Salivary glands express multiple isoforms of P2X and P2Y nucleotide receptors, but their \textit{in vivo} physiological roles are unclear. P2 receptor agonists induced salivation in an \textit{ex vivo} submandibular gland preparation. The nucleotide selectivity sequence of the secretion response was BzATP \textgreater ATP \textgreater ADP \textgreater UTP, and removal of external Ca\textsuperscript{2+} dramatically suppressed the initial ATP-induced fluid secretion (~85%). Together, these results suggested that P2X receptors are the major purinergic receptor subfamily involved in the fluid secretion process. Mice with targeted disruption of the P2X\textsubscript{7} gene were used to evaluate the role of the P2X\textsubscript{7} receptor in nucleotide-evoked fluid secretion. P2X\textsubscript{7} receptor protein and BzATP-activated inward cation currents were absent, and importantly, purinergic receptor agonist-stimulated salivation was suppressed by more than 70% in submandibular glands from P2X\textsubscript{7}-null mice. Consistent with these observations, the ATP-induced increases in [Ca\textsuperscript{2+}] were nearly abolished in P2X\textsubscript{7-/-} submandibular acinar and duct cells. ATP appeared to also act through the P2X\textsubscript{7} receptor to inhibit muscarinic-induced fluid secretion. These results demonstrate that the ATP-sensitive P2X\textsubscript{7} receptor regulates fluid secretion in the mouse submandibular gland.

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4 The abbreviations used are: [Ca\textsuperscript{2+}], intracellular free calcium concentration; BzATP, 2’,3’-O-(4-benzoylbenzoyl)adenosine 5’-triphosphate; SMG, submandibular gland(s); CCh, carbachol; GPCR, G protein-coupled receptors; SLG, sublingual; PG, parotid; ANOVA, analysis of variance; BSA, bovine serum albumin.
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animals had dramatically impaired ATP-activated Ca\textsuperscript{2+} signaling, consistent with this being the mechanism responsible for the reduction in ATP-mediated fluid secretion in these mice. Together, these results demonstrated that ATP regulates salivation, acting mainly through the P2X\textsubscript{7} receptor. Activation of the P2X\textsubscript{7} receptor may play a major role in non-adrenergic, non-cholinergic stimulated fluid secretion.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Mice were housed in microisolator cages with ad libitum access to laboratory chow and water during 12-hour light/dark cycles. An equal number of gender- andagematched (2–6-month-old) animals were utilized. Black Swiss/129 Svj hybrid (Rochester colony) and C57BL/6 strain mice were obtained from Jackson Laboratories (Bar Harbor, ME), while P2X\textsubscript{7}−/− C57BL/6 mice were obtained from Pfizer (Benton, CT) and used as indicated. All experimental protocols were approved by the University of Rochester Animal Resources Committee. Reagents were obtained from Sigma unless otherwise specified.

**Ex Vivo Submandibular Gland (SMG) Perfusion**—Ex vivo SMG perfusion was performed as previously reported (12, 13). In brief, mice were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg body weight). Following ligation of all branches of the common carotid artery except the SMG artery, the SMG was removed, cannulated, and perfused. The ex vivo perfusion solution contained (in mM): 4.3 KCl, 120 NaCl, 25 NaHCO\textsubscript{3}, 5 glucose, 10 HEPES, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, pH 7.4. Extracellular Ca\textsuperscript{2+}-free solutions were made by removing CaCl\textsubscript{2}. Solutions maintained at 37 °C were gassed with 5% O\textsubscript{2}, 5% CO\textsubscript{2}, and perfused at 0.8 ml/min using a peristaltic pump. When stimulating with only purinergic receptor agonists, muscarinic and β-adrenergic receptor antagonists were included (0.5 μM atropine and 20 μM propranolol, respectively).

Once the gland began to secrete fluid (defined as time 0), stimulation was continued for an additional 10 min. Saliva was collected in pre-calibrated capillary tubes (Sigma-Aldrich), and volumes were recorded every 0.5 or 1 min to calculate the flow rate (μl/min). Following saliva collection, the gland was blotted dried and weighed. Saliva samples were stored at −86 °C until further analysis. Na\textsuperscript{+} and K\textsuperscript{+} concentrations were analyzed by atomic absorption using a Perkin-Elmer 3030 spectrophotometer. The Cl\textsuperscript{−} concentration was analyzed with an Expandable Ion Analyzer EA 940 (Orion Research), and the pH and the osmolality were measured with a pH-sensitive electrode (Thermo Scientific, Beverly, MA) and a Wescor 5500 Vapor Pressure Osmometer (Logan, Utah), respectively.

**In Vitro [Ca\textsuperscript{2+}]\textsubscript{i} Measurement**—SMG ductal and acinar cells were prepared by enzyme digestion as previously reported (14). In brief, mice were euthanized by 100% CO\textsubscript{2} exposure followed by cardiac puncture. Acinar cells were dispersed in Minimum Essential Medium (SMEM, Invitrogen) supplemented with 1% bovine serum albumin (BSA), 0.17 mg/ml Liberase-RI (Roche Applied Science), and 2 mM l-glutamine, whereas duct cells were dispersed in Minimum Essential Medium (SMEM, Invitrogen) supplemented with 1% BSA, 0.012% trypsin, 0.05 mM EDTA, and 2 mM l-glutamine. Trypsin digestion was stopped with 2 mg/ml of soybean trypsin inhibitor. Duct cells were further dispersed by additional digestion in Minimum Essential Medium (SMEM) supplemented with 1% BSA, 0.15 units/ml Liberase-RI, and 2 mM l-glutamine. Following digestion, acinar and duct cells were rinsed in Basal Medium Eagle (BME, Invitrogen) supplemented with 1% BSA.

The fluorescent dye Fura-2 was used for [Ca\textsuperscript{2+}], measurement. Cells were loaded by incubation with 2 μM Fura-2 AM (Invitrogen) for 20 min at room temperature. Imaging was performed using an inverted microscope (Nikon Diaphot 200) equipped with an imaging system (Till Photonics, Pleasanton, CA). Images from Fura-2-loaded cells were acquired at a rate of 1 Hz by alternate excitation of light at 340 nm and 380 nm, and emission was captured at 510 nm using a high speed digital camera (Till Photonics). Chamber volume was maintained at ~400 μl. Cells were superfused at a rate of 4 ml/min with the ex vivo perfusion solution (37 °C). The fluorescence ratio of 340 nm over 380 nm was calculated, and all data are presented as the change in ratio units.

**Enrichment of Biotinylated Plasma Membrane Proteins**—Isolated SMG acinar cells (14) were biotinylated according to the manufacturer’s instructions (Pierce), and the plasma membrane proteins enriched as previously described (15). In brief, biotinylated cells were collected by centrifugation at 1,000 × g for 45 s, and the pellet homogenized twice in ice-cold homogenizing buffer containing: 250 mM sucrose, 10 mM triethanolamine, 1 μg/ml leupeptin, and 0.1 mg/ml phenylmethylsulfonyl fluoride. Unbroken cells and nuclei were pelleted at 4,000 × g for 10 min at 4 °C and discarded. The supernatants were centrifuged at 22,000 × g for 20 min at 4 °C. The resulting pellet was resuspended in homogenization buffer, centrifuged at 46,000 × g (Beckman SW28 rotor) for 30 min at 4 °C, and the crude pellet was resuspended in 1 ml of hypotonic buffer (100 mM NH\textsubscript{4}HCO\textsubscript{3}, pH 7.5, 5 mM MgCl\textsubscript{2}) followed by incubation overnight with 200 μl of Dynabeads M-280 streptavidin (Invitrogen Dynal AS; Carlsbad, CA) at 4 °C. Beads were collected with a magnetic plate and washed with hypotonic buffer. Streptavidin beads carrying the enriched plasma membrane fractions were suspended in 100 μl dithiothreitol for 2 h, centrifuged at 10,600 × g for 3 min, and the supernatants collected and used for immunoblotting.

**Electrophoresis and Immunoblot Analysis**—Protein samples (30 μg) were boiled for 5 min prior to separation in a 10% SDS-PAGE Tris-glycine mini-gel (Bio-Rad). Protein was transferred overnight at 4 °C onto polyvinylidene difluoride membranes (Invitrogen) as described previously (15). Membranes were blocked overnight at 4 °C with 5% nonfat dry milk in 25 mM Tris pH 7.5, 150 mM NaCl (TBS) and then incubated at 4 °C overnight with primary antibody raised against the 576–595 C-terminal of the rat P2X\textsubscript{7} receptor (Millipore-Chemicon International Inc., Temecula, CA) at a dilution of 1:300 in a 2.5% nonfat dry milk solution. After washing with TBS containing 0.05% Tween-20 (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Pierce) at a dilution of 1:2,500 in TBS-T/2.5% nonfat dry milk for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence (GE-Amersham Biosciences, Piscataway, NJ).
**RESULTS**

**Purinergic Receptor Agonists Evoke Fluid Secretion from the ex Vivo SMG**—Salivary glands express several types of purinergic receptors which belong to both the P2X (P2X1 and P2X2) and P2Y (P2Y1 and P2Y2) receptor subfamilies (10, 11). Given that activation of P2X and P2Y receptors evokes an increase in \([\text{Ca}^{2+}]_i\), in salivary gland cells, purinergic stimulation of either subfamily might be expected to result in fluid secretion (2). However, purinergic receptor activation has not been previously performed in the intact gland. To test this hypothesis we employed an *ex vivo*, perfused mouse SMG organ system. This *ex vivo* technique allows for precise control of the content of the vascular perfusate and ameliorates the rapid degradation of purinergic receptor agonists observed *in vivo*.

Most G protein-coupled P2Y receptors are sensitive to UTP whereas the ionotropic P2X receptors are not, while both classes of P2 receptors are generally activated by ATP and ADP (6). To determine if purinergic receptor activation produced saliva secretion in the *ex vivo* SMG preparation we initially utilized the ubiquitous P2 receptor activator ATP. Fig. 1A shows that the time course of the secretion generated by stimulation of the *ex vivo* SMG by ATP (1 mM) was best described as having an initial peak during the first 2 min followed over the next 8 min by a gradual decline to a relatively sustained fluid secretion rate (Fig. 1A, *black squares*). A relatively rapid loss of secretion was observed after the removal of ATP from the perfusate. To further characterize which P2 receptor was likely involved we also tested the ability of ADP and UTP to stimulate secretion. ADP (1 mM) produced a similar pattern as observed during ATP stimulation (Fig. 1A, *gray squares*), but the total amount of saliva produced during a 10-min stimulation was significantly less (Fig. 1B, ADP; 15.2 ± 2.8 µl/10 min versus ATP; 43.7 ± 3.5 µl/10 min, *p < 0.001*). Stimulation with UTP (1 mM) produced a transient flow (Fig. 1A, *white squares*) which generated markedly less fluid, ~6% of the saliva secreted with an identical concentration of ATP (Fig. 1B, UTP; 2.7 ± 0.7 µl/10 min versus ATP; 43.7 ± 3.5 µl/10 min, *p < 0.001*).

**Extracellular Ca²⁺ Depletion Suppresses ATP-induced Salivation by the ex Vivo SMG**—UTP does not activate P2X receptors (6). Accordingly, because UTP caused minimal fluid secretion (Fig. 1), it appeared that P2Y receptors do not play a major role in the purinergic-induced production of saliva. Therefore, we next evaluated the role of P2X receptors in ATP-mediated, Ca²⁺-dependent fluid secretion. Although both couple to an increase in \([\text{Ca}^{2+}]_i\), one fundamental difference between P2Y and P2X receptors is that P2X receptors mediate extracellular Ca²⁺ entry, whereas P2Y receptors initially trigger intracellular Ca²⁺ release. Consequently, acute removal of extracellular Ca²⁺ effectively eliminates Ca²⁺ influx via P2X channels, but the intracellular Ca²⁺ release mediated by P2Y receptors is resistant to this maneuver.

Using the muscarinic agonist carbachol (CCh), which activates an identical Ca²⁺ release GPCR pathway as P2Y receptors, we show that initial secretion by the *ex vivo* SMG was not significantly affected by acute removal of extracellular Ca²⁺. CCh-evoked (0.3 µM) SMG fluid secretion in the absence of extracellular Ca²⁺ (*black squares*) was similar to secretion in Ca²⁺-containing experiments (*gray squares*) during the first 10 min of stimulation (Fig. 1C). However, no secretion was observed following removal of Ca²⁺ from the perfusate (Fig. 1C, *white squares*).

**Electrophysiological Recordings**—Single SMG acinar cells were prepared by enzymatic digestion (13). In brief, SMG acinar cells were digested for 15 min in SMEM containing 0.02% trypsin (Invitrogen), then centrifuged and resuspended in medium containing soybean trypsin inhibitor (Type 1-5, Sigma), followed by 2 sequential digestions for 25 min each in 0.17 mg/ml Liberase RI Enzyme (Roche Applied Science, Indianapolis, IN). The cell suspension was gently centrifuged and the supernatant filtered through a 53-µm nylon mesh. Finally, the suspension was centrifuged, and the cell pellet was resuspended in BME supplemented with 2 mM L-glutamine (Invitrogen), then centrifuged and resuspended in 10 ml of BME. Cells were maintained at 37 °C in a 5% CO₂ humidified incubator until use.

Electrophysiological data were acquired at room temperature using a PC-501A amplifier (Warner Instrument, Hamden, CT) or an Axopatch 200b amplifier (Molecular Devices, Sunnyvale, CA). Voltage pulses were generated with Clampex 9 software through a Digidata 1320A interface (Molecular Devices), which also served to acquire the currents. Voltage clamp experiments were performed using the standard whole-cell configuration of the patch clamp technique. Glass pipettes (Warner Instrument) were pulled to give a resistance of 2–3 MΩ in the solutions described below. The external solution contained (in mM): 130 NaCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 20 sucrose, pH 7.4. The internal pipette solution contained (in mM): 150 NaCl, 1 MgCl₂, 1.5 EGTA, 10 HEPES, pH 7.3. Single mouse SMG acinar cells were voltage clamped at Eₐₜ (−63.8 mV). Liquid junction potential was calculated to be 17.3 mV, and the correction was applied to the voltage.

**Statistical Analysis**—Results are presented as the mean ± S.E. Statistical significance was determined using Student’s *t* test or ANOVA analysis, followed by a Bonferroni’s test for multiple comparisons with Origin 7.0 Software (OriginLab, Northampton, MA). *p* values of less than 0.05 were considered statistically significant. All experiments were performed using three or more separate preparations.

**FIGURE 1.** Purinergic P2 receptor agonists evoked fluid secretion in the *ex vivo* SMG. Agonists (1 mM) were applied to the *ex vivo*, perfused SMG for 10 min as indicated by the bar. *A,* flow rate following stimulation with ATP (*black squares*), ADP (*gray squares*), or UTP (*white squares*). *B,* summary of the results shown in *panel A* as the total volume of fluid secreted over the 10-min stimulation period. Data were from an equal number of male and female animals for ATP, ADP, and UTP (*n = 12, n = 10, and n = 10 glands, respectively*, *p < 0.001*). Black Swiss/129 Sv1 mice were used for these experiments.

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FIGURE 2. Extracellular Ca\(^{2+}\) removal abolished ATP-induced fluid secretion in the ex vivo SMG. Agonists were applied to the ex vivo, perfused SMG for 10 min as indicated by the gray bar. For Ca\(^{2+}\)-free experiments (black squares), glands were perfused with extracellular Ca\(^{2+}\)-free solution for 2 min prior to stimulation and extracellular Ca\(^{2+}\) was re-introduced to the perfusate following 5 min of stimulation as indicated by the black bar. A, 0.3 \(\mu M\) CCh-evoked fluid secretion in the presence (gray squares) or absence (black squares) of extracellular Ca\(^{2+}\). B, 1 mM ATP-evoked fluid secretion in the presence (gray squares) or absence (black squares) of extracellular Ca\(^{2+}\). CCh- and ATP-evoked fluid secretions were from an equal number of male and female animals (n = 8 glands for each condition). Black Swiss/129 SvJ mice were used for these experiments.

FIGURE 3. Enhanced SMG fluid secretion evoked by the P2X\(_7\)-selective agonist BzATP. Agonists were applied to the ex vivo, perfused SMG following stimulation with 0.25 mM of either BzATP (black squares) or ATP (gray squares). B, summary of the total volume of fluid secreted over a 10-min stimulation period. Data were from an equal number of male and female animals (BzATP and ATP, n = 12 and n = 8 glands, respectively; *, \(p < 0.001\)). Black Swiss/129 SvJ mice were used for these experiments.

minute of stimulation (Fig. 2A). However, without extracellular Ca\(^{2+}\) influx to replenish the rapidly depleted intracellular Ca\(^{2+}\) stores, the flow rate was eventually reduced to near zero (Fig. 2A, black trace at 4-min stimulation). Replacing extracellular Ca\(^{2+}\) after 5 min of CCh stimulation restored the flow rate to control values (Fig. 2A, black trace at 6–10 min stimulation). Secretion rapidly returned to basal levels upon removal of CCh from the perfusate. Using the same paradigm, the ATP-evoked (1 mM) fluid secretion was nearly abolished except for a very small volume secreted during the first minute of stimulation (Fig. 2B, black trace; ~85% decrease). Re-addition of extracellular Ca\(^{2+}\) resulted in a modest recovery of the fluid secretion flow rate (Fig. 2B, black versus gray trace at 6–10 min stimulation). Because UTP stimulated little secretion and because the initial ATP-mediated saliva secretion was dependent upon extracellular Ca\(^{2+}\), our results suggested that ATP activated a member of the P2X family of channels. To determine which P2X isoform was involved, both pharmacological and genetic approaches were utilized in the following experiments.

The P2X\(_7\) Receptor-selective Activator BzATP Stimulates Fluid Secretion—Salivary glands have been shown to express both P2X\(_4\) and P2X\(_7\) receptors (10). To differentiate between potential activation of P2X\(_4\) or P2X\(_7\) receptors in our ex vivo SMG fluid secretion experiments we utilized the ATP-derivative BzATP. P2X\(_7\) receptors are activated preferentially by BzATP over ATP (16); thus, BzATP has served as a classical tool to evaluate P2X\(_7\) receptor function. Concentrations of ATP and BzATP were selected to avoid maximum flow rates. Fluid secretion was often detectable at agonist concentrations as low as 0.1 mM for ATP (data not shown); however, fluid secretion was always observed at agonist concentrations of 0.25 mM and higher. Fig. 3 shows that either BzATP (black squares) or ATP (gray squares) can induce SMG fluid secretion; however, at the submaximal concentration tested, BzATP was much more effective (>6-fold at 0.25 mM). In addition, the ATP response was not typically sustained at this concentration of agonist; i.e. the majority of the glands quit secreting after 5 min of stimulation, whereas secretion was sustained with BzATP (Fig. 3A). Fig. 3B shows that the volume of BzATP-evoked SMG fluid secretion was significantly greater than the volume secreted by an equal concentration of ATP (0.25 mM BzATP; 54.2 ± 6.6 \(\mu\)l/min versus 0.25 mM ATP; 8.4 ± 1.0 \(\mu\)l/min, \(p < 0.001\)). Higher concentrations of ATP (1 mM) resembled the results using a lower BzATP concentration (compare Figs. 1A and 3A), suggesting that ATP-stimulated fluid secretion is mediated by the BzATP-sensitive, P2X\(_7\) purinergic receptor.

Loss of the BzATP-induced Cation Current in P2X\(_7\)-Receptor-null Mice—Collectively, the observations that extracellular Ca\(^{2+}\) was required for fluid secretion (Fig. 2) and BzATP was the most potent agonist (Fig. 3) implied that P2X\(_7\) receptor activation was primarily responsible for the sustained ATP-mediated fluid secretion. One caveat of pharmacological inhibitors of purinergic P2 receptors is that they are not selective and may affect other channels and transporters required for fluid secretion (16). Thus, to confirm the critical role of P2X\(_7\) receptor activation in stimulating fluid secretion we utilized P2X\(_7\) gene-disrupted mice.

Western blot analysis using a P2X\(_7\) receptor-specific antibody demonstrated that plasma membrane proteins isolated from P2X\(_7^{-/-}\) mice lacked P2X\(_7\) receptor protein expression in SMG, parotid (PG), and sublingual (SLG) salivary glands (Fig. 4A, see arrow). As an additional positive control and to confirm protein size, the cell lysate from HEK-293 cells transiently over-expressing the P2X\(_7\) receptor was included in the analysis (Fig. 4A, lane 7). Fig. 4 also shows that the large BzATP-induced inward cation current present in SMG acinar cells from wild-type mice was absent in P2X\(_7^{-/-}\) SMG acinar cells (Fig. 4, B and C; P2X\(_7^{-/-}\); ~349.8 ± 88.5 pA versus P2X\(_7^{+/+}\); ~2.2 ± 2.3 pA, \(p < 0.01\)). Together, these data demonstrated that disruption of
the P2X7 gene results in loss of both P2X7 receptor protein expression and nucleotide-gated channel activity.

P2X7 Receptor Disruption Has No Effect on CCh-evoked Fluid Secretion and [Ca2+]i Signals—We next determined if disruption of P2X7 receptors in the SMG had nonspecific effects on the fluid secretion machinery. To evaluate this we utilized the muscarinic receptor agonist CCh, the response to which should be unaffected in P2X7-null mice. Fig. 5A shows that the ex vivo salivary flow rates in P2X7+/+ (black squares) and P2X7−/− (gray squares) SMG were effectively identical in response to CCh stimulation. There were only subtle differences in the total volumes secreted in 10 min (P2X7+/+; 135 ± 5 μl/10 min versus P2X7−/−; 144 ± 6 μl/10 min, p = 0.25), saliva ion compositions (Table 1), or osmolalities (P2X7+/+; 183.9 ± 4.5 mOsm versus P2X7−/−; 186.0 ± 3.3 mOsm, p = 0.74). The lack of effect of P2X7 receptor ablation on the CCh-stimulated response demonstrated that ion channels and transporters responsible for fluid secretion in SMG are not affected when the P2X7 gene is disrupted.

Given that salivary gland fluid secretion is dependent on an elevation of [Ca2+]i, we expected that disruption of P2X7 receptors would also have no effect on CCh-evoked Ca2+ signals in SMG acinar cells. To confirm this, SMG acini were isolated and loaded with the Ca2+-sensitive dye Fura-2. In support of the fluid secretion data, Fig. 5B shows that the CCh-induced Ca2+ signals in P2X7+/+ (black trace) and P2X7−/− (gray trace) isolated SMG acinar cells were essentially identical. Analysis of the data showed that the average peak value over baseline of the CCh-induced Ca2+ signal for P2X7+/+ cells was not significantly different from that for P2X7−/− cells (Fig. 5B, P2X7+/+; 0.30 ± 0.02 ratio units versus P2X7−/−; 0.33 ± 0.03 ratio units, p = 0.41). In addition, the average plateau value over baseline (taken at 1.5 min into the 3-min stimulation) of the CCh-induced Ca2+ signal for P2X7+/+ cells was also similar to that for P2X7−/− cells (P2X7+/+; 0.17 ± 0.01 ratio units versus P2X7−/−; 0.21 ± 0.02 ratio units, p = 0.07). Thus, disruption of P2X7 receptors had no significant effect on either CCh-mediated fluid secretion or Ca2+ signaling.
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Disruption of P2X Receptors Decreases ATP-evoked Fluid Secretion and [Ca\(^{2+}\)] \textit{versus} P2X\(_7\)-mediated signaling—We next determined if loss of P2X, receptor expression had an effect on purinergic receptor agonist-evoked fluid secretion. Fig. 6 shows that the secretion response to the purinergic receptor agonist ATP was markedly reduced in the P2X\(_7\)-/- SMG (gray squares). The total volume of saliva collected over a 10-min period from the P2X\(_7\)-/- SMG was reduced 71% when stimulated with 1 mM ATP (Fig. 6A, P2X\(_7\)+/+; 28.9 ± 4.3 \(\mu\)l/10 min versus P2X\(_7\)-/-; 8.4 ± 1.5 \(\mu\)l/10 min, \(p < 0.001\)). A similar result was obtained when stimulating with the P2X\(_7\)-sele-
and secrete KHCO₃ (1, 2). ATP stimulated a very modest increase in [Ca²⁺] in SMG granular duct cells (Fig. 6C) suggesting that ATP has little influence on duct cell function. Indeed, in P2X₇³⁻/⁻ mice, ATP did not significantly alter the Na⁺ or Cl⁻ concentration of the saliva induced by CCh stimulation (Table 1); while the pH and the K⁺ concentration of saliva increased slightly. Consistent with these observations, ATP induced only modest changes in the ion composition of saliva from P2X₇⁻/- submandibular glands. Taken together, these results suggested that the ATP-stimulated increase in [Ca²⁺] in SMG duct cells is unlikely to play a major role in the regulation of ductal function.

**DISCUSSION**

Salivary tissues express multiple types of purinergic P2 receptors. Activation of these receptors increases [Ca²⁺], suggesting a possible role for P2 receptors in fluid secretion (4, 7, 8, 10, 18, 19), although this has never been directly tested. Here we show that purinergic stimulation results in a sustained, extracellular Ca²⁺-dependent secretion of saliva in an intact mouse submandibular salivary gland preparation. The sensitivity of this response to different purinergic agonists was consistent with P2X₇ receptor activation (BzATP >> ATP >> ADP >> UTP). Indeed, a >70% decrease was observed in BzATP- and ATP-evoked fluid secretions from the SMG in P2X₇-null animals. These results demonstrate that P2X₇ receptors are essential for ATP-mediated saliva production in the mouse submandibular gland.

Our results highlight the significant contribution purinergic receptors may have on the regulation of salivary gland fluid secretion. Earlier reports describe a non-adrenergic, non-cholinergic mediated fluid secretion (3, 20–24). In sheep, electrical stimulation of the parotid parasympathetic nerve in the presence of atropine produced a significant increase in the flow rate (25). The authors concluded that the flow rate was produced by vasoactive intestinal peptide (VIP) release (25), however this seems unlikely since VIP receptor activation is linked to an increase in cAMP, not [Ca²⁺]. Indeed, Ekstrom et al. (26) reported that VIP treatment caused little to no fluid secretion in feline parotid glands. Interestingly, the electrically evoked “atropine-resistant” fluid secretion was ~30% of the response prior to muscarinic receptor inhibition in the ferret submandibular gland (3). This observation is essentially identical to our results; i.e. the maximal volume of ATP-induced fluid secretion was about 30% of that produced by muscarinic receptor stimulation (Fig. 1 versus Fig. 5). Thus, our results are consistent with ATP-mediated fluid secretion playing a physiological role in non-adrenergic, non-cholinergic mediated salivation.

Although both P2X₄ and P2X₇ receptors are expressed in salivary glands, the greater secretion induced by BzATP (Fig. 3) and the severe impairment of secretion in P2X₇-null mice (Fig. 6) implied that the P2X₇ receptor channel contributes most to salivation. Adding to P2X receptor signaling complexity, functional P2X receptors form heteromeric channels comprised of three P2X subunits (16, 27, 28). Until recently P2X₇ receptors were the only P2X isomorph thought not to form a heteromeric channel. However, recent reports have identified P2X₄/P2X₇ heteromeric channels (29, 30). These latter observations suggest that salivary gland cells might contain both P2X₄ and P2X₇ homotrimers, as well as P2X₄/P2X₇ heterotrimers. The functional significance of P2X₄/P2X₇ heterotrimer formation is unclear, but genetic disruption of P2X₇ receptors might also interfere with P2X₄ targeting and/or overall function. Nevertheless, while a P2X₄/P2X₇ heterotrimer is possible, there is no evidence of P2X₄/P2X₇ heterotrimer formation in native tissue. Future studies should be directed to determine the relationship, if any, between P2X₄ and P2X₇ homo- and heterotrimers in salivary gland function.

P2X₇ receptors have been previously localized to the apical membrane of mouse parotid acinar and duct cells (8). P2X₇ receptor immunostaining was also noted in mouse SMG duct cells, which suggests that this ligand-gated channel may participate in modification of the electrolyte content (31). Li et al. (8) proposed that preassembled P2X₇ receptors in duct cells sense upstream secretion by acinar cells (via ATP release) and respond by augmenting HCO₃⁻ secretion. In agreement with this model, we found that ATP produced a modest increase in the pH of CCh-induced secretions, suggesting an increase in the [HCO₃⁻], and this effect disappeared in P2X₇-null mice (Table 1). Given that ATP is not likely to be released independent of other agonists in vivo, ATP might be expected to modulate the response of salivary glands to muscarinic receptor stimulation. Indeed, we also found that the volume of fluid secreted during muscarinic receptor activation was decreased by co-stimulation with ATP and CCh in the *ex vivo* SMG. These results are consistent with the previous observation that ATP inhibits musca-
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mobilization in rat parotid and submandibular gland acinar cells (32–34). The inhibition of the cholinergic receptor-induced response of salivary glands. More-with a muscarinic agonist, ATP likely modulates the musca-rinic receptor-stimulated response of salivary glands. Moreover, the in vivo physiologically relevant source of ATP release is unknown. It is well known that ATP is co-released from nerve terminals with acetylcholine and other neurotransmitters (35). Alternatively, ATP is also released from the secretory granules of exocrine acinar cells in response to agonists that stimulate granule fusion (36). The release of ATP from these two distinct sites is likely to have very different functional consequences.

In summary, our results demonstrate that ATP regulates fluid secretion in the mouse submandibular gland. This mechanism is extracellular Ca2+-dependent, sensitive to BzATP, and insensitive to UTP, suggesting activation of a P2X receptor family member. Indeed, ablation of the P2X7 gene resulted in a dramatic reduction in the amount of fluid secreted during ATP exposure. These new findings contribute to our understanding of the role of purinergic P2 receptors in salivary glands and provide insight into future studies. ATP-mediated fluid secretion is likely an important mechanism for “tuning” the resultant fluid secretion under different physiological conditions.

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