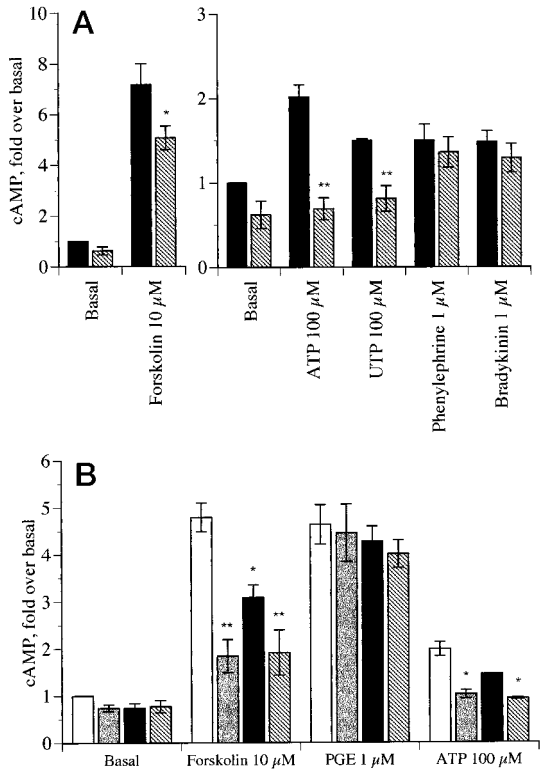

FIG. 2. **Sensitivity of cAMP formation to the hydrolysis of extracellular nucleotides.** *A*, cAMP accumulation was measured in either the absence (*solid bars*) or the presence (*hatched bars*) of 2 units/ml apyrase. *B*, cAMP accumulation was measured in control (*open bars*), indomethacin-treated (1 μM, *shaded bars*), apyrase-treated (2 units/ml, *solid bars*), or both indomethacin- and apyrase-treated (*hatched bars*) MDCK-D_1 cells. Each bar represents the mean ± S.E. of 3–6 experiments. *, $p < 0.05$; **, $p < 0.01$ by paired *t* test as compared with control condition.

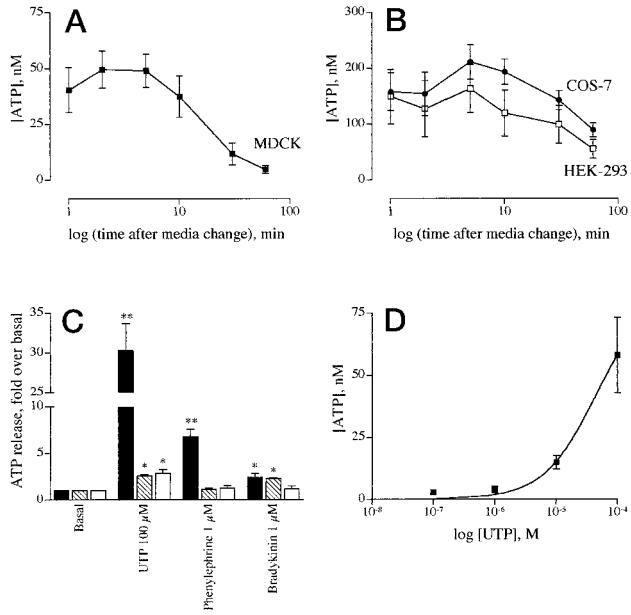

FIG. 3. **ATP release by various cell types in response to mechanical or biochemical perturbation.** *A* and *B*, concentration of ATP in the extracellular medium assayed at various times following a medium change in MDCK-D_1 (*A*) and HEK-293 or COS-7 cells (*B*, HEK-293 (*squares*), COS-7 (*circles*)). *C*, ATP release stimulated by UTP, phenylephrine, and bradykinin expressed as fold over basal in MDCK-D_1 (*solid bars*), HEK-293 (*hatched bars*), and COS-7 cells (*open bars*). *D*, concentration of ATP in the extracellular medium following addition of various concentrations of UTP in MDCK-D_1 cells. Line represents fit of the data using nonlinear regression analysis with a fixed B_{max}. Each point or bar represents the mean ± S.E. of 3–6 experiments. *, $p < 0.05$; **, $p < 0.01$ by paired *t* test as compared with control condition.

pendent manner. Such results define a mechanism, other than activation of particular adenylyl cyclase isoforms directly via Gβγ subunits, Ca^{2+}, or protein kinase C, whereby agonists that couple to G_q can increase cAMP levels.

Effect of Apyrase—We investigated whether extracellular nucleotides contribute to the indomethacin sensitivity of basal, forskolin, and hormonal agonist-stimulated cAMP by assessing cAMP accumulation in cells incubated with apyrase, a nucleotidase that can act extracellularly to dephosphorylate ATP and ADP. Both basal and forskolin-stimulated cAMP accumulation were substantially (38 and 29%, respectively) inhibited by incubation of cells with apyrase (2 units/ml) while, as expected, ATP- and UTP-mediated responses were abolished (Fig. 2*A*). Apyrase did not significantly inhibit cAMP generated in response to phenylephrine or bradykinin, indicating that extracellular nucleotides do not mediate the increases in cAMP levels induced by these $cPLA_2$-activating agonists. Combined treatment with indomethacin and apyrase had no further effect compared with indomethacin alone on basal or forskolin-mediated cAMP accumulation, while completely abolishing cAMP generated by exogenous ATP and having no effect on that stimulated by PGE_2 (Fig. 2*B*). The nonadditivity of inhibition by apyrase and indomethacin indicates that extracellular nucleotides act proximal to cyclooxygenase to increase basal and forskolin-stimulated cAMP accumulation. We also tested whether inhibition of other molecules involved in P2Y receptor signal transduction would reduce basal and forskolin-stimulated cAMP accumulation. The P2Y receptor antagonist suramin (100 μM) and protein kinase C inhibitor calphostin C (0.1 μM) each reduced basal cAMP levels as did indomethacin (data not shown).

ATP Release from MDCK-D_1, COS-7, and HEK-293 Cells

To assess directly whether ATP was released from cells under basal conditions, we measured ATP release into the extracellular medium by using a sensitive luciferin/luciferase assay. A gentle medium change released sizable quantities of ATP from MDCK-D_1 cells, reaching a concentration of 45 ± 12 nM within 2 min. The concentration of extracellular ATP decreased to 2.3 ± 0.5 nM in medium from cells incubated at 37 °C undisturbed for 60 min following medium change, presumably due to ecto-ATPase/nucleotidase activity (Fig. 3*A*). Our assays for cAMP accumulation were determined approximately 5 min following a medium change, a time at which extracellular ATP concentrations were at a peak. Thus, release of ATP in response to a change of media likely accounts for the sensitivity to indomethacin and apyrase of both basal and forskolin-stimulated cAMP accumulation (Figs. 1*A* and 2*A*). In cells incubated undisturbed for 60 min, ATP release increased 3.3 ± 0.5-fold (to 8.6 nM) when the plate was tilted to sample extracellular medium. COS-7 and HEK-293 cells also released large amounts of ATP following a gentle media change, with ATP concentrations remaining as high as 66 nM after 60 min (Fig. 3*B*). These and other data (9, 28) indicate that ATP release occurs in many cells types. We hypothesize that signal transduction in these widely utilized cell models is effected by mechanical stimulation and release of nucleotides.

Release of ATP in MDCK cells was stimulated 30 ± 3-fold over basal by the addition of UTP (100 μM) and 6.8 ± 0.8- and 2.4 ± 0.5-fold by the addition of phenylephrine (1 μM) and bradykinin (1 μM), respectively (Fig. 3*C*). Addition of forskolin (10 μM) inhibited ATP release slightly (26 ± 19%, $p = 0.18$) while PGE_2 (1 μM) had no effect (data not shown). UTP and bradykinin also increased extracellular levels of ATP in COS-7

and HEK-293 cells (Fig. 3*C*), albeit to a lesser extent than in MDCK cells. Extracellular ATP concentrations were stimulated by UTP in a concentration-dependent manner (Fig. 3*D*) and with a potency similar to the activation of PGE_2 production in MDCK cells (20). The effect of UTP in increasing ATP release in these experiments may also represent an ability of UTP to convert ADP to ATP via nucleoside diphosphokinases or to act as a competitive inhibitor for the catalysis of ATP, thereby increasing the amount of ATP detected. Irrespective of the mechanism for the effect of UTP, the increase in ATP levels adds an important complexity to interpretation of studies that assess effects of UTP on signal transduction and cellular responses (10, 29). As UTP-promoted ATP transport has recently been reported (30), we speculate that aspects of cellular responses previously attributed to UTP may be, in part, secondary to increases in extracellular ATP.

Arachidonic Acid Release from MDCK-D_1 Cells: Effect of Apyrase

Because both phenylephrine and bradykinin elicited ATP release, we sought to determine whether ATP (or other nucleotide) release might contribute to basal release of AA or the ability of those agents to enhance AA release (26, 27). Varying concentrations of phenylephrine and bradykinin elicited release of AA and its metabolites in control conditions with EC_{50} values of 0.2 μM and 9.9 nM, respectively, and with maximal responses of 101 and 204% over basal, respectively (values comparable with those observed for each agonist in mediating cAMP responses shown in Fig. 1*B*). Addition of apyrase (2 units/ml) did not significantly alter phenylephrine- or bradykinin-stimulated AA release, indicating that release of extracellular nucleotides does not mediate AA release promoted by α_1-adrenergic or bradykinin receptors (data not shown). By contrast, basal AA release was inhibited 32% by incubation of cells with apyrase, implicating nucleotide release as a critical determinant of basal AA production.

DISCUSSION

The present studies were designed to test the hypothesis that nucleotide release and, in turn, activation of P2 receptors are important for the autocrine/paracrine regulation of signal transduction mechanisms in native cells. The results document that such release plays a major role in determining basal cAMP levels and that release can be promoted by both physical and chemical means, the latter findings consistent with other data (as recently reviewed in Ref. 31). In the case of epithelial cells, such as MDCK cells, release of ATP may be of particular importance both physiologically and pathophysiologically because P2Y receptors can influence ion conductance and volume regulation and may contribute to chloride conductance in cells with mutations of the cystic fibrosis transmembrane conductance regulator (31–33). In addition, extracellular ATP may augment cyst enlargement in autosomal dominant polycystic kidney disease through stimulation of epithelia that line the cyst lumen (34). Despite the potentially key importance of ATP/nucleotide release with respect to cell signaling and function, the precise mechanism for such release, other than by exocytosis at synapses and blood platelets or by cell injury, remains poorly defined (31).

The effects of extracellular ATP, as well as nucleotides such as UTP, are dependent upon several factors. The type of P2 receptors expressed on a cell will determine the nature of the resulting signal. Thus far, six G protein-coupled P2Y receptors (coupled predominantly to the G_q family of heterotrimeric G proteins but also to $G_{i/o}$ and possibly G_s) and seven P2X receptors (ATP-gated ion channels permeable to Ca^{2+}) have been identified (35, 36). Also important is the expression of ecto-

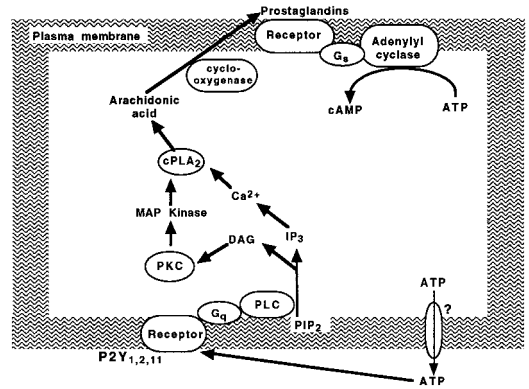

FIG. 4. **Schematic diagram of ATP release and action in MDCK-D_1 cells.** Released ATP can promote cAMP production via P2Y receptors through both calcium- and mitogen-activated protein (*MAP*) kinase-dependent activation of cPLA2, leading to the generation of prostaglandins that activate receptors coupled to the stimulation of adenylyl cyclase (19, 20, 26, 27). These pathways are activated by release of endogenous nucleotides in response to mechanical stimulation. *PKC*, protein kinase C; *PLC*, phospholipase C; *DAG*, diacylglycerol; *PIP_2*, inositol 1,4,5-bisphosphate.

ATPases and other ecto-nucleotidases. Two ecto-ATPase forms have been defined biochemically, one that hydrolyzes ATP to ADP and another that breaks down both triphosphate and diphosphate nucleotides (37, 38). Given that different isoforms of the P2Y receptor display different affinities for ADP and ATP and their analogs, the form of ecto-ATPase/nucleotidase expressed by a given cell likely confers different response profiles. In addition, released ATP can be hydrolyzed to adenosine, which can activate P1 receptors (39).

The concentrations of released ATP that we measured may be underestimated. Recent studies using a membrane-anchored method to detect ATP release in platelets (luciferase was tethered to an ATP-binding cassette protein using an antibody) indicate that ATP concentrations near the cell surface are many times higher than that detected in the bulk phase (40). The rapid action of ecto-nucleotidases on the cell surface, as well as membrane trapping and unstirred layer effects, likely account for differences between assays of ATP in bulk medium and those determined in the local environment of the cell membrane. Our measurements would not detect this local membrane concentration of ATP, as we sampled a small portion of the cell medium for the luciferase assay. Furthermore, membrane trapping and unstirred layer effects might also explain the lower efficacy of apyrase in inhibiting basal and forskolin-stimulated cAMP levels as compared with indomethacin, because apyrase in the bulk phase may not degrade all cellularly released ATP prior to its activation of P2 receptors.

We demonstrate that both mechanical and chemical perturbations can alter the release of ATP, initiate P2Y receptor signaling, and contribute to "basal" levels of second messengers (Fig. 4). Because P2 receptors are widely expressed and ATP release occurs in many cell types (3, 5–9, 28), our results imply that such release is important in establishing the basal state of cell signaling, in particular related to increases in calcium and activation of calcium- and protein kinase C-mediated events and, at least in certain cells as shown here, arachidonic acid release and cAMP formation. Our findings extend to native cells previous results that demonstrate mechanical stimulation can increase phosphoinositide hydrolysis and calcium mobilization in cells that overexpress P2Y receptors (4). Moreover, the ability of indomethacin or apyrase to substantially blunt cAMP generation in response to forskolin is akin to the observed decrease in broken cell adenylyl cyclase activity assays

as compared with cAMP generated in intact cell assays (25). ATP release by intact cells may contribute to such differences in forskolin response.

Considering that mild mechanical stimulation occurs during virtually every experimental protocol with cultured cells as well as *in vivo*, evidence that such stimulation can release ATP and alter second messenger systems implies that release of nucleotides is a critical factor in such systems and events they regulate. These findings have even more profound implications when one considers that released nucleotides may sensitize or desensitize signaling pathways (29). Thus, release of ATP (or other nucleotides (9)) likely provides an important means by which cells regulate responsivity not only to nucleotides themselves but also to agonists that act via other hormone and neurotransmitter receptors.

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