ATP as a Signaling Molecule: the Exocrine Focus

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Why and how do cells release ATP? It is not spilled energy. ATP becomes an extracellular regulator. Various cellular responses are initiated by purinergic receptors and signaling processes and are terminated by breakdown of ATP by ectonucleotidases. In epithelia, ATP regulates salt and water transport; other effects may be longer lasting.

In times of plenty, need, or stress, cells can release ATP and other nucleotides into their surroundings. This ATP can potentially, via specific purinergic receptors, influence the ATP-releasing cells or neighboring cells by the processes of neural transmission, paracrine signaling, and autocrine signaling. The many types of purinergic receptor that stud surfaces of various cells are thought to be involved in a spectrum of physiological and pathophysiological processes, including synaptic transmission, pain and touch perception, vasomotor responses, platelet aggregation, endothelial release of vasorelaxants, immune defense, cell volume regulation, cell proliferation and mitogenesis, apoptosis, and epithelial ion and water transport, just to name a few (Fig. 1).

Whether and how ATP will act on the cell depends on several interrelated and dynamic processes: storage and release of ATP, stimulus for the release, population of the purinergic receptors on cells, breakdown of ATP by ectonucleotidases, cellular signaling pathways, and effector processes within the stimulated cells (Fig. 1). Due to many years of intense research, much is known about the purinergic receptors and ectonucleotidases, but the other components in these complex reactions are less clear and subject to current investigations. After a general overview of major themes depicted in Fig. 1, this review will concentrate on the role of ATP as a signaling molecule in epithelia, especially in exocrine glands. Exocrine glands are one of the first epithelia where ectonucleotidases, and other nucleotides were identified in the study of epithelia is the luminescence detection of photon generation from reactions of luciferin and ATP that is catalyzed by luciferase. Supernatants from cell suspensions have been widely used for the assay, and now methods have been adapted for in situ determination of ATP release directly in cell samples (see Refs. 15 and 17 for references). However, the latter technique is tricky, because one must take into account dilution artifacts, ATP release due to mechanical disturbances.
during the assay, and the exquisite sensitivity of luciferase to ionic composition and inhibitors. The above techniques are very sensitive, and ATP concentrations measured in epithelia in response to mechanical stimulation or a hypotonic shock are in the picomolar-to-nanomolar range, depending on the number of cells. Other new applications of the luciferase method are emerging, and one of them is surface-attached luciferase on the cell membranes that can detect nearby ATP release. Another method utilizes the fluorescent properties of the substrate luciferin (Fig. 2). The biosensor method employs model cells expressing specific fast-responding purinergic receptors, which detect ATP release from other adjacent cells by using a patch-clamp setup. Atomic force microscopy with a myosin sensor-covered probe can potentially demonstrate very focal release of ATP from single transporters/vesicles. The latter methods yield extracellular ATP concentrations in the 10^{-10.220.33.6} to 20-μM range. These higher ATP concentration estimates are probably due to the fact that the detection is shifted close to the site of release and ATP has not yet been hydrolyzed by various ectonucleotidases. In fact, the micromolar ATP concentrations are in the working range of most purinergic receptors.

**Facing ATP: purinergic receptors and ectonucleotidases**

To understand the diversity and complexity of cellular answers to ATP and other nucleotides, it is important to consider types of purinergic receptors and possible signaling pathways in a given cell. It is not unusual to find that one cell is equipped with several receptor types, and for epithelia it is important to consider their localization. P2 receptors, the purinergic receptors preferring nucleoside di- and triphosphates, consist of two large families: P2Y and P2X (14) (Fig. 3). P2Y receptors are seven-transmembrane-spanning proteins coupled to G proteins. The mammalian receptors are P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11, as well as newly cloned P2Y_{12} and P2Y_{13}. Regarding their selectivity, P2Y_{1}, P2Y_{11}, P2Y_{12}, and P2Y_{13} are purinoceptors, P2Y_{2} is a pyrimidinoceptor, and P2Y_{4} receptors have mixed selectivity. All genuine P2Y receptors (except P2Y_{12}) activate phospholipase C (PLC), phosphoinositide hydrolysis, mobilization of intracellular Ca\(^{2+}\), and activation of PKC. P2Y_{11} stimulates PLC and adenylylate cyclase, whereas P2Y_{12} and P2Y_{13} inhibit adenylylate cyclase. Other P2Y receptors can also couple to several distinct G proteins, e.g., P2Y_{1} and P2Y_{2} via G_{i} stimulate PLC, but via G_{i} they inhibit adenylylate cyclase. P2Y_{2} can also stimulate phospholipase D (PLD) and breakdown of phosphatidylcholine. A multitude of receptors and signaling pathways offers a rich possibility for a cross-talk mechanism, including various isoforms of adenylyl cyclases (activated by βγ-subunits released from G proteins), Ca\(^{2+}\)-calmodulin and/or PKC, and adenosine receptors after ATP is split into adenosine by ectonucleotidases and 5’-nucleotidase. In addition, new pathways that may be involved long-term regulation, such as metabolism, cell adhesion, gene expression, and growth, are being described, so far for nonepithelial cells. For example, in astrocytes UTP/ATP can stimulate a mitogen-activated protein kinase (MAPK) cascade, such as extracellular signal-regulated kinase (ERK)1/2, which involves Ca\(^{2+}\)-independent PKC and PLD, and in renal mesangial cells, UTP activates the stress-

**FIGURE 1.** Communicating via ATP: the basic steps.
Excitation
364 nm
Luciferase

Luciferin + MgATP + O₂ → oxyluciferin + PP_i + AMP + CO₂ + light

Emission
540 nm

FIGURE 2. Luciferin fluorescence can be used to monitor ATP release into the medium. A pancreatic acinus surrounded by luciferin (left), a pseudocolor image of the same acinus (right), and the corresponding transmission image (middle) are shown. Methods were derived from Ref. 17.

activated protein kinase (SAPK) via G protein.

The second family of purinergic receptors includes the ionotropic P2X receptors (P2X1, P2X4, and P2X7) that have two transmembrane domains connected by a large extracellular loop and a COOH terminal of variable length (7). These receptors/channels are nonspecific cation channels, usually having greater permeability to Ca²⁺ than Na⁺, and they are modulated and inhibited by divalent cations. Single subunits are related in topology to the epithelial Na⁺ channel, nematode degenerin, and inwardly rectifying K⁺ channels but have little sequence homology with other ion channels. The subunits form multimers (homo- or heteromers), but the stoichiometry of functional receptors/channels is unclear and may be trimeric or tetrameric.

It was earlier believed that P2X receptors were confined to excitable cells, but they are now found to be ubiquitous. One unusual member of this family is the P2X2 receptor, which seems only to form homomers, has a long COOH terminal, and in some cells and expression systems is associated with formation of a large lytic pore. For this reason, this earlier-denoted P2Z receptor that was originally detected in immunoreactive cells (e.g., mast cells, macrophages, lymphocytes) was known as an ATP-permeabilizing or a killer receptor. It is not settled under which conditions this channel-to-pore formation occurs, whether it involves accessory signaling proteins, whether it is a pore-forming ability of only the multimeric receptor, or whether it is an association with a protein porin present in the membrane, usually in a closed state. In the case of a pore formation, ATP then becomes the killing molecule, causing necrosis or apoptosis in cells expressing P2X2, and being exposed to ATP, often at relatively high concentrations, and aggravated by altered ionic composition or pH. However, in its more peaceful edition the P2X2 receptor may be involved in epithelial transport, especially in exocrine glands (see below). Moreover, in some cells this receptor with “many talents” can even mediate proliferation signals, such as in lymphoid cells, and synthesis of chemoattractant protein in monocytes.

The P2X receptor’s variable effects probably depend on its ability to activate several specific signaling pathways. It is already known that some P2X receptors can stimulate PLD and SAPK, modulate ERK1/2 activation, and interact with cytoskeletal proteins (8). However, the P2X signaling pathways are relatively unknown and just now coming into research focus. It is becoming clear that native cells express a multitude of P2Y and P2X receptors, rendering the study of intracellular signaling quite difficult, and our first glimpses of signaling pathways are appearing from studies of P2 receptors in expression systems.

The lifetime of ATP is closely regulated by a number of proteins that have their catalytic site on the outer side of the plasma membrane (19). Extracellular nucleotides can be hydrolyzed by nonspecific enzymes, such as glycosylphosphatidylinositol-anchored ectoalkaline phosphatases and ecto-5’-nucleotidases or ectonucleotidases with more distinct characteristics that are now classified into two families. E-NTPDases (CD39 family) are ecto-nucleoside triphosphohydrolases that hydrolyze nucleoside 5’-triphosphates. Another family of enzymes, E-NPP, are ecto-nucleotide pyrophosphatase/phosphodiesterases with a broad substrate specificity. These can hydrolyze phosphodiester bonds of nucleotides and nucleic acids and pyrophosphatase bonds of nucleotides and nucleotide sugars, e.g., cleavage of ATP to AMP and PPI and conversion of cAMP to AMP. Some of these ectonucleotidases have distinct patterns of distribution in different cell types and are regulated during physiological and pathophysiological processes, probably in association with urine and pyrimidine signaling. The catalytic site of ectonucleotidases faces the extracellular medium, but some isoforms can be cleaved or released in a soluble form, in which case they can be regarded as exonucleotidases. There are also other ectoenzymes that contribute to levels of extracellular nucleotides, such as interconversion of nucleotides; ecto-nucleoside diphosphokinase (ecto-NDPK) and ecto-adenylate cyclase, possibly a production of ATP by F0-F1 ATP synthase, and use of ATP as a phosphate donor for ectoenzyme kinase synthesis.

ATP effects in epithelia

Purinergic agonists are widely investigated as possible regulators in epithelia, most diligently on airway tissues, various glands (see below). ATP may activate 1 or more G proteins. Mammalian P2X receptor monomers are 328 to 370 amino acids long, transverse the membrane 7 times, and activate 1 or more G proteins. Mammalian P2X receptor monomers are 328 to 370 amino acids long, transverse the membrane 7 times, and activate 1 or more G proteins. Mammalian P2X receptor monomers are 328 to 370 amino acids long, transverse the membrane 7 times, and activate 1 or more G proteins. Mammalian P2X receptor monomers are 328 to 370 amino acids long, transverse the membrane 7 times, and activate 1 or more G proteins.
gastrointestinal tissues, and kidney tubuli, often used as primary cultures or epithelial cell lines. In airway epithelia, ATP released onto the mucosal membranes by volume changes and by mechanical and shear stimulus (ciliary beat, surface liquid movement) can act directly on the P2Y2 receptors or after ectonucleotidase breakdown to adenosine on A<sub>1a</sub> receptors (13). In addition, ATP is released or synthesized from ATP and UDP by ecto-NDPK, and it can activate the predominant P2Y<sub>2</sub> receptors, or UTP/UDP can activate P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors, which may be more common in other epithelia. In a normal airway (proximal) epithelium cultured as monolayers, luminal ATP/UTP activation of P2Y<sub>2</sub> receptors leads to increase of cellular Ca<sup>2+</sup> due to opening of Ca<sup>2+</sup>-stores and Ca<sup>2+</sup> influx and subsequent, somewhat transient Cl<sup>-</sup> secretion resulting from activation of Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels (CaCC). In addition, CFTR-CI<sup>-</sup> channels can be stimulated by a Ca<sup>2+</sup>-independent PKC regulation via diacylglycerol or by local PKA activated via A<sub>1a</sub> receptors by adenosine generated on the apical surface. In CF tissue, the CFTR-CI<sup>-</sup> channel function is defective, but ATP/UTP stimulation can bypass this defect and secretion can be restored by activation of a Ca<sup>2+</sup>-dependent Cl<sup>-</sup> conductance. Due to their effects on Cl<sup>-</sup> transport, nucleotides were proposed as therapeutic agents for the treatment of CF (13).

Similar luminal effects of ATP/UTP via P2Y<sub>2</sub> and other P2Y receptors, on Ca<sup>2+</sup>-activated Cl<sup>-</sup> transport were demonstrated in cultured and native bronchi, intestinal cells, sweat gland cells, and medullary collecting ducts. In gallbladder, bile ducts, and pancreatic duct cell lines, UTP/ATP activates electrogenic HCO<sub>3</sub><sup>-</sup> secretion that can be dependent on Cl<sup>-</sup> (involving Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange) or be independent and proceed via Ca<sup>2+</sup>-dependent anion conductance. Notably in pancreatic duct cell lines with an intact CFTR-CI<sup>-</sup> channel, ATP/UTP has negligible effects on HCO<sub>3</sub><sup>-</sup> secretion. On the other hand, CF ducts (CFPAC-1 cells) are more sensitive to purinergic stimulation, and ATP activates CaCC, Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger, and K<sup>+</sup> channel, all leading to HCO<sub>3</sub><sup>-</sup> secretion (20). On the basolateral and luminal membranes of several epithelia, it was demonstrated that ATP/UTP stimulation increased K<sup>+</sup> conductances, often transiently, which could increase the driving force on Cl<sup>-</sup>/anion secretion. However, this would be to no avail if only the basolateral P2Y receptors were stimulated, because they do not lead to opening of CaCC. Thus apical and basolateral P2 receptors, even if they were the same, may not stimulate the same transporters in a polarized epithelium; this will depend on polarization of the signaling pathways. Moreover, net transepithelial transport will depend on coordinated action of ion channels and transporters on both sides of epithelia.

One very important effect of UTP/ATP stimulation is that, after a short activation of Na<sup>+</sup> absorption, there is a significant inhibition of Na<sup>+</sup> absorption in a Ca<sup>2+</sup>-dependent manner, and it may be regulated by a different P2Y receptor (5). This dual control of Cl<sup>-</sup> secretion and Na<sup>+</sup> absorption would, for example in respiratory epithelia, lead to effective hydration of respiratory surfaces and may be of importance to the clinical application of the P2Y receptor agonists in CF. In kidney, where ATP is filtered and released by proximal tubuli, its action on the distal nephron is to increase Cl<sup>-</sup> secretion and decrease Na<sup>+</sup><sup>+</sup> and Ca<sup>2+</sup> absorption, thus inhibiting salt and water reabsorption. In kidney, P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors are thought to be involved, although recent work in this area indicates that renal epithelium derived from polycystic kidneys, and respiratory and gastrointestinal cell lines also express several P2X receptor types (15). Summarizing the wealth of functional data on the above-described epithelia, the common denominator is the prevalence of P2Y<sub>2</sub> receptors, which increase secretion of Cl<sup>-</sup> (and anions), decrease Na<sup>+</sup> absorption, and increase or decrease K<sup>+</sup> conductance.

In exocrine glands, the story is quite different from the above-described epithelia, and much of the interesting work on exocrine glands was in fact pioneering in the P2 receptor field. In 1982 it had already been shown that, in parotid acini, nucleotides increased K<sup>+</sup> conductance and amylase release. During the following 10 years, it was shown that in parotid submandibular, and lacrimal acini ATP activated cation currents (Na<sup>+</sup> and Ca<sup>2+</sup> influx), K<sup>+</sup> currents, and apparently also Cl<sup>-</sup> efflux, which were independent of G proteins and phosphoinositide hydrolysis (16). Similar activation was achieved by ATP<sup>-</sup> and 2',3'-O-(4-benzoylbenzoyl)-ATP, and it became clear that the receptor was the P2Z type. Nevertheless, for a long time these findings were not appreciated, because from work on immunoreactive cells it was believed that such a receptor should have formed cytolytic pores. Clearly this was not the case in most preparations, and conditions and effects of ATP and BzATP are fully reversible. However, in some conditions (low extracellular Ca<sup>2+</sup>) P2X can also form pores in immunoreactive cells, and it seems that activation of PL<sub>A</sub> protective (1). Recently, it was shown that salivary gland acini indeed express functional P2X<sub>2</sub> receptors as well as high-affinity P2X<sub>7</sub> receptors (18).

Acinar cells from salivary and lacrimal glands also have high-affinity but low-capacity P2Y receptors, and several studies point out that the P2Y receptor expression and associated signal transduction pathways are not static but dynamic. For example, the expression of P2Y<sub>2</sub> is dramatically upregulated upon culture of salivary gland cells or ligation of the main duct. Similar upregulation of P2Y<sub>2</sub> receptors is also seen in cultured intestinal and respiratory epithelia. In contrast, the activity and expression of P2Y<sub>1</sub> receptors decreases during the gland development. Thus in the native gland epithelia, it is the P2X receptors (X<sub>2</sub> and X<sub>7</sub>) that may be predominant in a “normal function.”

What might be the function of ATP and P2X receptors in salivary glands? Apart from allowing Ca<sup>2+</sup> and Na<sup>+</sup> influx and stimulation of K<sup>+</sup> channels, it has been shown that they can stimulate the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) and Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup>...
cotransporter, cause volume changes, and stimulate amylase release. Therefore, on their own some P2 receptors could be involved in genuine secretory events in the glands. At this stage, however, studies elucidating where in salivary glands ATP might be released are missing. Masticatory muscles surround salivary glands, and myoepithelial cells surround secretory endpieces; thus one would predict that mechanical stress would lead to ATP release and autocrine/paracrine stimulation that could enforce secretion. In addition, since the parasympathetic or sympathetic nerve terminals might be the source of ATP, ATP is possibly a modulatory cotransmitter, and interaction between Ca\(^{2+}\) and inositol 1,4,5-trisphosphate signaling are already described. Interestingly, in acini of submandibular glands the number of P2X\(_4\) receptors increases about threefold in parasympathetically denervated glands, i.e., development of supersensitivity (18). In addition, it is postulated that the dramatic effects of P2 receptors on cellular ion composition, or possibly MAPK activation, could have other long-lasting effects.

The above-described data indicate that P2 receptors in acini should be positioned on the basolateral membrane. We do not know whether any of the P2 receptors are located on the luminal membrane, which comprises only a fraction of the cell surface membrane and has not been studied in native cells. Nevertheless, there is some information regarding P2 receptor distribution in excretory ducts of the salivary gland. Submandibular gland ducts have similar receptors to those in acini, e.g., P2Y\(_2\), P2X\(_4\), and P2X\(_7\) receptors. Studies on perfused main duct indicate that the P2X\(_7\)-type receptors are luminal, whereas P2Y\(_2\) are basolateral (9). Possibly, the duct distension and hypotonic saliva could be the eliciting factor for ATP release. The physiological response of ducts is not clear and might depend on the duct generation. In the smaller granular ducts, ATP causes activation of PLA\(_2\), and kallikrein release. In larger reabsorptive ducts, P2Y and P2X receptor activation was reported to stimulate Cl\(^{-}\) currents and NHE activity, both potentially contributing to reabsorption of NaCl from luminal fluid.

**ATP: a paracrine regulator in pancreas**

In pancreas, the main function of the excurrent ducts is secretion of NaHCO\(_3\)-rich fluid, and there are a number of P2 receptors that could regulate this process. In a recent study on rat pancreatic acini, it was considered whether acini could be a source of releasable ATP that could then regulate pancreatic ducts (17). As in other epithelia, mechanical stimuli (and to a smaller extent hypotonic shock) induced ATP release in the acinar cell suspension. Most importantly, cholinergic stimulation released ATP. By using confocal laser-scanning microscopy, ATP release in single acini was visualized by using luciferin fluorescence in a conventional luciferin/luciferase assay (Fig. 2). This method showed that near the pancreatic acini ATP rises up to 10 \(\mu\)mol/l following cholinergic stimulation. It appears that some ATP can be released into the acinar lumen, and fluorescent markers for ATP further show that indeed ATP is stored in secretory granules (Fig. 4). Thus these studies support the theory of a vesicular pathway of ATP release raised above. Once released, ATP would have the opportunity to act on pancreatic acini. Compared with other exocrine acini, P2 receptors on pancreatic acini were not extensively studied. With RT-PCR it was shown that, collectively, pancreatic acini contain transcripts for P2Y\(_2\), P2Y\(_4\), P2X\(_2\), and P2X\(_7\) receptors but notably not for the P2X\(_7\) receptor. Monitoring intracellular Ca\(^{2+}\) signals and an organic anion uptake, it became apparent that <20% of pancreatic acinar cells expressed functional P2 receptors (12). A similar low percentage of functional receptors (P2X\(_4\)) was also observed in salivary gland acini (18). It is possible that the number of functional receptors depends on yet-unknown functional states of the gland. At this stage, it is tempting to postulate that by having a low number of functional P2 receptors, and the notable absence of the P2X\(_7\) receptor, acini might avoid autocrine stimulation and possible activation of digestive enzymes that would be catastrophic for pancreas.

In contrast to acini, the native ducts from rat pancreas show several types of functional P2 receptors, including P2Y\(_2\), P2Y\(_4\), P2X\(_2\), and P2X\(_4\).
P2X₄ and P2X₂ receptors. The small intralobular ducts are richest in carbonic anhydrase and CFTR and have all of the components of a HCO₃⁻-secreting epithelium (Fig. 4). The prime driving force in initiation of secretion is opening of CFTR-CI⁻ channels on the luminal membrane and opening of basolateral K⁺ channels to maintain the driving force (11). UTP and ATP, presumably by stimulating basolateral P2Y₂ and P2Y₄ receptors, cause a release of Ca²⁺ and Ca²⁺ influx, and notably the final event is the closure of K⁺ channels (3). Clearly, such an event would not initiate secretion; on the contrary, it would inhibit secretion. In fact, in guinea pig pancreatic ducts, basolateral ATP inhibits secretin-evoked secretion (6). The source of interstitial ATP might be from acini by back-leak or basolateral transporter and in addition from nerve endings and islet cells. Stimulation of presumably luminal P2X receptors leads to Ca²⁺ and Na⁺ influx (3). There is no increase in the Cl⁻ current, despite that fact that ducts do posses Ca²⁺-activated Cl⁻ channels. Thus P2X receptors would not initiate secretory processes, but they could upregulate secretion already initiated by secretin. It is not known whether this involves increased intracellular Na⁺ and/or Ca²⁺ or stimulation of other signaling pathways. Thus in small native ducts ATP would not be an initiator but rather a modulator of secretion. There are likely differences among generation of ducts regarding P2 receptor population and functional states of ducts. Notably, in pancreatic cell lines that are derived from short-term cultures, luminal P2Y2 expression is the most dominant, and here the effects are similar to those found in respiratory and gastrointestinal epithelia. In bile ducts that also secrete HCO₃⁻ by similar ion transporters in response to secretin, it seems that P2Y₂ receptors are luminal and ATP that originates from hepatocytes enhances secretion. The basolateral application of ATP decreases secretion, as it does in pancreatic ducts.

In pancreas, ATP might be the coordinating signal between acini and ducts (Fig. 4). Acini secrete digestive enzymes and ATP in response to acetylcholine. Given that divalent cations are low in normal pancreatic juice, there might be sufficient ATP in the lumen of at least the initial ducts, and ATP would upregulate secretion already elicited by secretin. ATP interplay between acini and ducts explains the long-standing observation that cholinergic stimulation of acini potentiates the duct secretion evoked by secretin. Should there be sufficient ATP reaching the distal ducts that possibly express luminal P2Y2 receptors, CaCC and other components of the transport pathways could add to or modify secretion arriving from the acini and upstream ducts. Lastly, although ectonucleotidases, such as CD39, are expressed in pancreas, it remains to be shown how they contribute to ATP signaling within.

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