ATP-dependent Activation of $K_{Ca}$ and ROMK-type $K_{ATP}$ Channels in Human Submandibular Gland Ductal Cells*  

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$[Ca^{2+}]_i$, and membrane current were measured in human submandibular gland ductal (HSG) cells to determine the regulation of salivary cell function by ATP. 1–10 μM ATP activated internal Ca$^{2+}$ release, outward Ca$^{2+}$-dependent K$^+$ channel ($K_{Ca}$), and inward store-operated Ca$^{2+}$ current ($I_{SOCC}$). The subsequent addition of 100 μM ATP activated an inwardly rectifying K$^+$ current, without increasing $[Ca^{2+}]_i$. The K$^+$ current was also stimulated by ATP in cells treated with thapsigargin in a Ca$^{2+}$-free medium and was blocked by glibenclamide and tolbutamide, but not by charybdotoxin. This suggests the involvement of a Ca$^{2+}$-independent, sulfonyleurea-sensitive K$^+$ channel ($K_{ATP}$). UTP mimicked the low [ATP] effects, while benzoyl-ATP activated internal Ca$^{2+}$ release, a Ca$^{2+}$ influx pathway, and $K_{Ca}$. Thus, ATP acts via $P_{2U}$ ($P_{2Y2}$) and $P_{2Z}$ ($P_{2X7}$) receptors to increase $[Ca^{2+}]_i$ and activate $K_{Ca}$ but not $K_{ATP}$. Importantly, (i) ROMK1 and the cystic fibrosis transmembrane regulator protein (but not SUR1, SUR2A, or SUR2B) and (ii) cAMP-stimulated Cl$^-$ and K$^+$ currents were detected in HSG cells. These data demonstrate for the first time that a ROMK-type $K_{ATP}$ channel is present in salivary gland ductal cells that is regulated by extracellular ATP and possibly by the cystic fibrosis transmembrane regulator. This reveals a potentially novel mechanism for K$^+$ secretion in these cells.

Receptors for ATP are widely expressed in mammalian tissues and are reportedly involved in regulating a variety of cellular functions (1). Pharmacological studies with various ATP analogues have demonstrated two major groups of purinergic (P2) receptors: ionotropic receptors (P2X), associated with ligand-gated nonselective cation channel activity, and metabotropic receptors (P2Y), associated with activation of G-proteins (2–5). In nonexcitable cells, extracellular ATP induces an elevation of cytosolic $[Ca^{2+}]_i$ ($[Ca^{2+}]_c$) by two distinct mechanisms, either by activation of Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores or via activation of Ca$^{2+}$ influx from the external medium (5, 6). The P2Y subtype is coupled via $G_{αq/11}$ to the activation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase C. Thus, activation of these receptors by ATP leads to an increase in $[Ca^{2+}]_i$, due to increase in inositol 1,4,5-trisphosphate (IP$_3$) and IP$_3$-mediated internal Ca$^{2+}$ release.

Activation of the P2X subtype has been shown to be coupled to the activation of a nonspecific cation channel(s), resulting in increased permeability of the plasma membrane to Na$^+$, K$^+$, and Ca$^{2+}$.  

P2 receptors have been found in many epithelial cells, including those from various salivary glands. Four P2 receptors have been identified in salivary gland cells: $P_{2Y1}$, $P_{2Y2}$ ($P_{2U}$), $P_{2X4}$, and $P_{2X7}$ ($P_{2Z}$) (6). In rat parotid acinar cells, it has been reported that ATP primarily activates a Ca$^{2+}$ and Na$^+$-permeable cation channel via stimulation of $P_{2Z}$ receptors. While transcripts for P2Y receptors have been detected in rat parotid acinar cells, ATP does not appear to strongly induce IP$_3$ generation or internal Ca$^{2+}$ release (6–9). However, in rat submandibular ductal cells, evidence for both $P_{2U}$ and $P_{2Z}$ receptors have been presented (6, 10–13). Interestingly, these P2-purinergic receptors have distinct cellular localizations in these cells; $P_{2Z}$ receptors were found in the luminal membrane, while $P_{2U}$ receptors were detected in basolateral membrane (10–13). Presently, the exact mechanisms and ion channels involved in P2 receptor-mediated regulation of salivary gland function are poorly understood (6). The human submandibular ductal cell line (HSG) has been widely used to study the mechanism(s) of Ca$^{2+}$ signaling in salivary gland cells (6, 14–18). Previous studies with HSG cells have demonstrated that activation of the muscarinic receptor leads to the generation of IP$_3$, which causes intracellular Ca$^{2+}$ release and activation of store-operated Ca$^{2+}$ influx (15–18). HSG cells have also been reported to have P2 receptors (6, 14). ATP, the $P_{2U}$ receptor, was shown to increase $[Ca^{2+}]_i$, and Ca$^{2+}$-activated $^{86}$Rb$^+$ influx (14). It was also reported that in HSG cells ATP-stimulated IP$_3$ formation is coupled to the $P_{2U}$ activation, while ATP-stimulated $^{45}$Ca$^{2+}$ influx is mediated via activation of P2X receptors (19). However, the types of ion channels involved in these ATP-induced ion fluxes have not yet been identified.  

This study examines the mechanisms involved in the regulation of salivary gland cell function by ATP, by measuring $[Ca^{2+}]_i$, and the membrane conductance of HSG cells. The data show that ATP acts via multiple P$_2$ purinergic receptors, including $P_{2U}$ ($P_{2Y2}$) and $P_{2Z}$ ($P_{2X7}$), to activate the Ca$^{2+}$-activated K$^+$ channel ($K_{Ca}$), the store-operated Ca$^{2+}$ influx channel, and probably a Ca$^{2+}$-permeable cation channel. Importantly, we report here that ATP also activates an inwardly rectifying, Ca$^{2+}$-independent K$^+$ current. The inhibition of this current by sulfonyleurea compounds and the presence of ROMK1 and cystic fibrosis transmembrane regulator (CFTR) proteins in HSG cells, suggests for the first time that a ROMK-type epithelial K$_{ATP}$ channel is present in salivary gland epithelial cells. As has been suggested for kidney epithelial-
lial cells (20, 21), the putative ROMK channel in HSG cells might also be regulated by a CFTR-dependent mechanism. These data reveal a potentially novel mechanism for the regulation of K+ secretion in salivary epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**HSG cells were a kind gift from Dr. Mitsunabo Sato (Tokushima University, Japan). The conditions for cell culture were similar to those described previously (16, 17). Briefly, cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Biofluids, Rockville, MD) under 5% CO2 at 37 °C. Cells were passaged when confluent by detaching from the tissue culture dish with 0.25% trypsin, 1.0 mM EDTA (Biofluids). A single-cell suspension was resuspended on coverslips and used after about 24 h.

**Patch Clamp Measurements—**Patch clamp in a whole cell configuration was performed on single HSG cells attached to coverslips using the standard patch-clamp technique described previously (16, 17). The resistance of the pipette was about 3–6 MΩ. The chamber was connected with an Ag-AgCl pellet through a 150 mM NaCl-containing agar bridge. Membrane currents were measured with an Axopatch 200A amplifier in conjunction with pClamp 6.1 software and a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA). The currents were filtered (low-pass beta filter) and sampled at an interval of 10 ms. In the step protocol, the cell was held at 0 mV for 312 ms, 1200 A/D converter (Axon Instruments, Foster City, CA). The currents were digitized and recorded directly onto the hard drive of a Dell Pentium computer. The I-V relationship was calculated using the peak amplitude of the current during the step protocol and exported to Origin 5 (Microcal Software, Inc., Northampton, MA). The currents were digitized and recorded directly onto the hard drive of a Dell Pentium computer. The I-V relationship was calculated using the peak amplitude of the current during the step protocol and exported to Origin 5 (Microcal Software, Inc., Northampton, MA) for further analysis.

A piece of coverslip (0.5 × 0.5 mm) with cells was placed in the perfusion chamber (Warner Instrument Corporation, Hamden, Connecticut). For measuring K+, the standard extracellular solution contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM CaCl2, 10 mM glucose, 0.2 mM EGTA, and 10 mM HEPEs, pH 7.4. The pipette was filled with 150 mM KCl, 2 mM MgCl2, 1 mM ATP, 10 mM HEPEs, pH 7.2. For measuring I<sub>Na</sub>, the extracellular solution contained 135 mM sodium glutamate, 1 mM MgCl2, 10 mM CaCl2, 10 mM glucose, and 10 mM HEPEs, pH 7.4 (NaOH). The pipette was filled with 135 mM NMDG-glutamate, 10 mM CsCl, 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 1 mM MgCl2, 1 mM ATP, 10 mM HEPEs, pH 7.2. The cell was held at 0 mV for 312 ms and stepped from −120 to −80 mV in 20-mV steps for 200 ms each step. The leakage subtraction function was used to minimize the leak currents. The currents were digitized and recorded directly onto the hard drive of a Dell Pentium computer. The I-V relationship was calculated using the peak amplitude of the current during the step protocol and exported to Origin 5 (Microcal Software, Inc., Northampton, MA) for further analysis.

**RESULTS**

**ATP-induced Increase in [Ca<sup>2+</sup>]i, and Membrane Conductance in HSG Cells—**ATP, at concentrations ranging from 0.1 to 100 µM, was added sequentially to the cell as shown in Fig. 1. An outward current was detected in 95% (43/48) of the HSG cells tested at a holding potential of 0 mV in a whole cell patch clamp mode. Typically, the cells showed a response, i.e. an increase in the outward current, at 1 µM ATP with a few cells responding at 0.1 or 10 µM. A second response was seen at 100 µM ATP. However, a subsequent addition of 1 mM or higher ATP failed to induce any further increase in the membrane current. The current was blocked by replacing intracellular K+ with Cs+ (data not shown), strongly indicating that it was due to an increase in K+ ion conductance. Further, this pattern of response was similar in the presence or absence of external Ca<sup>2+</sup> (Fig. 1, compare A and B). However, in the presence of external Ca<sup>2+</sup>, the outward current seen at 1 µM ATP was more sustained, presumably due to the involvement of a Ca<sup>2+</sup>-influx component in this response. Most cells showed a quick rundown of the membrane conductance with a duration of about 2–3 min at any given ATP concentration. This is consistent with a previous report showing that ATP induced transient 45Ca<sup>2+</sup>-influx and IP3 production (19) in HSG cells. Measurement of [Ca<sup>2+</sup>]<sub>i</sub>, (expressed as the 340/380-nm fluorescence ratio) demonstrated that an increase in [Ca<sup>2+</sup>]<sub>i</sub>, was induced upon the addition of ATP in 94% of HSG cells (254 of 270). Most cells (64%, 212 of 254) showed a response at 1 µM ATP, with a few cells responding at 0.1% (9%, 24 of 254) or 10 µM ATP (7%, 18 of 254). Importantly, while a response to lower [ATP] was detected, a higher [ATP] added subsequently to the same cell failed to evoke a second response. A typical dose response in the presence or absence of external Ca<sup>2+</sup> is shown in Fig. 1C. In the absence of external Ca<sup>2+</sup>, the initial peak increase in [Ca<sup>2+</sup>]<sub>i</sub>, was not changed, although the response was more transient in nature. This suggests that both intracellular Ca<sup>2+</sup>-release and Ca<sup>2+</sup>-influx account for the ATP-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, Further, this pattern of [Ca<sup>2+</sup>]<sub>i</sub>, increase was not changed when Na<sup>+</sup> was removed from the external medium (n = 35, data not shown). However, when Mg<sup>2+</sup> was removed from the medium as shown in Fig. 1D, a small increase in [Ca<sup>2+</sup>]<sub>i</sub>, was induced with 100 µM ATP in a Ca<sup>2+</sup>-containing medium. These data suggest that, at 100 µM ATP induces a Ca<sup>2+</sup>- influx component that is inhibited by external Mg<sup>2+</sup>. This is also consistent with studies in pancreatic duct cells (20) and other salivary gland cells (6), showing that removal of external Mg<sup>2+</sup> increased the ATP-induced peak [Ca<sup>2+</sup>]<sub>i</sub>. However, since the K+ current measurements (shown in Figs. 1A and B) were made in the presence of external Mg<sup>2+</sup>, the K+ current-induced at 100 µM ATP is probably not associated with Ca<sup>2+</sup>-influx.

UTP, a potent agonist of several P2Y receptors, stimulates IP3 generation and release of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> stores (1–4). Unlike with ATP, sequential additions of increasing [UTP] induced an increase in [Ca<sup>2+</sup>]<sub>i</sub>, and outward current.
only at 1 μM (Fig. 2, A and B). This pattern was not changed in the absence of external Mg2⁺ or with 10 mM external Mg2⁺ (n = 43, data not shown). However, prior stimulation of cells with ATP attenuated the [Ca2⁺]i increase induced by a subsequent addition of ATP to the same cell, and vice versa (see Fig. 2, C and D). As seen with UTP, only low [2MeS-ATP] induced a response. However, the response was lower than that induced by ATP or UTP (data not shown).

We have recently reported that depletion of intracellular Ca²⁺ stores in HSG cells by muscarinic receptor stimulation with CCh, introduction of IP6 into the cell, or treatment with the Ca²⁺ pump inhibitor Tg, activated I_{SOC}, which was dependent on external [Ca²⁺] and inhibited by La³⁺ and Gd³⁺ (17). The data in Fig. 3 show that ATP (1 μM) also induced an inward Ca²⁺ current in HSG cells. This inward current was dependent on external [Ca²⁺] and was eliminated by changing the external [Ca²⁺] from 10 to 0 mM (Fig. 3A). ATP-induced I_{SOC} had a larger amplitude (25 ± 7 pA), but a shorter duration (3–4 min), compared with that induced by IP₆ or CCh (17) and was inhibited by La³⁺ (Fig. 3B). Notably, ATP (1 μM)-induced inward Ca²⁺ and outward K⁺ currents displayed similar characteristics i.e. dependence on external Ca²⁺ and inhibition by La³⁺ (Fig. 3, compare A and B with C and D). Further, stimulation of cells with ATP in a Ca²⁺-free medium inhibited the response to a subsequent addition of CCh (data not shown). This is the first report of ATP-induced I_{SOC} in a salivary cell. Taken together,
Fig. 3. Inhibition of ATP-activated I_{SOC} and outward K^{+} current by La^{3+} in HSG cells. Store-operated inward current (I_{soc}, A and B) stimulated by 10 μM ATP (perfused during the entire experiment) was measured by whole cell configuration at 0 mV holding potential as described under “Experimental Procedures.” A, [Ca^{2+}] in the external medium was changed from 10 to 0 mM, shown by the corresponding bar. B, cells were exposed to external medium containing 10 mM Ca^{2+} and 1 mM La^{3+}, shown by the corresponding bar. These are the representative traces from five (A) or four (B) cells, respectively. K^{+} currents (C and D) were measured as described for Fig. 1 in the presence of 10 μM ATP in the perfusion medium. C, [Ca^{2+}] in the perfusion medium was changed to 0 Ca^{2+} for the period shown by the corresponding bar; D, 1 mM LaCl_{3} was included in the external solution where indicated.

these data demonstrate that in HSG cells low [ATP] (<10 μM) stimulates P_{2U} (P2Y_{2}) receptors to cause the release of Ca^{2+} from internal Ca^{2+} stores, which results in the activation of K_{Ca} and I_{SOC}.

ATP-induced Ca^{2+}-independent K^{+} Current—Fig. 1 shows that high [ATP] activates a K^{+} current in HSG cells that is not accompanied by any changes in [Ca^{2+}]. To determine the nature of this K^{+} current, we examined the effect of depletion of the intracellular Ca^{2+} store with Tg. [Ca^{2+}], was monitored in the absence of external Ca^{2+}, and data are shown in Fig. 4A. Tg induced an increase in [Ca^{2+}], due to Ca^{2+} “leakage” from the internal stores. The subsequent addition of ATP (10–100 μM) to the cells failed to induce any further increase in [Ca^{2+}], (Fig. 4A). This suggests that the [Ca^{2+}]), increase induced by ATP is due to Ca^{2+} release from Tg-sensitive stores. Similarly Tg-induced increases in [Ca^{2+}], or the K_{Ca} current were diminished or greatly reduced after the cell was first exposed to ATP (n = 56 or n = 4 for [Ca^{2+}], and the K_{Ca} currents, respectively; data not shown). In contrast to [Ca^{2+}], increases shown in Fig. 4A, 100 μM ATP induced an increase in the outward current in Tg-treated cells (Fig. 4B). When external Ca^{2+} was reintroduced, a further increase in the outward current was seen, probably due to the activation of K_{Ca} as a result of Ca^{2+} entry. These results clearly indicate that two kinds of outward K^{+} currents are stimulated by ATP in HSG cells. One is associated with [Ca^{2+}], increase, and the other appears to be independent of [Ca^{2+}], increase. Consistent with the [Ca^{2+}], measurements shown in Fig. 4A, Tg induced a transient activation of the K^{+} current. In aggregate, the data presented above indicate that in HSG cells ATP (100 μM) activates a K^{+} current that is not (i) due to activation of K_{Ca} or (ii) accompanied by increases in [Ca^{2+}],.

To further characterize the apparent Ca^{2+}-independent K^{+} current, the effect of charybdotoxin (ChTx) was examined. We reported earlier that ChTx, a large conductance Ca^{2+}-dependent K^{+} channel antagonist, inhibits the carbachol-stimulated K_{Ca} current in HSG cells (16). As seen in Fig. 5A, 25 nM ChTx also effectively blocked the K^{+} current induced by 1 and 10 μM ATP but not that induced by 100 μM ATP (Fig. 5A). The I-V curve of the outward current induced by 100 μM ATP in ChTx-treated cells shows a weak inward rectification with a reversal potential of −75 mV, which is close to the K^{+} reversal potential (Fig. 5B). Furthermore, replacing intracellular K^{+} ion with Cs^{+} eliminated ATP-induced outward currents at all concentrations tested (from 1 μM to 1 mM, n = 5, data not shown). These data suggest that high [ATP] activates an inwardly rectifying, Ca^{2+}-independent, K^{+} current in HSG cells.

Bz-ATP-induced Ca^{2+} Influx and K^{+} Currents—In addition to the P_{2U} receptor, a P_{2Z} subtype of P_{2} receptor (P2X_{7}) has also been found in the salivary gland cells (6). To determine whether the Ca^{2+}-independent ChTx-insensitive K^{+} channel was stimulated by the activation of a P_{2Z} receptor, we examined the effects of Bz-ATP, a potent agonist of the P_{2Z} receptor, on [Ca^{2+}], and whole cell current. The data are shown in Fig. 6. Fig. 6, A and B, shows whole cell currents following the sequential additions of 1, 10, 100, and 1000 μM Bz-ATP to HSG cells in Ca^{2+}-containing and Ca^{2+}-free medium, respectively. In the presence of external Ca^{2+} (Fig. 6A), Bz-ATP activated a K^{+}
current at all concentrations tested, with the largest response, a somewhat sustained increase, obtained at 100 μM. No significant response was seen at 1 mM Bz-ATP. When cells were stimulated in the absence of external Ca2+ (Fig. 6B), the response at 1 μM Bz-ATP was relatively more transient, while that at 10 μM was greatly attenuated, and that at 100 μM was barely detectable. Thus, Bz-ATP, at higher concentrations, appeared to stimulate K+ currents only in the presence of external Ca2+. Similar effects of Bz-ATP were seen on [Ca2+]i (Fig. 6, C and D). P2Z-associated cation channels in salivary gland cells are reportedly permeable to external Na+ and inhibited by external Mg2+ (6). However, the Bz-ATP-induced [Ca2+]i increase did not appear to be affected by replacing Na+ in the external medium with NMDG (Fig. 7A). Further, as shown in Fig. 7B, external Mg2+ attenuated the [Ca2+]i increases induced at all concentrations of Bz-ATP but especially that at 100 μM. In the presence of 3 mM external [Mg2+]i, attenuated [Ca2+]i increases were observed in 35% of cells tested (25 of 65), while [Ca2+]i increases were not detected in 65% of the cells. Furthermore, 10 mM Mg2+ totally eliminated Bz-ATP (1 μM to 1 mM) induced responses (data not shown, n = 35). In aggregate, these data demonstrate the presence of a P2Z (P2X7) receptor in HSG cells, which can be activated by Bz-ATP to stimulate Ca2+ influx, via an as yet unidentified ion channel. Low Bz-ATP also stimulates internal Ca2+ release, probably via IP3 generation or by an as yet unidentified mechanism (6, 19). The resulting increase in [Ca2+]i activates KCa. However, the data are not consistent with the involvement of the P2Z receptor in the activation of the ChTx-insensitive, Ca2+-independent, inwardly rectifying K+ channel by high [ATP].

Effects of Sulfonylurea Compounds on the ATP-induced Ca2+-independent K+ Current—To further characterize the ChTx-insensitive K+ current activated by higher [ATP], we examined the effects of the sulfonylurea compounds, which have been reported to block a family of inwardly rectifying K+ channels, Kir (20, 21, 25). Fig. 8 shows that glibenclamide (10 μM) and tolbutamide (1 mM) inhibited the outward current induced by 100 μM ATP. Importantly, the K+ current stimulated by 1 μM ATP was not significantly affected by these compounds. Further, when ATP was substituted by Bz-ATP, 2MeS-ATP, UTP, or ADP no currents were detected in charybdotoxin-treated HSG cells (data not shown).

These data suggest that the ATP-activated ChTx-insensitive K+ current might belong to the KATP family of Kir channels. However, since the pipette solution in these experiments contained 1 mM ATP, the KATP channel in HSG cells appears to have a lower sensitivity for inhibition by intracellular ATP. Consistent with this, increasing internal [ATP] to 5 or 10 mM (in the presence of 1 mM Mg2+) significantly reduced the K+ current (data not shown).

Presence of CFTR and ROMK1 Proteins in HSG Cells—It has been suggested that the sulfonylurea sensitivity of KATP channels might be conferred via interaction with sulfonylurea-binding proteins such as SUR or CFTR protein (20, 21, 25). The
membrane fraction of HSG cells (Fig. 9). CFTR antibody. A strong reactivity was detected in the plasma
ductal cell line, by Western blotting using an anti-human
presence of CFTR in HSG cells, a human submandibular gland
of salivary gland ductal cells (26–28). Thus, we examined the
ationally and by immunofluorescence, in the luminal membrane
of the CFTR protein was previously demonstrated, both func-
tional and with a protein of with an approximate molecular mass of 180
kilobase RT-PCR product was strongly detected (lane 1), compared with the crude membrane fraction (lane 3).
These data demonstrate for the first time the presence of the
ROMK1 protein in salivary gland cells.

To confirm the presence of ROMK in HSG cells, RT-PCR was
performed using ROMK-specific primers and HSG cell mRNA
(Fig. 9C; see “Experimental Procedures” for details). A ~1.2-
kilobase RT-PCR product was strongly detected (lane 1). Fur-
ther, when RT-PCR was performed using SUR1-, SUR2A-, or
SUR2B-specific primers, SUR-homologous sequences were not
detected in HSG cell mRNA (lanes 2, 4, and 6). However, as
reported earlier (31), SUR1 (lane 3) and SUR2B (lane 7), but
not SUR2A (lane 5), were detected in control human pancreatic
mRNA.

One of the suggested criteria for the possible functional as-
association of the classical K\textsubscript{ATP} channel (Kir 6.2) in pancreatic
\(\beta\) cells with SUR has been well established. This K\textsuperscript{+} channel
demonstrates high sensitivity for intracellular ATP and gibl-
enclamilide (25). In contrast, the ROMK family of weakly in-
wardly rectifying epithelial K\textsuperscript{+} channels have been reported to
have a lower sensitivity for inhibition by intracellular ATP.
These K\textsuperscript{+} channels also have a relatively lower sensitivity to
glibenclamide (20, 21). The K\textsubscript{ATP} channels in kidney epithelial
cells (Kir 1.1a and 1.1b, ROMK1 and ROMK2, respectively) have been suggested to be regulated via interaction with the
CFTR protein, although presently there are no data that con-
clusively demonstrate this molecular interaction. The presence
of the CFTR protein was previously demonstrated, both func-
tional and by immunofluorescence, in the luminal membrane
of salivary gland ductal cells (26–28). Thus, we examined the
presence of CFTR in HSG cells, a human submandibular gland
ductal cell line, by Western blotting using an anti-human
CFTR antibody. A strong reactivity was detected in the plasma
membrane fraction of HSG cells (Fig. 9A, lane 2) associated with a protein of with an approximate molecular mass of 180
kDa, which co-migrated with the control CFTR protein (see
lane 1, plasma membrane preparation of HEK cells infected
with an adenovirus encoding the CFTR). Additionally, consistent with the reported intracellular localization of the CFTR
protein (29, 30), more reactivity was detected in a crude mem-
brane fraction (lane 3), which probably also includes intracel-
lar organelles. These data suggest that HSG cells, similar to
rat submandibular gland ductal cells and kidney cells, have the
CFTR protein.

Further, an antibody against the ROMK protein was used to
examine the presence of this protein in HSG cells. The data are
shown in Fig. 9B. Three prominent bands were detected in the
plasma membrane fraction of HSG cells with estimated molecu-
lar masses of about 49, 47, and 42 kDa. We have not yet
identified the higher molecular weight proteins that react with
the antibody, which supposedly recognizes all isoforms of
ROMK. However, the 42-kDa band comigrated with the control
ROMK1 protein (lane 3, ECL reaction; lane 4, Amido Black
staining of the region of the blot shown in lane 3). Importantly,
this protein was enriched in the plasma membrane fraction
(lane 2) compared with the crude membrane fraction (lane 1).
These data demonstrate for the first time the presence of the
ROMK1 protein in salivary gland cells.

The current was of smaller magnitude, and relatively more sus-
duited to a lower level. It was blocked by glibenclamide (data not shown), and further, when RT-PCR was performed using
SUR1-, SUR2A-, or SUR2B-specific primers, SUR-homologous sequences were not
detected in HSG cell mRNA (lanes 2, 4, and 6). However, as
reported earlier (31), SUR1 (lane 3) and SUR2B (lane 7), but
not SUR2A (lane 5), were detected in control human pancreatic
mRNA.

One of the suggested criteria for the possible functional as-
association between the CFTR and ROMK proteins in a cell has been the demonstration that cAMP can stimulate both Cl\textsuperscript{−} and
K\textsuperscript{+} currents in the cells. The data in Fig. 10 show that HSG
cells fulfill this criterion. Inclusion of 10 \(\mu\)M cAMP in the
pipette solution stimulated a small inward Cl\textsuperscript{−} current (250 ±
34 pA, \(n = 5\), at −60 mV holding potential with 150 mM
NMDGCl in the external and internal solutions). The current
developed slowly, remained stable for a short time, and de-
creased to a lower level. It was blocked by glibenclamide (data not shown). These data provide evidence for the presence of a
functional CFTR protein in HSG cells. Fig. 10B shows the
activation of outward K\textsuperscript{+} current by 10 \(\mu\)M cAMP. The outward
current was of smaller magnitude, and relatively more sus-
tained, than that seen with ATP. The current also showed a
decreased sensitivity toward glibenclamide (data not shown),
as has been previously reported for the ROMK channels in
kidney cells (20).
DISCUSSION

The data presented above demonstrate that ATP activates multiple P2 receptors to increase [Ca\(^{2+}\)], and activate distinct cation channels in HSG cells. Importantly, the data provide evidence that external ATP activates a novel glibenclamide-sensitive, Ca\(^{2+}\)-independent, inwardly rectifying K\(^+\) current that is mediated by a K\(_{ATP}\) channel. Further, we have shown that a ROMK-like channel and CFTR, but not SUR, are present in HSG cells. Thus, we suggest that this K\(_{ATP}\) channel belongs to the ROMK subfamily of K\(_{ATP}\) channels, which might be regulated via an interaction with the CFTR. These data reveal a potentially new mechanism for K\(^+\) secretion in salivary epithelial cells.

P2Y\(_1\), P2Y\(_2\) (P\(_{2U}\)), P2X\(_4\), and P2X\(_7\) (P\(_{2Z}\)) receptors are expressed in salivary glands (6). We have shown here that low [ATP] and [UTP] are equally potent in activating internal Ca\(^{2+}\) release. Further, the response to low [ATP] was greater than the response to low [2MeS-ATP] (data not shown). These data suggest the presence of a P\(_{2U}\) receptor in HSG cells and are consistent with previous studies showing that in HSG cells the P\(_{2U}\) receptor has a nucleotide selectivity in the order ATP > UTP > ADP (14). Additionally, P\(_{2U}\)-stimulated depletion of the internal Ca\(^{2+}\) store resulted in the activation of the store-operated Ca\(^{2+}\) influx current (I\(_{SOC}\)) that was inhibited by La\(^{3+}\) and Gd\(^{3+}\), but not by Zn\(^{2+}\). Thus, I\(_{SOC}\) activated by ATP has similar characteristics as that activated by CCh or Tg (Fig. 3–5; also see Ref. 17). Low (0.1–10 \(\mu\)M) ATP also activated an outward K\(^+\) current with characteristics similar to those previously reported for K\(_{Ca}\) in HSG cells (15, 16); (i) inhibition by charybotoxin, but not by apamin, and (ii) dependence on [Ca\(^{2+}\)]. Notably, this is the first report describing ATP-dependent activation of K\(_{Ca}\) and I\(_{SOC}\) in human submandibular gland cells. The present data demonstrate that P\(_{2Z}\) receptors are also present in HSG cells. Bz-ATP, a potent P\(_{2Z}\) receptor agonist, induced responses that were distinct from that induced by either UTP or ATP. Increasing concentrations of Bz-ATP produced dose-dependent increases in both [Ca\(^{2+}\)], and the K\(^+\) current. At lower concentrations, Bz-ATP induced increases in [Ca\(^{2+}\)] and K\(^+\), in the absence of external Ca\(^{2+}\). As has been suggested earlier, this could be due to (i) release of Ca\(^{2+}\) from intracellular stores by Na\(^+\) that might enter the cells when Ca\(^{2+}\) is removed from the external medium or (ii) stimulation of IP\(_3\) generation (6). However, the effects at higher Bz-ATP were acutely dependent on the presence of external Ca\(^{2+}\) and were more sensitive to high external Mg\(^{2+}\). These data suggest that higher [Bz-ATP] induces a Ca\(^{2+}\) influx component that is apparently not associated with internal Ca\(^{2+}\) store depletion.
(Fig. 5, compare A and B with C and D). 2MeS-ATP did not induce a similar Ca$^{2+}$ influx response, even at higher concentrations, while 100 μM ATP induced a small increase in Ca$^{2+}$ influx, in the absence of external Mg$^{2+}$. In aggregate, these data are consistent with the presence of a P2X receptor in HSG cells. However, it is unlikely that ATP induces any significant effects via this receptor in a normal Mg$^{2+}$-containing medium.

The important, and novel, finding of this study was that high [ATP] (>10 μM) induced an outward current that was not accompanied by an increase in [Ca$^{2+}$]. We have shown that the current was detected in cells previously stimulated with low [ATP] (Fig. 1), Tg (Fig. 4), or CCh (not shown) and was not affected by the removal of Ca$^{2+}$ from the external medium (Fig. 1) or treatment of cells with charybotoxin. Furthermore, the current measured in the presence of high [ATP] and charybotoxin (i) displayed a weak inward rectification with a reversal potential of ~75 mV, a value close to the K$^+$ equilibrium potential, and (ii) was blocked by replacing intracellular K$^+$ with Cs$^+$. Taken together, these data provide strong evidence for the activation of a Ca$^{2+}$-independent K$^+$ channel by high [ATP] in HSG cells. Some previous studies with salivary gland cells have suggested that ATP induces a Ca$^{2+}$-independent component of K$^+$ efflux (24, 32). It was suggested that this component of K$^+$ release could be due to a direct action of ATP on nonspecific cation channels on the plasma membrane. We have not yet determined whether ATP activates the Ca$^{2+}$-independent K$^+$ channel directly or via binding to an ATP receptor. However, our data rule out the possibility that the known P2X or P2Y receptors are involved in the ATP-dependent activation of the Ca$^{2+}$-independent, inwardly rectifying K$^+$ channel. The charybotoxin-insensitive K$^+$ current was not activated by 2MeS-ATP, Bz-ATP, UTP, or low [ATP]. Thus, the Ca$^{2+}$-independent K$^+$ current appears to be selectively activated by high [ATP] via a mechanism that is distinct from that associated with the P2X or P2Y receptors. Significantly, the sulfonlurea compounds, glibenclamide and tolbutamide, inhibited the K$^+$ current induced by high [ATP] but not that induced by low [ATP], in contrast to charybotoxin, which inhibited only the current induced by low [ATP]. These data provide evidence for the presence of at least two pharmacologically distinct K$^+$ channels in HSG cells: a Ca$^{2+}$-dependent maxi-K$^+$ channel that is sensitive to charybotoxin and an inwardly rectifying K$^+$ channel that is sensitive to sulfonlurea compounds, probably a K$\text{ATP}$ channel (20, 21, 25).

Two classes of K$\text{ATP}$ channels are modulated by sulfonlurea compounds: the ROMK channels and the classical K$\text{ATP}$ channels (20, 21, 25). Classical K$\text{ATP}$ channels (Kir 6.0 subfamily) have been shown to be present in pancreatic β cells, cardiac and skeletal myocytes, vascular smooth muscle, and neurons and are suggested to regulate the electrical activity of these cells (25). These K$\text{ATP}$ channels have been shown to be associated with the sulfonlurea receptors (SUR1, SUR2A, or SUR2B), which confer the sensitivity to the sulfonlurea compounds (see Ref. 25 for a review). The ROMK family of K$\text{ATP}$ channels (Kir 1.1 family) are expressed in the nephron, where they have been proposed to regulate K$^+$ secretion (20, 21, 33, 34). In the case of the ROMK channels, the sensitivity to sulfonlurea compounds has been suggested to depend on an interaction with the CFTR protein, although conclusive experimental data for a molecular interaction between these two proteins is yet lacking (20, 21, 35). However, several observations are consistent with this suggestion. For example both the CFTR and the ROMK proteins were shown to be localized in the apical membrane of the nephron (33, 34). Co-expression of the ROMK1 protein and CFTR in oocytes conferred glibenclamide sensitivity to the K$^+$ current (35). Further, CAMP stimulated both the CFTR-associated Cl$^-$ current and the inwardly rectifying, glibenclamide-sensitive, K$^+$ current, and CAMP plus ATP decreased the sensitivity of the K$^+$ channel for glibenclamide (20).

The present data demonstrate that HSG cells have (i) a Ca$^{2+}$-independent, sulfonlurea-sensitive, inwardly rectifying K$^+$ channel (K$\text{ATP}$) activity, (ii) the ROMK channel and CFTR, and (iii) cAMP-stimulated Cl$^-$ and K$^+$ currents. Thus, the K$\text{ATP}$ activity detected in HSG cells fulfills the criteria associated with the ROMK family of K$\text{ATP}$ channels. Based on measurements of electrolytes in saliva and 86Rb$^+$ flux studies, it has been suggested that salivary gland ducts mediate K$^+$ secretion into the lumen (36). Thus, the currently accepted model for ion fluxes in the salivary gland ductal cell depicts a pathway for K$^+$ efflux via the apical membrane. However, presently there are no data to demonstrate the identity or function of this K$^+$ conductance. Based on the data discussed above, we suggest that the ROMK-type K$\text{ATP}$ channel detected in HSG cells might be a likely candidate for this K$^+$ secretory function. Further, as has been proposed for the regulation of the ROMK channels in kidney cells, the present data suggest that the ROMK channel in HSG cells might also be regulated via interaction(s) with the CFTR protein. Importantly, we have clearly demonstrated that CFTR, but not SUR, is present in HSG cells. Thus, HSG cells provide an excellent experimental system for further studies to examine the putative mechanism by which CFTR regulates the ROMK channel. Notably, the CFTR protein has also been shown to be present in the luminal membrane of rat submandibular gland ductal cells (27, 28), and studies reported by Muallem and co-workers have demonstrated cAMP-dependent activation of CFTR-associated Cl$^-$ current (i.e. Ca$^{2+}$-independent, glibenclamide-sensitive) in rat submandibular gland ductal cells (7). However, the exact physiological role of CFTR in the ductal luminal membrane of salivary epithelial cells is still unclear.

In summary, ATP triggers two distinct Ca$^{2+}$ signaling pathways in HSG cells through the P2X and P2U receptors, respectively. The Ca$^{2+}$ signaling pathway linked to the P2U receptor, is independent of external Na$^+$ and Mg$^{2+}$ and is associated with activation of K$\text{ATP}$ and SOC. In contrast, the Ca$^{2+}$ signaling pathway via the P2X receptor does not appear to be activated by ATP under normal conditions (i.e. with 1 mM external Mg$^{2+}$). However, it is associated with Ca$^{2+}$ influx, probably via an as yet unidentified Ca$^{2+}$-permeable cation channel, and activation of K$\text{ATP}$ when cells are exposed either to an agonist stronger than ATP or to ATP in low external [Mg$^{2+}$]. Importantly, we have shown here that ATP also activates a novel, Ca$^{2+}$-independent, weakly inwardly rectifying K$^+$ channel in HSG cells, which is blocked by sulfonlurea compounds but not by charybotoxin. Together with our demonstration of the presence of CFTR, a ROMK channel, and the activation of both Cl$^-$ and K$^+$ currents in HSG cells by CAMP, the present data strongly suggest that this K$^+$ channel belongs to the ROMK family of K$\text{ATP}$ channels. This putative salivary ROMK channel provides a potentially novel mechanism for the regulation of K$^+$ secretion in salivary epithelial cells.

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