Characterization and Localization of P$_2$ Receptors in Rat Submandibular Gland Acinar and Duct Cells*

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Min Goo Lee, Weizhong Zeng, and Shmuel Muallem‡

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

[Ca$^{2+}$], and the Cl$^{-}$ current were measured in isolated submandibular gland acinar and duct cells to characterize and localize the purinergic receptors expressed in these cells. In both cell types 2'-3'-benzoylbenzoyl (Bz)-ATP and ATP increased [Ca$^{2+}$], mainly by activation of Ca$^{2+}$ influx. UTP had only minimal effect on [Ca$^{2+}$], at concentrations between 0.1 and 1 mM. However, a whole cell current recording showed that all nucleotides effectively activated Cl$^{-}$ currents. Inhibition of signal transduction through G proteins by guanyl-5'-β-thiophosphate revealed that the effect of ATP on Cl$^{-}$ current was mediated in part by activation of a G protein-coupled and in part by a G protein-independent receptor. BzATP activated exclusively the G protein-independent portion, whereas UTP activated only the G protein-dependent portion of the Cl$^{-}$ current. Measurement of [Ca$^{2+}$], in the microperfused duct showed that ATP stimulated a [Ca$^{2+}$], increase when applied to the luminal or the basolateral sides. BzATP increased [Ca$^{2+}$], only when applied to the luminal side, whereas UTP at 100 μM increased [Ca$^{2+}$], only when applied to the basolateral side. The combined results suggest that duct and possibly acinar cells express P$_2$Z receptors in the luminal and P$_2$U receptors in the basolateral membrane.

The submandibular salivary gland (SMG)$^1$ secretes fluid rich in K$^-$–HCO$_3$$. Acinar cells secrete the primary, NaCl-rich fluid into the duct, which reabsorbs most of the NaCl in exchange for K$^-$–HCO$_3$$. As in any other CFTR-expressing tissues (2–4) and Na$^+$-transporting epithelia (5), Cl$^{-}$ channels play a central role in transcellular ion transport in the two cell types of SMG (1). However, unlike that of other epithelia, little is known of the regulation of Cl$^{-}$ channels in salivary gland cells, in particular regulation by purinergic (P$_2$) receptors.

In the intact salivary gland secretion is regulated by several agonists, which include cholinergic, α-adrenergic, and β-adrenergic agonists (6–8). The agonists act on both acinar and duct cells but modulate different activities in the two cell types. In acinar cells the agonists stimulate secretion primarily by stimulation of the basolateral NaKCl$_2$ cotransporter (9). In the duct the same agonists reduce the transepithelial potential and Na$^+$ reabsorption (6–8). Work on the cellular level showed that cholinergic and α-adrenergic agonists act on salivary acinar and duct cells to increase [Ca$^{2+}$], (10–15), and β-adrenergic agonists increase cAMP (11, 16).

Salivary acinar and duct cells also respond to purinergic stimulation by a change in [Ca$^{2+}$], (15, 17–22). However, the identity of the receptors and their membrane localization is not clear. Response of duct cells to BzATP and several other nucleotides suggested that duct cells express P$_2$Z and P$_2$Y$_1$ receptors (37). Response of acinar cells to BzATP was interpreted to suggest that acinar cells express P$_2$Z receptors (20, 22). More recently it was shown that acinar and duct cells in long term culture up-regulate the P$_2$Y$_2$ receptors (29). However, whether native and freshly isolated cells express P$_2$Y$_2$ receptors and how these and other purinergic receptors regulate cellular activity is not known.

P$_2$Z receptors are believed to play a central role in regulating Cl$^{-}$ transport in CFTR-expressing tissues (3, 4, 24–27). Because of our recent findings of CFTR expression in SMG duct and acinar cells (28) and the expression of P$_2$ receptors in SMG cells (15, 17–22), in the present work we studied the regulation of Cl$^{-}$ channel activity by P$_2$ receptors in SMG duct and acinar cells. For the purpose of these studies it was necessary to characterize and localize the P$_2$Z receptors in the two cell types. Measurements of [Ca$^{2+}$], and Cl$^{-}$ current were used to show that SMG acinar and duct cells express at least two types of P$_2$ receptors, a P$_2$Z and a P$_2$U receptor. The action of the P$_2$U receptors required the activation of G proteins, whereas that of the P$_2$Z receptors was independent of G proteins. Microperfusion of the SMG duct localized the P$_2$Z receptors in the luminal membrane and the P$_2$U receptors in the basolateral membrane. In a companion study (29) we show that the two P$_2$ receptors activate different Cl$^{-}$ channels that reside in the same membrane as the respective receptor.

**MATERIALS AND METHODS**

Cell Preparation—The extralobular duct of the SMG was microdissected, cannulated, and prepared for perfusion as described previously (15, 30, 31). The cannulated duct was removed to a Petri dish containing PSA buffer and immediately loaded with Fura 2 (see below). The composition of PSU is (in mM): NaCl 140; KCl 1; MgCl$_2$ 1; Hepes 10 (pH 7.4 with NaOH); glucose 10; pyruvate 10; bovine serum albumin 0.1%, and soybean trypsin inhibitor 0.02%. Acini and duct fragments were prepared by collagenase digestion as described previously (15). In brief, the SMGs of one rat were removed into PSA, finely minced, and incubated in 8 ml of PSA containing 2.5 mg of collagenase P for 10–12 min at 37°C. The dissociated cells were washed three times with PSA and kept on ice until use. To prepare single acinar and duct cells (for details, see Zeng et al. (28)) the finely minced SMGs were incubated in 5 ml of PSA containing 4 mg of collagenase CLS4 (254 units/mg from Worthington) for 20 min at 37°C. The partially digested tissue was washed twice in phosphate-buffered saline and incubated in phosphate-buffered saline containing 0.05% trypsin, 0.02% EDTA (Sigma) for 8 min at 37°C. The tissue was washed twice with PSA and incubated in 6 ml of PSA containing 3.2 mg of collagenase for 20 min at 37°C. The digest containing small acinar clusters, duct fragments, and many single cells was washed with PSA and kept on ice until use. Acinar and duct cells were distinguished by size and by their capacitance which, in a typical
series of experiments, averaged 16.3 ± 0.1 picofarads (n = 47) in acinar and 4.2 ± 0.1 (n = 39) picofarads in duct cells.

**Loading with Fura 2 and Fluorescence Measurement**—For [Ca\(^{2+}\)]\(_i\), measurement, cells were suspended in 4 ml of PSA containing 5 μM Fura 2/AM and incubated for 20–30 min at room temperature. The cells were then washed once with 50 ml of PSA, resuspended in 2 ml of PSA, and kept on ice until use. The lumen of the extralobular duct was perfused with PSA containing Fura 2/AM. After the Fura 2 perfusion, the bath solution was changed twice to remove external dye. After 10–15 min of incubation at room temperature, the duct lumen was perfused with 0.2 ml of PSA, and the duct was mounted in the perfusion chamber.

The Ca\(^{2+}\)-loaded acini and duct fragments were plated on coverslips that formed the bottom of a perfusion chamber. After 2–3 min of incubation at room temperature, unattached cells were removed by perfusion with solution A (PSA without soybean trypsin inhibitor and pyruvate). The cells were perfused for at least 10 min with warm (37°C) solution A before exposure to agonists.

Perfusion was at a rate of 20 chamber volumes/min with warm solutions to maintain constant temperature. Fluorescence was measured with an image acquisition and analysis system from PTI as detailed elsewhere (15). Fura 2 fluorescence occurred at 355 and 380 nm and calibrated by exposing the cells to solutions containing high and low concentrations of Ca\(^{2+}\) and 10 μm ionomycin as described previously (15).

Fura 2 fluorescence in the perfused extralobular duct was measured as described (15). The lumen and the bath were continuously perfused, and agonists were employed by inclusion in the respective perfusion solution. In the case of bath stimulation with BzATP, the high cost of the agonist dictated application of the agonist to the bath by perfusion with 10 chamber volumes over 0.5 min and then stopping the perfusion until removal of bath stimulation by perfusion. Alternatively, the bath solution was aspirated and replaced with a solution containing 100 μM BzATP.

**Electrophysiology**—Cl\(^{-}\) currents were recorded using the whole-cell configuration of the patch clamp technique (32). Cl\(^{-}\) currents were isolated by using Cl\(^{-}\) as the only permeant ion in the pipette and bath solutions. In all experiments the bath solution contained (all in mM) 140 N-methyl-D-glucamine chloride, 10 Hepes (pH 7.4 with Tris), 1 MgCl\(_2\), and 1 CaCl\(_2\). In some experiments this solution was supplemented with 1 mM CaCl\(_2\) (Ca\(^{2+}\)-containing) or 0.2 mM EGTA (Ca\(^{2+}\)-free). Unless otherwise indicated, the pipette solution contained 140 N-methyl-D-glucamine chloride, 10 Hepes (pH 7.2 with Tris), 0.5 or 5 EGTA, 1 Tris-ATP, and 1 MgCl\(_2\). All recordings were made at room temperature. The glass pipettes used had a resistance of 2–3 MΩ. The access resistance for acinar cells was around 10 MΩ and for duct cells about 13 MΩ. Seals of 5–9 MΩ were obtained on the cell surface prior to establishing the whole-cell configuration by gentle suction and/or voltage pulses of 0.5 V for 0.3–1 ms. Macroscopic currents were recorded using the Axopatch-1B patch clamp amplifier (Axon Instruments). Results were collected at 5 kHz after filtering at 2 kHz. The membrane potential was held at −40 mV to record the outward currents. Periodically current-voltage relationships were recorded from −100 to +100 mV in 20-mV steps for a 800-ms duration from a holding potential of 0 mV. The potential was held at 0 mV for 1200 ms between voltage steps to record any tail currents.

**RESULTS**

**Regulation of [Ca\(^{2+}\)]\(_i\), by Purinergic Receptors**—To identify the purinergic receptors expressed in SMG acinar and duct cells and the role of [Ca\(^{2+}\)]\(_i\) in Cl\(^{-}\) channel regulation, we characterized the effect of ATP and other nucleotide triphosphates on [Ca\(^{2+}\)]\(_i\). Previous studies reported the effect of ATP and BzATP on [Ca\(^{2+}\)]\(_i\) of SMG acinar and duct cells (15, 17–22, 37). We showed that SMG duct cells respond to luminal ATP (15). Fig. 1, a and b, extends these findings to show that, in SMG duct cells, ATP and BzATP evoked a small Ca\(^{2+}\) release from internal stores and a marked increase in Ca\(^{2+}\) influx. UTP had no apparent effect on [Ca\(^{2+}\)]\(_i\) in the absence of external Ca\(^{2+}\) and caused a small increase in Ca\(^{2+}\) influx (Fig. 1c). The P\(_2\) agonists had similar effects in SMG acinar cells except that the absolute changes in [Ca\(^{2+}\)]\(_i\) were about 40–60% of those measured in duct cells (Figs. 1, d–f). Additional experiments showed that SMG acinar and duct cells failed to respond to other specific P\(_2\) agonists, which include 2-methylthio-ATP, α,β-methylene ATP, 2-chloro-ATP, ATPγS, ADP, and UDP (not shown). In a previous study (38) several of these nucleotides were shown to have a small effect on [Ca\(^{2+}\)]\(_i\), of duct cells. Despite persistent attempts we were unable to demonstrate the effects of the above nucleotides on [Ca\(^{2+}\)]\(_i\) or Cl\(^{-}\) current.

**Measurement of the dependence of [Ca\(^{2+}\)]\(_i\)**, increase on the concentration of nucleotides in both cell types is shown in Fig. 2. In both cells BzATP was the most potent agonist in terms of the maximal increase in [Ca\(^{2+}\)]\(_i\), and the apparent affinity. The effect of UTP on [Ca\(^{2+}\)]\(_i\), was very small, and the signal/noise ratio precluded accurate measurement of the potencies of these nucleotides. As expected, omission of Mg\(^{2+}\) from the incubation medium increased the apparent affinity for ATP and BzATP but did not change the relative potency of the nucleotides acting on the same cell type or the differences between the two cell types (not shown).

The finding that high concentrations of UTP were needed to observe any increase in [Ca\(^{2+}\)]\(_i\), and the ability of high concentration of UTP to interact with the P\(_2\) receptor (33) raised the question of whether each SMG cell expresses one or two types of P\(_2\) receptors. To address this question we tested the involvement of G proteins in the response to each nucleotide. For this we measured the activation of Cl\(^{-}\) current by the nucleotides using the whole cell configuration of the patch clamp technique. Transduction of signaling by G proteins was inhibited by including 2 mM GDPβS in the pipette solution. Fig. 3e shows that stimulation of duct cells with 25 μM epinephrine (Epi) caused a large increase in Cl\(^{-}\) current. Removal of Epi resulted in return of the current to base line. The same cell responded to stimulation with 1 mM ATP in a marked increase in Cl\(^{-}\) current. The control experiment for acinar cells is shown in Fig. 3c in which the cell was stimulated with carbachol and inhibited with atropine prior to stimulation with ATP. Epi and carbachol were used to stimulate duct and acinar cells because of their respective potency in increasing [Ca\(^{2+}\)]\(_i\), in the two cell types (10, 15). Fig. 3, b and d, shows that 2 mM GDPβS completely inhibited activation of Cl\(^{-}\) current by the G protein-coupled
The effect of different concentrations of ATP and BzATP on \([\text{Ca}^2+]\) was tested. Previous studies showed that stimulating ducts through the basolateral membrane with up to 100 \(\mu\text{M}\) ATP had no effect on \([\text{Ca}^2+]\). Inhibition of the effect of UTP by GDP\(\beta\)S was not an artifact, since the same cells responded to subsequent stimulation with ATP. The results of several experiments are summarized in Table I, which shows that 2 \(\text{mM}\) GDP\(\beta\)S inhibited the effect of BzATP by less than 10% and that of ATP by more than 85%.

To further address the question of the number of \(P_2\) receptors in SMG cells and localize the receptors to specific membranes, we used micropipetted ducts to measure the effect of the active nucleotides on \([\text{Ca}^2+]\). Previous studies showed that stimulating ducts through the basolateral membrane with up to 100 \(\mu\text{M}\) ATP had no effect on \([\text{Ca}^2+]\). In additional experiments with 1 \(\text{mM}\) and in three experiments with 5 \(\text{mM}\) GDP\(\beta\)S, the same effect as that shown in Fig. 3. In additional experiments with duct cells, in six experiments with acinar cells using 2 \(\text{mM}\) GDP\(\beta\)S, and in two experiments with 1 \(\text{mM}\) and in three experiments with 5 \(\text{mM}\) GDP\(\beta\)S with each cell type.

\(P_2\) receptors were expressed in SMG acinar and duct cells in both cell types. The later finding indicates that UTP acts without affecting the ability of BzATP to stimulate the current in both cell types. The latter finding indicates that UTP acts through a G protein-coupled receptor to mobilize \(\text{Ca}^2+\) and activate the \(\text{Cl}^-\) current.

**DISCUSSION**

The purpose of this study was to define and localize the \(P_2\) receptors expressed in SMG acinar and duct cells. The overall evidence supports the presence of at least two separate types of \(P_2\) receptors that are expressed in specific membranes of SMG cells.

Measurement of \([\text{Ca}^2+]\), and \(\text{Cl}^-\) current in the present and companion study (29) clearly shows the presence of \(P_2\) receptors in SMG acinar and duct cells. Thus, BzATP increases \([\text{Ca}^2+]\), in intercalated, granular, and the main duct and in acinar cells. BzATP also activated the \(\text{Cl}^-\) current in both cell types. The only \(P_2\) receptor responsive to BzATP and ATP but not to all the other nucleotides tested described so far is the \(P_2\) receptor (33).

The situation was less clear concerning the presence of \(P_2\) receptors since UTP had minimal effect on \([\text{Ca}^2+]\) in SMG cells. However, expression of \(P_2\) receptors in these cells is supported by the findings that at low concentrations UTP activated the \([\text{Ca}^2+]\)–activated \(\text{Cl}^-\) channel (29), 100 \(\mu\text{M}\) UTP acted only from the basolateral membrane of the perfused duct, and, most importantly, inhibition of G proteins with GDP\(\beta\)S almost completely inhibited the effect of UTP on the \(\text{Cl}^-\) current without affecting the ability of BzATP to stimulate the current in both cell types. The latter finding indicates that UTP acts through a G protein-coupled receptor to mobilize \(\text{Ca}^2+\) and activate the \(\text{Cl}^-\) current.

The expression of more than one \(P_2\) receptor that can increase \([\text{Ca}^2+]\), and activate the \(\text{Cl}^-\) current in SMG cells and the relatively small signals stimulated by UTP prevented definitive identification of the receptor activated by UTP. Unlike a previous study (38), pharmacological analysis with various nucleotides was not successful in our study to show the expression of \(P_2\) receptors in SMG duct cells. A recent study reported the induction of the \(P_2\) receptor type in SMG acinar and duct cells when maintained in primary culture (23).
molecular analysis (23) and the small effect of UTP on \([Ca^{2+}]_i\) reported here support the possibility that the P2Y2 receptors are present at low levels in freshly isolated SMG cells. However, activation of the P2Y2 receptors must have increased \([Ca^{2+}]_i\) to sufficiently high levels next to the plasma membrane to activate the Ca2+-dependent Cl− channels (29).

The use of the perfused duct allowed us to localize the P2Z receptors to the luminal membrane of SMG duct cells. Although ATP stimulated duct cells when applied to the apical or basolateral membrane, BzATP affected \([Ca^{2+}]_i\) only from the luminal side. UTP up to 100 μM acted only from the basolateral side, suggesting that SMG duct cells express the P2Y2 receptors in the basolateral membrane. The expression of these receptor subtypes in the respective membranes is quite specific to SMG and maybe to all salivary glands. Other CFTR-expressing epithelia, such as the airway and nasal epithelia, express P2Y2 or P2Y6 receptors in the luminal membrane and P2Y3 receptors in the basolateral membrane (3, 4, 24–27). The unique property of the P2Z receptor expressed in the luminal membrane of SMG cells is that this receptor also acts as an ion channel that can conduct Ca2+, Na+, and K+ (33, 34). Our study is the first to report activation of a Cl− current by the P2Z receptor. The cation selectivity of the P2Z receptor (33, 34) makes it unlikely that the receptor directly mediates the Cl− current. It would therefore be of interest to determine the type of Cl− channels activated by the P2Z receptor and the role of [Ca2+]i in such

### Table I

| Cell type | ATP (n = 4) | BzATP (n = 4) | UTP (n = 3) |
|-----------|------------|--------------|------------|
| Acini     |            |              |            |
| Duct      | 29.7 ± 3.6 | 19.9 ± 1.9\* | 18.0 ± 2.6 |
|           | 48.8 ± 4.4 | 16.7 ± 1.4   | 8.5 ± 0.7  |
|           | 51.8 ± 4.1 | 19.8 ± 0.9   | 26.2 ± 1.8 |
|           | 24.5 ± 0.4 | 3.2 ± 2.2    |            |

* pF = picofarads.

† a indicates p < 0.05 and b indicates p < 0.01 relative to control.
activation. This, together with the characterization of the Cl−
channels activated by the basolateral P2u receptors, is de-
scribed in our companion study (29).

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