Membrane-specific Regulation of Cl\textsuperscript{−} Channels by Purinergic Receptors in Rat Submandibular Gland Acinar and Duct Cells*  

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Weizhong Zeng, Min Goo Lee, and Shmuel Muallem‡

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Salivary glands, in particular the submandibular gland (SMG),\textsuperscript{1} have been extensively used as a model system for fluid and electrolyte secretion by secretory epithelial cells (1–3). Salivary secretion occurs in two steps. Acinar cells secrete the primary isotonic, NaCl-rich fluid. The duct changes electrolyte composition and to some extent the osmolarity of the primary fluid by absorbing the NaCl and secreting KHCO\textsubscript{3}\textsuperscript{−} (2, 3).

The central feature of the accepted model of fluid and electrolyte secretion by salivary acinar cells is transepithelial Cl\textsuperscript{−} movement as the driving force for fluid and electrolyte secretion (1, 3). Functional (4) and immunofluorescence localization (5, 6) point to NaKCl\textsubscript{2} cotransport as the Cl\textsuperscript{−} entry mechanism in the basolateral membrane (BLM). A Ca\textsuperscript{2+}-activated, outward rectifying Cl\textsuperscript{−} channel, found in many salivary acinar cells (1, 2), is believed to be the Cl\textsuperscript{−} exit pathway in the luminal membrane. This model, however, cannot account for a large fraction (\textasciitilde 40%) of Cl\textsuperscript{−} transport by SMG acinar cells. A search for alternative Cl\textsuperscript{−} pathways revealed the presence of at least three Cl\textsuperscript{−} channels in parotid acinar cells; Ca\textsuperscript{2+}-activated, volume-sensitive and hyperpolarization-activated Cl\textsuperscript{−} channels (7, 8). Recently, we demonstrated the expression of CFTR in the luminal membrane (LM) of SMG acinar cells (9). Except for CFTR, the membrane localization, possible role in Cl\textsuperscript{−} secretion, and regulation by agonists of the various Cl\textsuperscript{−} channels are not known.

Electrolyte transport by salivary ducts, including Cl\textsuperscript{−} reabsorption, is not well understood on the molecular level. The bulk of Cl\textsuperscript{−} entry in the LM and Cl\textsuperscript{−} efflux across the BLM are assumed to be mediated by Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange and Cl\textsuperscript{−} channels, respectively (2). Indeed, Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange activity was found in the LM of SMG duct cells (10). Additional electrogenic Cl\textsuperscript{−} transport, which is needed to balance the electrogenic Na\textsuperscript{+} reabsorption, may occur through luminal and basolateral Cl\textsuperscript{−} channels (2). This prediction is based on the finding that changing LM and BLM Cl\textsuperscript{−} concentration affects the transepithelial potential and resistance of the excretory SMG duct (11). Luminal Cl\textsuperscript{−} permeability is likely to be in part mediated by CFTR. Duct cells of all salivary glands express CFTR (12) in the LM (13). Another Cl\textsuperscript{−} channel found in SMG duct cells is a CIC2-like channel (14). The membrane localization and physiological function of this channel are not known, although it may participate in cell volume regulation, as in other cell types (15).

How agonists regulate Cl\textsuperscript{−} channels and Cl\textsuperscript{−} transport in SMG and other salivary glands is only partially understood. In acinar cells Ca\textsuperscript{2+} mobilizing agonists activate the Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channel (2, 3). Agonists acting through cAMP elevation can activate CFTR in acinar and duct cells (9, 16).

Salivary acinar and duct cells also respond to purinergic stimulation by a change in [Ca\textsuperscript{2+}] (17–22). However, the identities of the receptors and whether they regulate Cl\textsuperscript{−} channels in salivary gland cells are not known. In other epithelia, in particular airway and nasal epithelia, regulation of Cl\textsuperscript{−} channels by purinergic receptors emerged as an important physiological activity with possible therapeutic implications (23–27). Different P\textsubscript{2} receptors are expressed in the LM and BLM of these cells and appear to regulate multiple and different Cl\textsuperscript{−} chan-  

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1To whom correspondence should be addressed: Dept. of Physiology, University of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235. Tel.: 214-648-2493; Fax: 214-648-8685; E-mail: smual@mailmednet.swmed.edu.

2The abbreviations used are: SMG, submandibular gland; BLM, basolateral membrane; LM, luminal membrane; CFTR, cystic fibrosis transmembrane regulator; GLM, glibenclamide; BzATP, 2′-3′-benzoylbenzoyl-ATP; SPQ, 6-methoxy-N-(3-sulfopropyl)quinolinium; ORCC, outward rectifying Cl\textsuperscript{−} channel; MAb, monoclonal antibody; ATP\textsuperscript{S}, adenosine 5′-O-(thiophosphosphate).
FIG. 1. ATP regulates [Cl\textsuperscript{−}], in SMG duct and acinar cells. SMG acini and duct fragments loaded with SPQ were stimulated with 1 mM ATP (a–f) in the presence of 0.1 mM bumetanide (b and c) or 0.1 mM diphenylamine-2-carboxylic acid (DPC) (c and f). In each experiment fluorescence was recorded from at least two acinar clusters and duct fragments present in the same recording field. Similar results were obtained in at least three experiments under each condition.

nals. Thus, apical ATP acting through \(P_2\) receptors activates the \(Ca^{2+}\)-dependent Cl\textsuperscript{−} channels, CFTR, and probably indirectly the outward rectifying Cl\textsuperscript{−} channels (ORCC). Basolateral ATP acting through \(P_2\) receptors activates only CFTR in a \(Ca^{2+}\)- and cAMP-independent manner (27). More recently it was reported that the LM of nasal epithelial cells express UDP-sensitive receptors different from \(P_2Y_2\), which are coupled to inositol 1,4,5-trisphosphate signaling and activate Cl\textsuperscript{−} secretion in these cells (28).

Due to our recent discovery of the expression of CFTR in SMG acinar cells, the localization of \(P_2\) receptors in the LM and BLM of SMG cells (29) and the importance of purinergic regulation of Cl\textsuperscript{−} secretion in epithelia, in the present study we used [Cl\textsuperscript{−}], measurements, immunoanalysis, and recording of Cl\textsuperscript{−} currents to characterize and evaluate the contribution of Cl\textsuperscript{−} channels and the NaKCl\textsubscript{2} cotransporter to Cl\textsuperscript{−} transport during purinergic stimulation of SMG acinar and duct cells. The combined results point to the central role of CFTR and the \(Ca^{2+}\)-activated Cl\textsuperscript{−} channel in regulating Cl\textsuperscript{−} transport in SMG duct cells and their contribution to Cl\textsuperscript{−} transport relative to that by the NaKCl\textsubscript{2} cotransporter in acinar cells.

MATERIALS AND METHODS

The general methods are identical to those in our companion study (29), except for the following.

**Measurement of [Cl\textsuperscript{−}], with SPQ**—SPQ-loaded acini and duct fragments were plated on coverslips and perfused in a manner similar to that described for Fura 2-loaded cells (29). SPQ fluorescence was measured at an excitation wavelength of 380 nm and was calibrated by incubating the cells with high K\textsuperscript{+} solutions containing different concentrations of Cl\textsuperscript{−} (0–100 mM), 10 \(\mu\)M tributyltin, and 2.5 \(\mu\)M nigericin. A maximal fluorescence quench was obtained by exposing the cells to a solution containing 150 mM SCN\textsuperscript{−} (29), except for the following.

**Results**

**Measurements of [Cl\textsuperscript{−}], with SPQ**—To evaluate the role of different Cl\textsuperscript{−} transporters in [Cl\textsuperscript{−}], regulation we first measured [Cl\textsuperscript{−}], with SPQ. Fig. 1 shows that [Cl\textsuperscript{−}], was differently regulated in the SMG duct and acinar cells. In resting duct cells [Cl\textsuperscript{−}], averaged 26 ± 7 \(n = 34\) and in acinar cells 56 ± 8 mM \(n = 29\). Stimulation of duct cells with 1 mM ATP caused a rapid reduction in [Cl\textsuperscript{−}], to about 13.5 ± 4.5 mM. Subsequently [Cl\textsuperscript{−}], increased over 1.5–2 min and stabilized at 36 ± 6 mM \(n = 11\) (Fig. 1a). Stimulation of acini in the same recording field with 1 mM ATP caused a reduction in [Cl\textsuperscript{−}], to 17 ± 5 mM, which was then increased to about 52 ± 5 mM \(n = 11\) (Fig. 1d). The absence of HCO\textsubscript{3} in the perfusion medium ensured that these changes were not affected by Cl\textsuperscript{−}/HCO\textsubscript{3} exchange (10).

Another potential transporter mediating some of the agonist-dependent [Cl\textsuperscript{−}], changes is the NaKCl\textsubscript{2} cotransporter (33, 34). Fig. 1e shows that in SMG acinar cells 0.1 mM bumetanide reduced the rate of Cl\textsuperscript{−} uptake by about 46 ± 9% and [Cl\textsuperscript{−}], stabilized at 38 ± 8 mM \(n = 9\). In duct cells bumetanide failed to affect the [Cl\textsuperscript{−}], changes evoked by ATP stimulation \(n = 9\) (Fig. 1b). On the other hand, Fig. 1, c and f, shows that 100 \(\mu\)M of the general Cl\textsuperscript{−} channel blocker diphenylamine-2-carboxylic acid largely inhibited Cl\textsuperscript{−} efflux, and thus all [Cl\textsuperscript{−}], changes evoked by ATP \(n = 4\) for each cell type.

**NaKCl\textsubscript{2} Cotransport in SMG Acinar and Duct Cells**—In view of a recent report of the expression of the secretory NaKCl\textsubscript{2} cotransporter in SMG duct cells (6) and the results in Fig. 1, we proceeded to examine more directly the expression and activity
of NaKCl₂ cotransport in the two cell types. Western blot analysis with the mAb T₄, which recognizes multiple isoforms of the NaKCl₂ cotransporter (5), showed that acinar cells of various exocrine glands express different forms and levels of cotransporter protein (Fig. 2a). Parotid acinar cells express a 185–190-kDa protein. Interestingly, SMG acinar cells expressed at least 10- and 150-fold more NaKCl₂ cotransporter protein than did parotid and pancreatic acinar cells, respectively. The T₄ mAb detected only low levels of cotransporter protein in the SMG ductal preparation. Densitometric analysis showed that intensity in the duct lane was about 6.8 ± 5% (n = 3) of that in SMG acinar lane. After correction for the amount of protein loaded in each lane, this value is within the contamination of the SMG duct preparation with acini (32).

The results of the Western blot analysis were confirmed by immunolocalization studies. Fig. 2b shows that the T₄ mAb detected high levels of NaKCl₂ cotransport in the BLM of acinar cells. The LM of acinar cells and both membranes of duct cells did not stain with this antibody. Further evidence for low NaKCl₂ cotransport activity in SMG duct cells was obtained by measuring ⁸⁶Rb uptake. In SMG acinar cells bumentamidine alone inhibited ⁸⁶Rb uptake by about 60% and nearly all the ⁸⁶Rb uptake in the presence of ouabain (Fig. 2c). In duct cells bumentamidine alone had no measurable effect on ⁸⁶Rb uptake, ouabain alone inhibited the uptake by about 60%, and bumentamidine further reduced this uptake by about 8% (Fig. 2d). Although the latter fraction was consistently observed (n = 6), it never exceeded 10%.

Cl⁻ Channels in SMG Acinar and Duct Cells—Excluding Cl⁻/HCO₃⁻ exchange and NaKCl₂ cotransport in duct cells and finding a component of Cl⁻ transport not mediated by these transporters in acinar cells stimulated with ATP suggested a role of Cl⁻ channels in both cell types. To identify the Cl⁻ channels participating in the [Cl⁻] changes induced by ATP we attempted to characterize the various Cl⁻ channels expressed in freshly isolated SMG duct and acinar cells. Following the voltage protocol of Ludewig et al. (35) (Fig. 3a) revealed the presence of a voltage gated Cl⁻ current with properties similar to those reported for CICO (36) (Fig. 3, traces 1 and 5). For the present studies the most characteristic feature of this current was the fast gating observed after maximal channel opening by hyperpolarizing pulses. Typically, channel inactivation was faster at −140 than at −80 mV. Another channel found in both cell types is the volume-sensitive Cl⁻ channel. Thus, swelling the cells revealed the presence of an outward rectifying Cl⁻ current with time dependent inactivation in positive potentials (Fig. 3, traces 2 and 6), similar to that described in several other cell types (15). As reported before in SMG duct cells (14), SMG acinar cells also showed the presence of an inwardly rectifying Cl⁻ current with voltage- and time-dependent activation (not shown). Elevating [Ca²⁺], with the Ca²⁺-ionophore A23187 activated an outwardly rectifying Cl⁻ current with a typical time-dependent activation and tail currents (Fig. 3, traces 3 and 7). Finally, elevation of cellular cAMP activated a CFTR-like Cl⁻ current in SMG duct and acinar cells (Fig. 3, traces 4 and 8) (see also Zeng et al. (9)). For the purpose of the present work the channels were not characterized further. However, as can be seen in Fig. 3 the Cl⁻ channels expressed in both cell types are similar and have sufficiently distinct kinetic characteristics to aid in their identification during agonist stimulation.

Effect of ATP on Cl⁻ Current—Stimulation with 1 mM ATP rapidly activated a Cl⁻ current in single duct and acinar cells. Fig. 4 shows the two patterns of Cl⁻ current activation. In about 20% of experiments, after rapid activation by ATP the current returned to near resting level. Subsequent removal of ATP resulted in transient reactivation of the current (Fig. 4, a and c). In most experiments the current remained activated, and removal of ATP resulted in a small current rebounding before its complete inactivation. Determination of the current-voltage relationship at various times during and after ATP stimulation did not result in distinctive patterns (Fig. 4, c and g, lanes 2–4 in each panel). However, subtracting the current at period 3 from that measured at periods 2 and 4 suggests that in
both cell types ATP activated at least two distinct Cl\(^{-}\) channels (Fig. 4, d and h).

Previous studies reported that ATP increases [Ca\(^{2+}\)], in SMG acinar (22) and duct cells (32). To evaluate the contribution of the Ca\(^{2+}\)-activated Cl\(^{-}\) channels to the current activated by ATP we tested the effect of extracellular Ca\(^{2+}\) and intracellular EGTA on the current. An example of such experiments is shown in Fig. 5 and the results of several experiments are summarized in Table I. In both cell types ATP activated Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent Cl\(^{-}\) currents. The Ca\(^{2+}\)-dependent current was about 60 and 50% of the total current in acinar and duct cells, respectively. This was the case whether the current was calculated as the portion sensitive to external Ca\(^{2+}\) (Fig. 5, a in Table I) or blocked by high intracellular [EGTA] (Fig. 5, b in Table I). The kinetic properties of this current are similar to those induced by A23187 (Fig. 3). That is, the current showed outward rectification, time-dependent activation, and substantial tail currents (Fig. 5, b and f).

The residual, Ca\(^{2+}\)-independent current measured in the absence of external Ca\(^{2+}\) (Fig. 5, c and e) and in the presence of 5 mM internal EGTA (Fig. 5, c and g) or 2 mM 1,2-bis(2-amino- phenoxy)ethane-N\(_2\)N\(_3\)N\(_3\)-tetraacetic acid (not shown) has kinetic properties resembling those of CFPTR. Since we previously showed that glibenclamide (GLM) inhibits the CFPTR-like current in SMG duct and acinar cells (9) we next tested the effect of GLM on the current stimulated by ATP. Fig. 6 and Table I show that GLM completely inhibited the Ca\(^{2+}\)-independent Cl\(^{-}\) current. Furthermore, the Cl\(^{-}\) current inhibited by GLM in the presence of low (Fig. 6, a and e) or high (Fig. 6, c and g) EGTA concentration in the pipette solution has a CFTR-like kinetic characteristic (current/voltage curves 2-3 in b and f and curves 1-2 in d and h). The remaining current (current/voltage curves 3-1 in b and f) showed strong outward rectification, as expected from the current carried by the Ca\(^{2+}\)-independent Cl\(^{-}\) channel. Regulations of Cl\(^{-}\) Currents by BzATP and UTP—Taking advantage of the membrane-specific action of BzATP and UTP (29) we studied regulation of Cl\(^{-}\) channels by P\(_{2}\) receptors localized in the LM and BLM, respectively. Fig. 7 shows the effect of 25 \(\mu\)M BzATP on Cl\(^{-}\) currents in SMG duct and acinar cells and Table I summarizes between four and six such experiments. Despite the finding that BzATP in both cell types was the most active nucleotide in increasing [Ca\(^{2+}\)], it activated largely the Ca\(^{2+}\)-independent Cl\(^{-}\) current. Thus, removal of external Ca\(^{2+}\) (Fig. 7, a and g) and including high [EGTA] in the pipette solution (Fig. 7, c, e and i) reduced the BzATP-activated current by only 30.1 ± 1.8%. Most of the current showed a linear current/voltage relationship, no time-dependent activation, and no tail currents (Fig. 7, b and h, traces 3-1). GLM almost completely inhibited the fraction of the Ca\(^{2+}\)-independent current (about 70%) under all conditions. In contrast with BzATP, UTP at 100 \(\mu\)M almost exclusively

**Fig. 3. Expression of multiple Cl\(^{-}\) channels in SMG duct and acinar cells.** In all experiments the N-methyl-D-glucamine chloride-based bath and pipette solutions are as indicated in our companion study (29), except that ATP and EGTA concentrations are as specified below. To record the CICO-like current (traces 1 and 5) the pipette solution contained 1 mM ATP and 5 mM EGTA. The voltage protocol used is shown in a. The slow gate was opened by holding the membrane potential for 3.3 s at −100 mV between test pulses. The fast gate was opened by stepping the membrane potential from −100 to +60 mV. Then a test potential from −140 to +60 mV in 20-mV steps was applied. The instantaneous current/voltage relation is shown to the right of the traces. The volume-sensitive Cl\(^{-}\) current (traces 2 and 6) was recorded by including 5 mM ATP and 5 mM EGTA in the pipette solution. The current was activated by reducing the osmolarity of the bath solution from 320 to 280 mOsm. The osmolarity was reduced by reducing the concentration of N-methyl-D-glucamine chloride. The Ca\(^{2+}\)-dependent Cl\(^{-}\) current (traces 3 and 7) were recorded by including 0.5 mM EGTA and 1 mM ATP in the pipette solution. The current was activated by incubating the cells for 2–3 min with 2 \(\mu\)M A23187. The voltage protocol used to record the volume-sensitive, and Ca\(^{2+}\)-dependent Cl\(^{-}\) currents are given in c. The potential was held at −40 mV for 1.2 s between pulse potentials of between −100 and +100 mV in increments of 20 mV. The CFTR-like Cl\(^{-}\) currents (traces 4 and 8) were recorded by including 2 mM EGTA and 5 mM ATP in the pipette solution. The current was activated by incubating the cells for 5–10 min with a cAMP-increasing mixture which included 2.5 \(\mu\)M forskolin, 10 \(\mu\)M isoprenaline, and 1 mM isobutylmethylxanthine. The voltage protocol used is shown in b. The potential was held at 0 mV for 1.2 s before test potentials from −100 to +100 mV with incremental steps of 20 mV.
activated the Ca$^{2+}$-dependent Cl$^{-}$ current. Fig. 8 shows that the Cl$^{-}$ current activated by UTP was eliminated by removal of external Ca$^{2+}$ (traces a and e) or including 5 mM EGTA in the pipette solution (traces d and h). The current had kinetic characteristics of the Ca$^{2+}$-activated, outward rectifying Cl$^{-}$ channel (traces 2-3 in panels b and f). Finally, the current was completely insensitive to 100 μM GLM (traces c and g). As was found for changes in [Ca$^{2+}$], (29), all other nucleotides tested did not activate the current at concentrations between 1 μM and 1 mM (not shown). However, preincubation of the cells with several of the nucleotides desensitized the cells to subsequent stimulation with ATP. Fig. 8, d and h, shows that ATP had only a small effect on the current after preincubation with 100 μM UTP. This desensitizing effect was not studied further in the present studies.

Considering the small effect of UTP on [Ca$^{2+}$], (29) and the relatively robust effect on Cl$^{-}$ current, it was of interest to measure the dependence of current activation on nucleotide concentrations. The results of four (BzATP and UTP) and five (ATP) such experiments are plotted in Fig. 9. In both cells ATP was the most potent agonist, activating the Cl$^{-}$ current to a calculated 1.82- and 1.6-fold higher than BzATP and 3.5- and 3.8-fold higher than UTP in SMG duct and acinar cells, respectively. In both cell types UTP activated the current with a similar apparent affinity ($K_{app}$) of between 10 and 12 μM. The $K_{app}$ for ATP was also similar in both cell types (between 0.9 and 1.5 mM). However, the $K_{app}$ for BzATP in duct cells was about 12 μM and that in acinar cells was about 70 μM. These values are quite different from those determined for Ca$^{2+}$ mobilization by the nucleotides.

**DISCUSSION**

Fluid and electrolyte secretion by salivary glands involves secretion of isotonic, NaCl-rich fluid by acinar cells and its modification by duct cells (2, 3), which closely resembles fluid and electrolyte secretion by sweat glands (37). In both systems transepithelial Cl$^{-}$ transport (secretion by acinar cells and reabsorption by duct cells) plays a central role in controlling the entire process, which is regulated by cholinergic, α-adrenergic (Ca$^{2+}$-dependent), and β-adrenergic (cAMP-dependent) stimulation. A reasonably well understood mode is that stimulated by Ca$^{2+}$-mobilizing agonists in acinar cells. In this case the bulk of Cl$^{-}$ influx across the BLM is mediated by the NaKCl$_2$ cotransporter and Cl$^{-}$ efflux across the LM by a Ca$^{2+}$-activated Cl$^{-}$ channel. In duct cells these agonists likely facilitate Cl$^{-}$ influx in the LM by activation of Ca$^{2+}$-dependent Cl$^{-}$ channels. The CAMP-dependent agonists are most likely to activate CFTR, which is expressed in the LM of both cell types (9).

A mode of regulation currently drawing much attention in epithelial transport is regulation by ATP acting on several purinergic receptors (23–27). Much of the work with P$_2$ agonists has been done with airway and nasal epithelia due to the potential implication of the findings to cystic fibrosis. Although expression of P$_2$ receptors in parotid and SMG acinar cells has been known for quite some time (17–22), the mechanism by which these receptors may regulate secretion is not known. The presence and action of P$_2$ receptors in duct cells is known to only a very limited extent (32). In the present studies we determined the Cl$^{-}$ channels activated by selective P$_2$ agonists to regulate [Cl$^{-}$], and, in turn, Cl$^{-}$ transport by the two cell types.

Under resting conditions and in the absence of HCO$_3^{-}$ rat SMG duct cells maintained [Cl$^{-}$], at about 26 mM, compared with 56 mM in acinar cells. This may be due to the different level of NaKCl$_2$ cotransport in the two cell types. Our estimation of [Cl$^{-}$], in rat SMG duct cells is different from those
reported for the striated intralobular ducts from the rabbit SMG (38). Large differences in mechanisms of fluid and electrolyte secretion among SMG of different species are well documented (2, 39). The higher level of $[\text{Cl}^-]$ in the rabbit SMG duct would suggest that $\text{Cl}^-$-loading mechanisms are more prominent in the rabbit duct compared with the rat duct.

Previous immunolocalization using a polyclonal antibody raised against a 22-amino acid peptide from the N-terminal of the secretory NaKCl$_2$ cotransporter, reported expression of the cotransporter in the BLM of all rat SMG acinar and about one-third to one-half of rat SMG duct cells (6). Since our functional studies did not support high activity of NaKCl$_2$ cotrans-
port in SMG duct cells (Fig. 2), we used the mAb that was used to localize the cotransporter in the parotid gland (5) to reevaluate the contribution of the cotransporter to Cl⁻ transport in the SMG. Western blot analysis and immunolocalization revealed the absence or low level of NaKCl₂ cotransporter in duct cells. Interestingly, SMG acinar cells express at least 10-fold

**FIG. 6.** The ATP-activated and Ca²⁺-independent Cl⁻ current is inhibited by glibenclamide. SMG duct (a–d) and acinar (e–h) cells were used to measure the effect of 100 μM glibenclamide (Gli) on the current activated by ATP when the pipette solution contained 0.5 (a and e) or 5 mM (c and g) EGTA. The glibenclamide-sensitive currents (traces 2-3 in b and f and 1-2 in d and g) under both conditions are summarized in Table I.

**FIG. 7.** Activation of Cl⁻ currents by BzATP. SMG duct (a–f) and acinar (g–l) cells were stimulated with 25 μM BzATP. The pipette solution contained either 0.5 (a, c, g, and i) or 5 mM EGTA (e and j). Where indicated, the cells were perfused with a Ca²⁺-free bath solution (a and g) or a bath solution containing 100 μM glibenclamide (Gli) (c, e, i, and j). Ca²⁺-dependent (b and h, traces 2 and 3) and Ca²⁺-independent, glibenclamide-sensitive currents (traces 3-1 in b and h, and traces 2-3 in d, f, k, and l) derived from the current/voltage relationships are summarized in Table I.
more cotransporter protein than parotid gland acinar cells, and the proteins have different apparent molecular weights in the two glands. Based on previous survey of NaKCl₂ cotransporters in a variety of cells and tissues (5), the different size of the proteins in the two glands is likely to be due to different degrees of glycosylation. The reason for the high levels of Na-KCl₂ cotransporter protein in SMG acinar cells and the differences in protein size is not known at present.

Despite the high level and activity of NaKCl₂ cotransport in SMG acini, the cotransporter was not the only mechanism responsible for Cl⁻ uptake in stimulated cells. Bumetanide had no effect on the initial ATP-evoked Cl⁻ efflux and the subsequent Cl⁻ influx. The absence of HCO₃⁻ in the solutions ensured that the Cl⁻/HCO₃⁻ exchangers in the cells will not be active (10). Hence, the only remaining pathways that can mediate the bumetanide-insensitive Cl⁻ fluxes are Cl⁻ channels. Indeed, the Cl⁻ channel inhibitor diphenylamine-2-carboxylic acid largely inhibited the ATP-mediated net [Cl⁻] changes (Fig. 1) and activation of Cl⁻ currents (not shown), and ATP activated at least two Cl⁻ channels in SMG duct and acinar cells.

Identification of the channels activated by ATP was aided by partial characterization of Cl⁻ currents under defined conditions. Following established protocols in several cell types (15, 35), including salivary gland cells (7, 8, 14, 16), we confirmed the presence of a CFTR-like (9) and voltage-activated inward rectifying (14) currents in SMG duct and acinar cells. Ca²⁺-activated, outward rectifying and volume-sensitive, outward rectifying currents were also found in both cell types. Finally, a Cl⁻ current with fast voltage-dependent gating similar to ClCO was recorded after a conditioning stepping of the holding potential from −100 to +60 mV. The five types of Cl⁻ currents may not be all the Cl⁻ channels expressed in SMG acinar and duct cells. However, for the purpose of the present study the channels were not characterized further since the partial characterization was sufficient to identify with reasonable certainty

**Fig. 8. Activation of the Ca²⁺-dependent Cl⁻ current by UTP.** SMG duct (a–d) and acinar (e–h) cells were stimulated with 100 μM UTP (a, c, e, and g) or UTP and then 1 mM ATP (d and h). The pipette solution contained 0.5 (a, c, e, and g) or 5 mM (d and h) EGTA, and the bath solution was either Ca²⁺-free (a and e) or contained 100 μM glibenclamide (c and g). The current traces were used to obtain the current/voltage curves (b and f) and the Ca²⁺-sensitive portion of the currents (traces 2-3 in b and f) are summarized in Table I.

**Fig. 9. Dependence of Cl⁻ current on nucleotide concentration.** The protocols of Figs. 5a, 7a, and 8a, and 5c, 7g, and 8e were used to determine the effect of ATP, BzATP, and UTP on Cl⁻ current of SMG duct and acinar cells, respectively. The mean ± S.E. of four to six experiments with acinar (panel A) and duct cells (panel B) are plotted.
the channels activated by ATP.

Partial characterization of Cl⁻ channels became necessary when we found that the overall Cl⁻ current activated by ATP did not follow any known Cl⁻ channel kinetic. This became evident when the contribution of Ca²⁺ to Cl⁻ current activation was eliminated by removal of external Ca²⁺ or buffering [Ca²⁺], with high EGTA concentrations. Both protocols inhibited about 50% of the current activated by ATP (Table I). This current has many of the characteristics of the Ca²⁺-activated Cl⁻ channel. The residual, Ca²⁺-independent Cl⁻ current activated by ATP had many of the CFTR-like characteristics (9).

Activation by ATP of multiple Cl⁻ channels has also been reported in tracheal and nasal epithelial cells (24–27). These included the Ca²⁺-dependent Cl⁻ channel, CFTR and a Ca²⁺-independent channel believed to be the ORCC (23, 24, 27, 40). Analyzing the properties of the Cl⁻ currents activated by ATP and other nucleotides, we could not observe activation of the ORCC in SMG acinar and duct cells. This may be due to differences in cell types and/or the purinergic receptors expressed in SMG cells. In airway epithelial cells the ORCC is directly activated by ATP (23) in the mM range (40). In SMG cells activation of Cl⁻ current required at least 10–100 μM ATP. In airway epithelia luminal ATP acted on P₂Y₁ receptors to activate Ca²⁺-dependent and Ca²⁺-independent Cl⁻ currents. This receptor responded to UTP > ATPP₂S = ATP >> ADP (27). The luminal membrane of nasal epithelia was shown recently to respond to UDP, probably through P₂Y₁ receptors (28). The BLM of these cells responded to ADP = ATPP₂S = ATP >> UDP probably through P₂Y₂ receptors (27). SMG cells responded to BzATP > ATP > UTP. Other nucleotides, including ATPP₂S, ADP, 2-methylthio-ATP, α,β-methylene-ATP, 2-chloro-ATP, and UDP at concentrations up to 1 mM had no effect on [Ca²⁺]i or Cl⁻ current. This profile is most like the one reported for the P₂ptide receptor (41).

An interesting finding, which may relate to the localization of the Cl⁻ channels activated by the P₂ receptors, is the lack of correlation between the effects of the various nucleotides on [Ca²⁺]i, (see our companion study (29)) and their ability to activate the Ca²⁺-dependent Cl⁻ channel. BzATP increased [Ca²⁺]i, at least 20-fold higher than did UTP (29), yet BzATP activated the Ca²⁺-dependent Cl⁻ current less than did ATP and UTP. UTP caused minimal increase in [Ca²⁺]i, while activating only the Ca²⁺-dependent Cl⁻ current. The simplest interpretation of these findings is that UTP increases [Ca²⁺]i, next to the plasma membrane to a level sufficient to activate the Cl⁻ channels. Indeed, previous studies already showed that plasma membrane localized events such as Ca²⁺-activated ion channels (42) and exocytosis (43) are more accurate near membrane Ca²⁺-sensors than are fluorescence probes. The relative potency of the P₂ agonists in activating the CFTR and the Ca²⁺-dependent Cl⁻ current can then be explained by expression of the P₂ receptors in the LM and P₂u receptors in the BLM. Polarized expression of Ca²⁺-signaling complexes have been demonstrated in SMG acinar and duct cells (31, 44). A similar polarized distribution of P₂z and P₂u receptors and their Ca²⁺-signaling complexes may account for the selective activation of Cl⁻ channels by purinergic stimulation.

Whereas activation of the Ca²⁺-dependent Cl⁻ channel by UTP can be explained by a local effect of UTP on [Ca²⁺]i, it is not clear how BzATP activated a CFTR-like Cl⁻ current. To the best of our knowledge, the present study is the first to describe Cl⁻ channel activation by a P₂z receptor. The P₂z receptor functions as a ligand-gated ion channel. This, however, cannot account for activation of the Cl⁻ current since the P₂z receptor conducts Ca²⁺ better than monovalent cations and is not permeable to anions like Cl⁻ (45). Furthermore, conductance of monovalent ions by the P₂z receptor is strongly inhibited in the presence of divalent cations (41, 45–47). The incubation medium in our experiments contained 1 mM Mg²⁺ and 1 mM Ca²⁺, and removal of Ca²⁺ did not enhance the Cl⁻ current. The latter, together with the experiments in which cytosolic Ca²⁺ was clamped at about 2 nM with 5 mM EGTA, also excludes [Ca²⁺], as the activator of the Cl⁻ current by BzATP. None of the other classical second messengers (cAMP, cGMP, protein kinase C) are likely to mediate activation of Cl⁻ channels by the P₂u receptor since the P₂u receptor in SMG cells is not coupled to G proteins (29). An intriguing possibility is that the P₂z receptor directly interacts with CFTR or a CFTR regulatory protein to regulate channel activity. Additional work is needed to clarify the mechanism by which P₂z receptors can activate Cl⁻ channels in SMG cells.

The physiological significance of our findings may be severalfold. The granules of SMG acinar and duct cells contain ATP that is likely to be discharged to the lumen during stimulation of exocytosis. Another potential source of luminal ATP is CFTR. At least in some cells CFTR appears to transport ATP (27, 40, 48) at sufficiently high concentrations to activate luminal P₂ receptors (40). In SMG ATP can regulate cellular activity by interacting with the luminal P₂ receptors. Release of ATP from nerve endings can activate the P₂u receptors in the basolateral membranes. The combination of luminal and basolateral Cl⁻ channels activated by ATP in conjunction with modulation of the respective membrane potentials can be used to mediate Cl⁻ secretion by acinar cells or Cl⁻ absorption by duct cells, in an equivalent manner to the pull-push model proposed by Kasai and Augustine (49) for pancreatic acinar cells. Cl⁻ absorption by a pull-push model may be responsible for the final concentration of Cl⁻ in salivary fluid. The initial part of Cl⁻ influx across the luminal membrane of duct cells is likely to be mediated by the potent luminal Cl⁻/HCO₃⁻ exchanger present in these cells (10). However, when Cl⁻ in luminal fluid is reduced to about 70 mM and HCO₃⁻ is increased to about 30–35 mM the exchanger is at equilibrium and an alternative mechanism is needed for further Cl⁻ reabsorption and HCO₃⁻ secretion. A pull-push model can be ideal for such a task. Because of the close similarity in mechanisms and regulation of fluid and electrolyte secretion between the SMG and other CFTR expressing secretory glands, the high expression of CFTR in the SMG duct, the accessibility of multiple experimental systems and the wealth of knowledge on the functioning of salivary glands (2), the SMG can be a prime experimental system to study the function of CFTR and its interaction with other proteins.

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