Involvement of apical P2Y2 receptor-regulated CFTR activity in muscarinic stimulation of Cl– reabsorption in rat submandibular gland

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Ishibashi K, Okamura K, Yamazaki J. Involvement of apical P2Y2 receptor-regulated CFTR activity in muscarinic stimulation of Cl– reabsorption in rat submandibular gland. Am J Physiol Regul Integr Comp Physiol 294: R1729–R1736, 2008. First published March 12, 2008; doi:10.1152/ajpregu.00758.2007.—Previously, we presented in vivo evidence for a physiological significance of cAMP-regulated CFTR Cl– channels in Ca2+-activated Cl– reabsorption in the ductal system of the rat submandibular gland. Here, we address the mechanism by which basal CFTR activation contributes to the transepithelial Cl– movement evoked by mucinergic stimulation. The Cl– concentration ([Cl–]) increased in the final saliva from rat submandibular gland during pilocarpine stimulation when a small interfering RNA for CFTR or a specific CFTR inhibitor, CFTRinh-172, was injected retrogradely into the gland’s own duct, indicating that basal CFTR activation is involved in Cl– reabsorption. Systemically administered propranolol failed to alter the [Cl–], suggesting little involvement of a β-adrenergic pathway in the Cl– movement that occurs through basal CFTR activation. Intraductal injection of suramin (a nonspecific P2-receptor antagonist) increased the salivary [Cl–], indicating the existence of endogenous purinergic activation. Upon separate intraductal injection, ATP and a P2Y2-receptor agonist, UTP, decreased the salivary [Cl–] almost equipotently, CFTRinh-172 and suramin each prevented these effects, whereas 2′,3′-O-(4-benzoylbenzoyl)-ATP (Bz-ATP), a P2X4 agonist, had no specific effect. Pilocarpine stimulation evoked ATP secretion into the salivary fluid. Immunohistochemistry revealed the partial coexistence of CFTR and P2Y2 receptors on the luminal surface of epithelial cells in the striated ducts of this gland. These results raise the possibility that muscarinic stimulation-induced Cl– reabsorption occurs through basal CFTR activity and that this is regulated by P2Y2 receptors in the ductal epithelium via stimulation by ATP secreted into the salivary fluid.

IN SALIVARY ACINAR AND DUCTAL cells, multiple classes of Cl– channels have been reported to be involved in electrolyte secretion and reabsorption (14). Specifically, CFTR, a cAMP-regulated Cl– channel, and a Ca2+-activated Cl– channel are present in rat salivary ductal cells, and they are likely to participate in Cl– reabsorption (24, 30, 31). Recently, we published direct in vivo evidence of a physiological significance for both CFTR and a Ca2+-activated Cl– channel (CLCA) in Cl– reabsorption from the salivary flow during muscarinic stimulation through the ductal system of the rat submandibular gland (9, 29). This implies that CFTR is involved in Ca2+-dependent Cl– movement during parasympathetic activation. Cross-talk between the Ca2+-activated Cl– channel and cAMP-regulated Cl– channel pathways is recognized, but much less is known about how CFTR is basally activated when Cl– is reabsorbed during parasympathetic stimulation.

The existing evidence suggests that the Ca2+- and cAMP-signaling systems are likely to communicate with each other (5, 11, 12, 15). Because CFTR is known to modulate other types of Cl– channels [e.g., outwardly rectifying Cl– channels, ORCC (20)], sympathetic activation of CFTR located on the apical membrane of duct cells may augment the proximally located Cl– channels used during muscarinic stimulation. Alternatively, since CFTR is known to be modulated not only by PKA, but also by PKC (10, 28), it is possible that the CFTR Cl– channel activated tonically by sympathetic signaling is regulated directly by the muscarinic receptor-PLC–PKC pathway. However, another mechanism may be involved in the latter scenario. In salivary glands, P2X4, P2X7 (P2Z), P2Y1, and P2Y2 have been reported to be present (25, and Zeng et al. (30, 31) suggested that P2-receptor activation stimulates both Ca2+-dependent Cl– channels and Ca2+-insensitive, CFTR-like Cl– channels in the rat submandibular gland. Further, a potentially important finding reported a few years ago was of a paracrine-like ATP-mediated secretion in pancreatic acini (22). An intriguing possibility is that, likewise, salivary acini might release an endogenous activator during muscarinic stimulation and that this might regulate CFTR-like Cl– conductance in that ductal epithelium by a similar mechanism.

To elucidate the above possibilities, we measured ion concentrations in the saliva collected during muscarinic receptor stimulation after retrogradely injecting one rat salivary duct either with one of several activators or inhibitors of P2 receptors or CFTR or with a short double-stranded small interfering RNA (siRNA) designed to knock down CFTR gene function. In these studies, we obtained evidence suggesting that 1) the basal CFTR activation contributes to the transepithelial Cl– movement evoked by muscarinic stimulation, 2) basal CFTR activity is regulated by luminal P2Y2 receptors located in the duct, and 3) ATP secreted into the salivary fluid is involved in this basal activity.

MATERIALS AND METHODS

Animals and administration of chemicals. Male Wistar rats (8 wk old) were anesthetized with pentobarbital sodium (50 mg/kg ip; Nembutal; Abbott Laboratories, Abbott Park, IL), permission for the procedures used having been granted by the Animal Research Committee of Fukuoka Dental College. One submandibular duct (referred

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to as the injected side) was cannulated intraorally via its orifice in the sublingual papilla.

ATP, UTP (both from Wako Pure Chemicals, Osaka, Japan), or 2’3’-O-(4-benzoylbenzoyl)-ATP (Bz-ATP) (Sigma-Aldrich, St. Louis, MO) was injected retrogradely (60 µl) into the submandibular duct 5 min before an intraperitoneal administration of pilocarpine HCl, a muscarinic-receptor agonist (Wako Pure Chemicals). (±)-Propranolol HCl (Wako Pure Chemicals) was administered intraperitoneally 30 min before pilocarpine injection. CFTR<sub>rab-172</sub> (Calbiochem, La Jolla, CA) or suramin Na (Wako) was injected retrogradely (100 and 60 µl, respectively) into the duct 5 min before administration of pilocarpine or a purinergic agonist. CFTR<sub>rab-172</sub> was dissolved in DMSO to make a stock solution. All compounds were finally dissolved in saline (final concentration of DMSO ≤ 0.1%). In the case of saline containing ATP, UTP, or Bz-ATP, the pH was adjusted using NaOH to 6.0–7.0.

A small interfering RNA (siRNA) was synthesized by B-Bridge International (Sunnyvale, CA) in a purified and annealed duplex form. The siRNA sequence was designed to target the rat CFTR gene, the accession number of the targeted mRNA sequence being XM_342645. For the rat isoform of CFTR (with the sense sequence 5’-GCCUUAAAGGAAGAGGAUAUdTdT-3’). For the negative siRNA control, the sequence is scrambled to yield a corresponding negative control with the same GC content and nucleic acid composition. According to a BLAST search (2), this siRNA possesses no significant homology to other mRNAs within existing databases for rats (GenBank, EMBL, DDBJ, and PDB; mismatching nucleotides ≥4).

Retrograde ductal injection with siRNA was performed as reported previously (9). Briefly, one submandibular duct (referred to as the injected side) was cannulated, and a total of 2 nmol siRNA (CFTR siRNA or negative siRNA) was suspended with a hemagglutinating virus of a Japan envelope vector (GenomONE-Neo; Ishihara Sango Kaisha, Osaka, Japan) and was injected retrogradely into the submandibular gland. Two days after the above injection, rats were again anesthetized with pentobarbital sodium, and the submandibular duct on the injected side was cannulated intraorally. The above methodology allows us to examine the local function of CFTR and P2 receptors in the epithelium facing the luminal space.

For validating experiments to show successful blockade of β-receptors by propranolol, a femoral artery was cannulated with a catheter connected to a pressure transducer, and arterial pressure and heart rate were measured (AP-621G and AT-601G, Nihon Kohden, Tokyo, Japan). A femoral vein was cannulated for administration of a heart rate were measured (AP-621G and AT-601G, Nihon Kohden, Tokyo, Japan). A femoral vein was cannulated for administration of a

Immunohistochemistry. For the immunohistochemical study, rat submandibular glands were dissected out and formalin-fixed; 5-µm-thick tissue sections were mounted on slides coated with silane. To enhance the immunoreactivity, the tissues on the slides were heated for 15 min in 0.1 M sodium citrate buffer (pH = 6.0) using a microwave. The tissues were then immersed in 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 20 min to block endogenous peroxidase activity, washed in PBS, and finally incubated in 10% normal rabbit serum. The sections were then incubated either with a polyclonal rabbit anti-CFTR antibody (ACL006; diluted 1:100; Alomone Labs, Jerusalem, Israel) or with a polyclonal rabbit anti-P2Y<sub>2</sub> antibody (P6612; diluted 1:100; Sigma-Aldrich, MO), in each case for 3 h at room temperature.

The bound antibody was detected by the universal immuno-enzyme polymer method (Histofine Simple Stain Rat MAX PO kit; Nichirei, Tokyo, Japan). After the above reactions, the sections were treated with 0.02% 3,3’-diaminobenzidine tetrahydrochloride (Dokin Laboratories, Kumamoto, Japan) in PBS together with 0.003% H<sub>2</sub>O<sub>2</sub> and then counterstained with 1% methylgreen.

For immunofluorescence detection, the heat-treated tissues were incubated in 10% normal goat serum-containing PBS and then were incubated for 2 h at room temperature with the same buffer containing two types of primary rabbit polyclonal antibodies (or only one type in the case of the negative control experiments), each of which had been conjugated separately with a different kind of fluorophore-labeled Fab fragment (Zenon Alexa Fluor 488 and 594 Rabbit IgG; Molecular Probes, Eugene, OR). The primary antibodies used were against either CFTR (ACL006; diluted 1:100) or the P2Y<sub>2</sub> receptor (P6612; diluted 1:100). The tissues were postfixed in 4% paraformaldehyde.

Fluorescence was observed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK) equipped with a Nikon 100 × 0.4 oil immersion lens and appropriate filters. ImageJ 1.37v (National Institutes of Health, Bethesda, MD) and Adobe Photoshop 7.0 (Adobe Systems Incorp., San Jose, CA) were used for image processing.

Statistics. All values are presented as means ± SE, where n is number of observations. A grouped or paired t-test was performed for the statistical analysis of two groups. A one-way ANOVA followed by a post hoc Bonferroni’s t-test was employed when three or more groups were to be compared. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Role of CFTR in Cl- reabsorption within the ductal system. First, we performed immunohistochemical analysis using anti-CFTR antibody to confirm the localization of CFTR Cl- channels in the rat submandibular gland. Immunostaining revealed intense CFTR signals in the apical plasma membranes of intralobular ductal epithelial cells and also faint staining in the acinar cells (Fig. 1A).

The Cl-, Na<sup>+</sup>, and K<sup>+</sup> concentrations in the final saliva secreted during stimulation with pilocarpine HCl (8 mg/kg ip) (Figs. 2 and 3) were comparable to those measured in our previous study (9). The values were markedly different from those reported for primary saliva ([Cl<sup>-</sup>] = 112 mM, [Na<sup>+</sup>] = 136 mM and [K<sup>+</sup>] = 8.4 mM, (21)), as expected if the ion concentrations are drastically modified, while the primary saliva is flowing down through the ducts. CFTR-siRNA significantly increased the Cl<sup>-</sup> concentration in the saliva secreted under the influence of pilocarpine (Fig. 2). In the same condition, pretreatment with CFTR-siRNA greatly diminished the CFTR immunoreactivity described above (Fig. 1C), but pretreatment with the negative control RNA did not (Fig. 1B).

The effect of CFTR-siRNA on the Cl- concentration is likely to be due to an inhibition of CFTR-mediated Cl- reabsorption from the primary saliva flowing down through the ducts (9). The Na<sup>+</sup> concentration was not different between the CFTR siRNA and negative control groups. The K<sup>+</sup> concentration was higher in the CFTR-siRNA group than in its control group [discussed in the paper by Ishibashi et al. (9)]. Flow rate was significantly lower in the CFTR-siRNA group than in the...
negative controls, while the protein concentration in the saliva was not different between these two groups (Fig. 2). This indicates that basal CFTR activity contributes mainly to salivary water movement, rather than to protein secretion.

Because the above data imply that basal CFTR activity induces Cl\textsuperscript{−} reabsorption, we initially thought that basal activation of the sympathetic nervous supply might be involved in the underlying mechanism. A previous report indicated that a β-adrenoceptor antagonist, propranolol, effectively inhibited the isoproterenol-induced protein release from the rat submandibular gland (1). To explore the possible involvement of the basal level of sympathetic activity in Cl\textsuperscript{−} reabsorption, we determined whether propranolol would increase the Cl\textsuperscript{−} concentration in the saliva flowing during pilocarpine stimulation. Our initial pilot experiment showed that systemic administration of (±)-propranolol HCl (10 mg/kg ip) caused a significant negative chronotropic effect (heart rate: before, 375 ± 17 beats/min; 30 min after, 236 ± 12 beats/min, \( P = 0.004, n = 4 \)) and also tended to cause a hypertensive effect (diastolic arterial pressure: before, 108 ± 6 mmHg; 30 min after, 119 ± 5 mmHg, \( P = 0.08, n = 4 \)). This effect of propranolol was sustained for more than 30 min, indicating a long-lasting inhibition of β-adrenergic receptors within the rat cardiovascular system. Propranolol also potently inhibited the (-)-isoproterenol HCl (1 μg/kg iv)-induced hypotensive effect (decrease in diastolic arterial pressure: before, 54 ± 3 mmHg; 30 min after, −5 ± 2 mmHg, \( P = 0.0006, n = 4 \)). Under the same conditions, however, propranolol HCl (10 mg/kg ip) failed to increase the salivary [Cl\textsuperscript{−}] (Fig. 3), suggesting little or no involvement of a β-adrenergic pathway in the Cl\textsuperscript{−} movement induced by basal activation of CFTR. In contrast, the same propranolol administration significantly decreased the protein concentration, suggesting that this compound was effective at inhibiting the basal sympathetically activated protein secretion from the salivary tissue. The K\textsuperscript{+} concentration was lower in the propranolol group than in the control group (Fig. 3). Previously, isoproterenol has been
reported to cause propranolol-inhibitable potentiation of the carbachol-induced $K^+$ current in the rat parotid (8). Because $K^+$ is known to be secreted into the luminal fluid as it passes through the ductal structure (21), inhibition of ductal $K^+$ permeability may explain the propranolol-induced decrease in $K^+$ concentration in the final saliva.

Involvement of P2Y2 receptors in CFTR-dependent Cl⁻ reabsorption. Certain bioactive compounds present in the luminal fluid need to be considered as candidates for the agents maintaining the CFTR activity needed for Cl⁻ reabsorption. Here, we examined whether or not released ATP might mediate CFTR-dependent Cl⁻ reabsorption. First, we tried to semi-quantify the ATP concentration in the final saliva evoked by administration of pilocarpine HCl (8 mg/kg ip). Luminometer detection of the luciferase-catalyzed reaction between luciferin and ATP gave an estimate for the ATP concentration of 38.4 ± 8.6 nM ($n$ = 5). Because this value was lower in saliva samples examined without prior boiling (−10 nM; see MATERIALS AND METHODS), intrinsic ATP hydrolysis presumably led to a substantial underestimation of the amount of ATP released.

Next, we examined whether or not exogenously applied ATP might alter the Cl⁻ concentration in the saliva elicited by pilocarpine administration. After intraductal injection of ATP, the amount of ATP remaining in the salivary fluid can be presumed to decrease drastically with time, probably because of its degradation and dilution in the saliva. The concentration, which was measured by the method of luciferin fluorescence, was found to decrease from 446 ± 45 μM ($n$ = 3) during a sampling time consisting of the first 1 min after an intraductal injection of ATP (60 nmol) to a minimum value (55 ± 6 nM, $n$ = 3) at more than 10 min after the intraductal injection. The salivary flow rate was also decreased from 15.7 ± 7.7 μl/min in the first 1 min to 3.4 ± 1.2 μl/min at more than 10 min after the injection. On the basis of the values obtained for ATP concentration and salivary flow rate in five different sampling periods, the mean value of the ATP concentration in the total salivary fluid (collected for 15 min) was estimated to be 79.5 ± 42.8 μM ($n$ = 3). This estimated ATP concentration appears to be within the physiological range for ATP responses in salivary glands and possibly does not reach the level needed for maximal activation (3, 30). We therefore injected ATP retrogradely in amounts of 6–600 nmol in the present study.

Intraductal injection of ATP decreased the salivary Cl⁻ concentration significantly in a dose-dependent manner (6 to 600 nmol, Fig. 4A). For a first estimation of the receptor subtype involved in this action, we tested UTP (a P2Y2/Y4 agonist) and Bz-ATP (a P2X7 agonist) since both P2Y2 and P2X7 receptors are known to be present in salivary tissues (25). Intraductal injection of UTP decreased the salivary Cl⁻ concentration significantly at 6 and 60 nmol (Fig. 4B). At both 6 and 60 nmol, ATP and UTP seemed to be almost equipotent at decreasing the Cl⁻ concentration, suggesting that P2Y2 is a candidate for the receptor subtype involved in this mechanism. In contrast, injection of Bz-ATP (6 and 60 nmol) had no
significant effect on the Cl\(^-\) concentration (Fig. 4C). In support of the above result, we next confirmed the presence of immunoreactivity for P2Y\(_2\) receptors in rat submandibular ducts. In the ductal epithelium, intense P2Y\(_2\) immunoreactivity was seen both on the apical membranes and intracellularly near the apical membrane, although it was not distributed evenly among all cells (Fig. 1D).

CFTRinh-172 is a highly selective CFTR inhibitor (13). Intraductal injection of CFTRinh-172 increased the Cl\(^-\) concentration significantly in a dose-dependent manner (0.3–6 nmol, Fig. 5A). This result strengthens our idea that CFTR is involved in Cl\(^-\) reabsorption from the saliva evoked by muscarinic stimulation. Suramin also increased the Cl\(^-\) concentration in a dose-dependent manner (6–60 nmol, Fig. 6A). Although suramin is a nonspecific antagonist of purinergic receptor subtypes, this result is consistent with an endogenous purinergic stimulation through P2 receptors being involved in this Cl\(^-\) reabsorption. After injection of CFTRinh-172 (6 nmol), exogenous administration of ATP (60 nmol) or UTP (60 nmol) failed to alter the salivary Cl\(^-\) concentration (Fig. 5, B and C). Similarly, after injection of suramin (6 nmol), ATP (60 nmol), or UTP (60 nmol) had no further effect (Fig. 6, B and C). These results suggest that activities of both CFTR and the P2Y\(_2\) receptor are likely to participate in ATP- and UTP-induced Cl\(^-\) movement in the rat submandibular salivary ducts.

**Colocalization of CFTR and P2Y\(_2\) in ductal epithelium of rat submandibular gland.** Examining immunofluorescence using confocal microscopy revealed some P2Y\(_2\) signals in close proximity to the CFTR immunofluorescence, presumably on the apical surface of a single ductal cell (Fig. 7, A and B). In the presence of either P2Y\(_2\) antibody or CFTR antibody alone, the corresponding signal could be detected without any nonspecific staining (Fig. 7, C and D).

**DISCUSSION**

We previously reported in vivo evidence in favor of CFTR having a physiological significance in Cl\(^-\) reabsorption within the ductal system of the rat submandibular gland (9). However, it has not been clarified how basal CFTR activation contributes to the transepithelial Cl\(^-\) movement present under muscarinic stimulation. The main aim of this current project was to determine what type of receptors might affect the basal state of the local CFTR function. To this end, we measured the Cl\(^-\) concentration in the final saliva flowing through the rat sub-
mandibular duct. In general, if a given inhibitor increases the Cl\textsuperscript−/H\textsubscript{1002}\textsuperscript{−} concentration in the luminal fluid or if a given activator decreases the Cl\textsuperscript−/H\textsubscript{1002}\textsuperscript{−} concentration in the luminal fluid, then the corresponding receptor/ion channel could play a role in Cl\textsuperscript−/H\textsubscript{1002}\textsuperscript{−} reabsorption in the duct. However, interpreting the actions of a given agent in the duct could be complicated if the volume of saliva secreted from the acini is also altered by the same intervention. Indeed, the Na\textsuperscript+/H\textsubscript{1001}\textsuperscript{+} and Cl\textsuperscript−/H\textsubscript{1002}\textsuperscript{−} concentrations of the rat sublingual saliva were reported to increase as flow rate increased (but only when the flow rate was <25 μl·mg\textsuperscript{−1}·min\textsuperscript{−1}) (21). In the present study, the salivary flow rate was the same before and after most of the interventions. In cases in which the volume did alter, the Na\textsuperscript{+} concentration appeared not to change in the same direction. Moreover, the salivary flow rate during pilocarpine stimulation (40 to 80 μl·mg\textsuperscript{−1}·min\textsuperscript{−1}) was as high as the level (>35 μl·mg\textsuperscript{−1}·min\textsuperscript{−1}) at which Na\textsuperscript{+} and Cl\textsuperscript− concentrations were at a plateau in the above report (21). Thus, we believe that the influence of salivary flow per se on ductal Cl\textsuperscript− reabsorption was minimal in the present study. Here, under in vivo conditions, inhibition of CFTR (either by its specific siRNA or by CFTR\textsubscript{inh-172}) caused an increase in the Cl\textsuperscript− concentration of the final saliva evoked by pilocarpine administration, strengthening the idea that a CFTR-mediated pathway plays a physiologically significant role in the modulation of the salivary Cl\textsuperscript− composition.

Previously, isoproterenol was reported to suppress the carbachol-induced Cl\textsuperscript− current in the perfused submandibular gland in rats (23). The present finding that propranolol failed to alter the salivary Cl\textsuperscript− concentration argues against the idea that tonic activation of the sympathetic nervous supply is required for basal CFTR activity. An alternative notion is that some bioactive compounds in the luminal fluid may serve to maintain this activity. Nucleotide triphosphates have been detected...
in the isolated rat submandibular gland in an NMR study using $^{31}$P (16). Moreover, there is evidence for the expression of two P2X ligand-gated ion channels (P2X$_1$/P2Z and P2X$_2$) and two P2Y G protein-coupled receptors, (P2Y$_1$ and P2Y$_2$) in rat salivary glands (25). In addition, a functional study on polarized monolayers of the ParC10 parotid epithelial cell line revealed the exclusive presence of P2Y$_2$ receptors on the luminal membranes (26), a finding supported by the present immunohistochemical demonstration that P2Y$_2$ is expressed on the apical side of the ductal cells of the rat submandibular gland. Furthermore, P2-receptor activation has been reported to stimulate both Ca$^{2+}$-dependent Cl$^{-}$ channels and Ca$^{2+}$-insensitive, CFTR-like Cl$^{-}$ channels in rat submandibular gland (30, 31). However, a conflicting view has been put forward, namely, that ATP-gated Cl$^{-}$ conductance in mouse parotid acinar cells is unlikely to be associated with any stimulation of purinergic receptors (3). Thus, to help determine whether any purinergic receptor is involved in Cl$^{-}$ movement across the ductal cells, it was of interest to examine whether or not ATP is released during muscarinic stimulation and whether it is involved in a paracrine fashion in a P2 receptor-mediated increase in CFTR Cl$^{-}$ conductance in the ductal epithelium, as reported for rat pancreatic acini (22).

A recent study showed that the major purinergic receptors expressed in mouse submandibular ductal cells are P2X$_2$ receptors (18). However, the P2X$_2$ receptor appears not to be involved in the present mechanism since Bz-ATP had little effect on the Cl$^{-}$ concentration of the saliva. In the present study, ATP and UTP induced almost equipotent, suramin-inhibitable increases in the Cl$^{-}$ concentration of the luminal fluid, and suramin itself increased the Cl$^{-}$ concentration. These results suggest that endogenous activation of P2Y$_2$ receptors is involved in mediating the transepithelial Cl$^{-}$ movement evoked by muscarinic stimulation (Fig. 8). A further question then arises as to the mechanism by which the P2Y$_2$ receptor might modulate CFTR activity. Phosphorylation of the CFTR Cl$^{-}$ channel by the cAMP-dependent protein kinase, PKA, regulates CFTR, and Ca$^{2+}$-dependent and Ca$^{2+}$-independent isoforms of PKC activate a recombinant CFTR Cl$^{-}$ channel (4). Moreover, the CFTR Cl$^{-}$ channel has been reported to be modulated by both PKA and PKC, acting synergistically, in a heterologous expression system (10, 28). In addition, in mouse heart, a purinergic receptor was shown to be coupled to CFTR through a PKC signaling pathway (6, 27). Furthermore, cAMP-independent activation of CFTR can occur via phosphorylation by kinases other than PKA, including a Ca$^{2+}$-independent PKC isoform, in native human sweat ducts (19). In porcine endometrial gland epithelial cells, UTP- and phorbol ester-induced activation of CFTR has recently been shown to occur in a manner that is independent of an increase in intracellular cAMP but dependent on PKC activation (17). Collectively, the above reports support the idea that both nucleotides (ATP and UTP) activate the CFTR Cl$^{-}$ channel through the P2Y$_2$ receptor-PLC-PKC pathway (Fig. 7). Using confocal microscopy, we confirmed the proximity of P2Y$_2$ receptors and CFTR Cl$^{-}$ channels on the luminal surface of the ductal epithelial cells, a finding consistent with a functional coupling of these membrane proteins.

ATP would be expected to be easily degraded by various ecto-ATPases. Sørensen and Novak (22) demonstrated that the concentration of ATP in the supernatant of rat pancreatic acini (22 nM) following stimulation by carbachol was 400 times lower than that (9 µM) released into the surrounding fluid (as monitored using confocal microscopy). Moreover, the local concentration of released ATP at the surface of a single pancreatic β cell was shown by a biosensor technique to be over 25 µM (7). The ecto-ATPase activity in saliva appears to be tremendously high, and probably for that reason, we could not detect (by the method of luciferin fluorescence) an ATP concentration high enough to have the physiological effect of 1) decreasing the salivary Cl$^{-}$ concentration (100 µM, the value estimated based on the injection volume of 60 µl and the ATP content of 6 nmol; in the present study) or 2) activating CFTR Cl$^{-}$ currents (0.1–100 µM) (27).

**Perspectives and Significance**

On the basis of the above discussion, it is tempting to argue in favor of the paracrine hypothesis [namely, that ATP released from acinar cells causes ductal Cl$^{-}$ reabsorption via activation of P2Y$_2$ receptors and CFTR Cl$^{-}$ channels (Fig. 8)], leaving aside the intrinsic limitations concerning the quantification of the easily degraded endogenous substance. Possibly, the released ATP may behave as a mediator by which the saliva volume directly regulates the hypotonicity of the final saliva via Cl$^{-}$ reabsorption. In addition to the well-known autonomic nervous regulation, such a local mediator could allow a tissue-wide coordination of distinct functions in the acinar and ductal regions of a given exocrine gland.

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