cAMP Potentiates ATP-evoked Calcium Signaling in Human Parotid Acinar Cells*

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In salivary acinar cells, intracellular calcium ([Ca\(^{2+}\)]\(_i\)) signaling plays an important role in eliciting fluid secretion through the activation of Ca\(^{2+}\)-activated ionic conductances. Ca\(^{2+}\) and cAMP have synergistic effects on fluid secretion such that peak secretion is elicited following activation of both parasympathetic and sympathetic pathways. We have recently demonstrated that cAMP exerts effects on Ca\(^{2+}\) release, through protein kinase A (PKA)-mediated phosphorylation of inositol 1,4,5-trisphosphate receptors (InsP\(_3\)R) in mouse parotid acinar cells. To extend these findings, in the present study cross-talk between Ca\(^{2+}\) signaling and cAMP pathways in human parotid acinar cells was investigated. In human parotid acinar cells, carbachol stimulation evoked increases in the [Ca\(^{2+}\)]\(_i\) and the initial peak amplitude was enhanced following PKA activation with ATP also evoked an increase in [Ca\(^{2+}\)]. The ATP-evoked Ca\(^{2+}\) elevation was largely dependent on extracellular Ca\(^{2+}\), suggesting the involvement of the P2X family of purinergic receptors. Pharmacological elevation of cAMP resulted in a 5-fold increase in the peak [Ca\(^{2+}\)]\(_i\), change evoked by ATP stimulation. This enhanced [Ca\(^{2+}\)], increase was not dependent on intracellular release from InsP\(_3\)R or ryanodine receptors, suggesting a direct effect on P2XR. Reverse transcription-polymerase chain reaction and Western blot analysis confirmed the presence of P2X4R and P2X7R mRNA and protein in human parotid acinar cells. ATP-activated cation currents were studied using whole cell patch clamp techniques in HEK-293 cells, a null background for P2X2R. Raising cAMP resulted in a 4.5-fold enhancement of ATP-activated current in HEK-293 cells transfected with P2X2R DNA but had no effects on currents in cells expressing P2X4R. These data indicate that in human parotid acinar cells, in addition to modulation of Ca\(^{2+}\) release, Ca\(^{2+}\) influx through P2X2R may constitute a further locus for the synergistic effects of Ca\(^{2+}\) and PKA activation.

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In salivary acinar cells, acetylcholine (ACH) released following parasympathetic stimulation is the primary regulator of a variety of physiological processes, including exocytosis of salivary proteins and fluid secretion. These processes are controlled in large part by the ACh-stimulated increase in [Ca\(^{2+}\)], as a result of the G\(_\alpha\)-coupled, phospholipase C-catalyzed increase in inositol 1,4,5-trisphosphate (InsP\(_3\)) and subsequent Ca\(^{2+}\) release from the endoplasmic reticulum (1–4).

The principal targets of the [Ca\(^{2+}\)]\(_i\) increase important for initiating fluid secretion are Ca\(^{2+}\)-activated conductances required for the transcellular movement of ions (5–9). Initially, Ca\(^{2+}\)-activated Cl\(^–\) conductances present in the apical plasma membrane of the acinar cell are activated by the release of Ca\(^{2+}\). The result of net Cl\(^–\) accumulation in the lumen leads to an electrical potential that allows Na\(^+\) movement via the paracellular pathway, and the subsequent osmotic movement of water creates an isotonic, NaCl-rich fluid, the primary saliva (10, 11). As a consequence of the Cl\(^–\) flux through Ca\(^{2+}\)-activated Cl\(^–\) conductance, the membrane depolarizes and the driving force for secretion diminishes as the membrane potential \(V_m\) approaches the equilibrium potential for Cl\(^–\) (\(E_{Cl}\)). To maintain the membrane potential and facilitate continued Cl\(^–\) efflux, basolaterally located Ca\(^{2+}\)-activated K\(^+\) channels open, allowing K\(^+\) efflux (7, 9, 12, 13) and membrane repolarization.

Fluid secretion evoked by muscarinic stimulation has been shown to be dramatically potentiated by activation of cAMP-raising pathways, for example by co-stimulation with vasoactive intestinal peptide or \(\beta\)-adrenoreceptor agonists (14–17). Recently, cAMP has been shown to modulate the cellular Ca\(^{2+}\) release machinery. Specifically in mouse parotid acinar cells, stimulation with cAMP-raising agents and subsequent activation of protein kinase A (PKA) results in phosphorylation of type II InsP\(_3\) receptors (InsP\(_3\)R), and increased sensitivity of the receptor to InsP\(_3\) (18, 19). This enhanced Ca\(^{2+}\) release appears largely responsible for the potentiated CCh-evoked Ca\(^{2+}\) response that is observed following exposure to cAMP-raising agonists and is consistent with this event contributing to potentiated fluid secretion observed upon co-stimulation with cAMP and Ca\(^{2+}\) raising agonists. An initial goal of this study was to confirm that this mechanism is relevant in salivary gland tissue of human origin. Indeed, using two different cAMP-raising agents, the present study demonstrated that CCh-evoked Ca\(^{2+}\) signaling in human parotid acinar cells was potentiated with characteristics similar to that observed in mouse parotid acinar cells.

In addition to ACh release following parasympathetic stimulation, extracellular ATP also functions as a regulator of salivary gland function. Indeed, various salivary gland acinar cells derived from an assortment of species express ATP-acti-

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* The abbreviations used are: ACh, acetylcholine; CCH, carbamylcholine; cAMP, cyclic adenosine 3′,5′-monophosphate; PKA, protein kinase A; R, receptor(s); [Ca\(^{2+}\)]\(_i\), intracellular calcium concentration; InsP\(_3\), inositol 1,4,5-trisphosphate; RyR, ryanodine receptor(s); RT, reverse transcription; Bz-ATP, 2′,3′-O-(4-benzoylbenzoyl)ATP.
vated purinergic receptors (20–29). The purinergic receptor gene family consists of two distinct groups based on the signal transduction pathway activated by ATP binding. P2Y receptors (P2YR) act as traditional Gα-coupled receptors, and thus activation results in an increase in [Ca^{2+}]_i. (30). P2X receptors (P2XR) conversely act as ligand-gated, cation-selective ion channels (31). Activation of P2XR consequently results in the influx of positively charged ions such as Na^+ and Ca^{2+} into the cell. Thus, P2XR represents an additional mechanism resulting in an increase in [Ca^{2+}]_i, which is independent of the InsP_3R pathway. Salivary tissue has been reported to express P2X_4 and P2X_2 receptors in human parotid acinar cells. Moreover, as synergism between cAMP and Ca^{2+} signaling is often observed in a number of cell types, ATP is a more effective stimulus than cAMP in elevating [Ca^{2+}]_i (20, 24, 28, 32). These receptors are thought to be activated by neuromodulators and modulate the release of zymogen granules or transit through gap-junction hemi-channels, acting in a paracrine manner (33–35). In terms of elevating [Ca^{2+}]_i, it has been suggested that activation of purinergic receptors by ATP is a more effective stimulus than activation of muscarinic receptors (21). Consistent with this assertion, ATP can effectively regulate secretory processes in human parotid acinar cells. Moreover, as synergism between cAMP and Ca^{2+} signaling is physiologically important in this tissue, the effects of raising cAMP on ATP-mediated Ca^{2+} signaling events were also evaluated. These latter experiments reveal a novel mechanism whereby raising cAMP can potentiate Ca^{2+}-signaling events via an effect on P2XR purinergic signaling in human parotid acinar cells.

**MATERIALS AND METHODS**

**Isolation of Human Parotid Acinar Cells**—Human parotid tissue was obtained from adult male and female subjects scheduled to have parotid surgery because their gland contained an adenoma or other type of tumor that required removal of all or a large portion of the gland. Not all of the normal tissue surrounding the tumor is typically used for diagnostic evaluation of the sample. This remaining tissue was collected immediately after surgical excision and transported in iced physiological saline to the laboratory for acute functional assays, and cell preparations were begun within 1 h of surgical removal. Informed consent was obtained, and the use of human tissue was approved by the University of Rochester Institutional Review Board. Small groups of human parotid acinar cells were isolated by collagenase digestion of surgically resected human parotid glands using a protocol similar to that described previously for the isolation of rodent parotid acinar cells (36, 37). Briefly, parotid glands were dissected from connective tissue and minced in Earle’s minimum essential medium (Biofluids, Rockville, MD) containing 2 mM glutamine, 0.1% bovine serum albumin, and 0.02% Type I trypsin inhibitor (Sigma). Minced tissue was placed in Earle’s minimum essential medium (Biofluids) containing 2 mM glutamine, 0.1% bovine serum albumin, and 0.3 mg/ml collagenase P (Roche Applied Science) and was dispersed by multiple triterations. Cells were resuspended in Basal Media Eagle (Invitrogen) supplemented with 2 mM glutamine, 0.1% bovine serum albumin, 100 units/ml penicillin/streptomycin and maintained at 37 °C and equilibrated with 95% O_2 and 5% CO_2 until use.

**RT-PCR**—Total RNA from three individual human parotid tissue preparations was prepared using the RNeasy kit (Qiagen). From these RNA preparations, cDNA preparations were made using a commercially available kit (Invitrogen). Primers were ordered from Integrated DNA Technologies (Corvalle, IA). Primers were diluted to a working solution ready for PCR using a high speed, digital charge-coupled device camera (TILL Photonics, Pleasanton, CA). The fluorescence ratio of 340 nm over 380 nm was calculated, and all data are presented as the change in ratio units. Images were acquired at a rate of 1 Hz with an exposure of 20 ms. All imaging experiments were performed at room temperature, essentially as previously described (8, 38, 39). Traces are from a single cell, representative of multiple individual cells (>3 cells) from an imaged acinus in a particular experimental run, and n represents the number of experimental runs.

**RT-PCR Analysis**—Total RNA from three individual human parotid tissue preparations was prepared using the RNeasy kit (Qiagen). From these RNA preparations, cDNA preparations were made using a commercially available kit (Invitrogen). Primers were ordered from Integrated DNA Technologies (Corvalle, IA). Primers were diluted to a working solution concentrated of 20 μg/ml. Each reaction contained (in μl): 2.5 μl 10× Power PCR Buffer, 2.0 μl 50 mM MgCl_2, 1.4 μl dNTP (25 mM), 1 μl cDNA of interest, 0.5 μl forward primer, 0.5 reverse primer, and 0.1 μl Taq polymerase. All standard reagents were from Invitrogen. The total reaction volume was 25 μl. The PCR cycle protocol was 94 °C for 3 min, 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min for 30 cycles, then 72 °C for 3 min, final hold 4 °C. Samples were separated on a 1–3% agarose gel for 1–2 h at 100 mV and visualized by ethidium bromide staining. A 100-bp DNA ladder was used as a molecular size marker. The primer sequences and expected sizes of the PCR products are shown in Table I.

**Transfection of HEK-293 Cells**—Rat P2X_4 or P2X_7 cDNA kindly provided by R. A. North (University of Manchester, UK) was transiently transfected into HEK-293 cells using LipofectAMINE 2000 (Invitrogen) following the instructions provided. Specifically, 5 × 10^4 cells were grown on 25-mm coverslips in 6-well culture plates and were co-transfected with 1 μg of the P2X_4 cDNA and 100 ng of pHRed 1-N1 cDNA (red fluorescent protein for visualization of positively transfected cells) per well. Just prior to transfection, cells were placed in 2 ml of Opti-MEM (Invitrogen) medium and incubated with the DNA mixture in 500 μl of Opti-MEM per well for 3–5 h in a 5% CO_2 incubator at 37 °C. After 3–5 h the media was removed and the cells were washed in phosphate-buffered saline and then placed back into 2 ml of MEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 units/ml; 100 μg/ml penicillin/streptomycin) at 37 °C overnight, and experiments were performed the next day. All standard reagents not mentioned were from Invitrogen. 

**Digital Imaging of Intracellular Ca^{2+}**—Human parotid acinar cells were loaded with the Ca^{2+}-sensitive dye Fura-2 AM (2 μM, TEFLABS, Austin, TX) by incubation for 30 min in a physiological saline solution at room temperature. Cells were removed from Fura-2-containing solution and resuspended in physiological saline solution used for imaging experiments that contained (mM): 137 NaCl, 0.56 MgCl_2, 4.7 KCl, 1 Na_2HPO_4, 10 HEPES, 5.5 glucose, 1.26 CaCl_2, pH 7.4. Fura-2-loaded cells were allowed to adhere to a 25-mm glass coverslip for 1 min before cells were locally superfused at a rate of at least 1 ml/min using a 0.5-mm diameter fused silica tube placed within 100 μm of the cells to be recorded. Rapid solution changes were performed utilizing an electronic solenoid controlled perfusion system and gravity-fed reservoirs (Warner Instruments, Hamden, CT). Imaging was performed using an inverted Nikon epifluorescence microscope with a 40× objective oil immersion objective lens (numerical aperture, 1.3). Fura-2-loaded cells were excited alternately with light at 340 and 380 nm using a monochromator-based illumination system, and the emission at 510 nm was captured using a high speed, digital charge-coupled device camera (TILL Photonics, Pleasanton, CA). The fluorescence ratio of 340 nm over 380 nm was calculated, and all data are presented as the change in ratio units. Images were acquired at a rate of 1 Hz with an exposure of 20 ms. All imaging experiments were performed at room temperature, essentially as previously described (8, 38, 39).

**TABLE I**

| Forward primer | Reverse primer | Expected size |
|----------------|----------------|---------------|
| P2X_4          | TGGTCAACTCGAACCATGCTCTCCTTCTCTCCCGCAACTCTCTGAGGAGAAATGCCAGCTCTGCGC | 291 |
| P2X_4          | TGGTCAACTCGAACCATGCTCTCCTTCTCTCCCGCAACTCTCTGAGGAGAAATGCCAGCTCTGCGC | 274 |
| P2X_6          | AGGATGCTGCCCCAACGGAGGAAAATTTTGAGGAGAAATGCCAGCTCTGCGC | 196 |
| P2X_5          | TGGTCAACTCGAACCATGCTCTCCTTCTCTCCCGCAACTCTCTGAGGAGAAATGCCAGCTCTGCGC | 294 |
| P2X_6          | AGGATGCTGCCCCAACGGAGGAAAATTTTGAGGAGAAATGCCAGCTCTGCGC | 176 |
| P2X_5          | TGGTCAACTCGAACCATGCTCTCCTTCTCTCCCGCAACTCTCTGAGGAGAAATGCCAGCTCTGCGC | 252 |
| P2X_7          | AGGATGCTGCCCCAACGGAGGAAAATTTTGAGGAGAAATGCCAGCTCTGCGC | 410 |
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pH 7.4. Samples were sonicated and left on ice for 30 min to solubilize. Protein concentrations were measured using Bio-Rad protein assay reagent. Samples were resolved on 7.5% SDS-PAGE and transferred at room temperature to nitrocellulose membranes using a semi-dry transfer system for 1 h at 0.08 A (Bio-Rad). Polyclonal α-P2X\(_7\)R and α-P2X\(_R\) primary antibodies were obtained from Chemicon (Temecula, CA) and used at a 1:250 dilution. When indicated, the control antigen was preincubated in the primary antibody for 1 h at room temperature prior to use (1 μg of antigen/1 μg of primary antibody). After 2 h of incubation in primary antibody at room temperature, the proteins were visualized using a 1:2000 dilution of goat anti-rabbit IgG horseradish peroxidase secondary antibody (Bio-Rad) and SuperSignal West Pico substrate (Pierce) exposed on XAR film (Eastman Kodak Co.).

Whole Cell Patch Clamp Recordings—ATP-activated cation currents were recorded at a sampling rate of 1 kHz using an Axopatch 200A patch clamp amplifier (Axon Instruments, Union City, CA). Axon digital interface, and pCLAMP version 9.0 software using the whole cell patch clamp technique. To measure ATP-activated currents in HEK-293 cells, cells were perfused with an extracellular solution containing (mM): 140 NaCl, 5 CsCl, 1.2 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES-CsOH, 0.1 EGTA, 10 NaCl, 0.0365 CaCl\(_2\), pH 7.2. Intervals of 2–3 min were allowed between patch rupture and stimuli to allow for equilibration with the patch pipette solution. HEK-293 cells were voltage-clamped at a holding potential of −30 mV. Experiments were performed at room temperature. All currents were normalized to cell size.

Statistical Analysis—Statistical significance was determined using either a paired or unpaired t test. Data from several cells in a particular experimental run were averaged, and experiment averages were used to calculate the mean ± S.E. Two-tailed p values of less than 0.05 were considered statistically significant.

RESULTS

Effects of cAMP on CCh-evoked Ca\(^{2+}\) Signals in Human Parotid Acinar Cells—Experiments were first performed to characterize Ca\(^{2+}\) signaling events in human parotid acinar cells following muscarinic receptor stimulation. Low, physiologically relevant concentrations of CCh (100–300 nM) elicited oscillatory-type responses, typical of signals observed in mouse exocrine acinar cells at similar concentrations of muscarinic agonist. In contrast, higher concentrations of CCh (5 μM) resulted in a peak and plateau-type response, characteristic of maximal concentrations of agonists in a variety of exocrine cell types (Fig. 1A) (18, 38, 40).

Recently, it has been reported that raising cAMP significantly potentiates CCh-induced Ca\(^{2+}\) release in mouse parotid acinar cells (18). This effect was blocked by the serine/threonine kinase inhibitor H89, and attributed to phosphoregulation of InsP\(_3\)R (18). Initially, experiments were performed to investigate if a similar phenomenon occurs in human parotid acinar cells. Prior incubation of human parotid acinar cells with forskolin (Fig. 1B) or the β-adrenergic agonist, isoprenaline (Fig. 1C) to raise cAMP levels, significantly enhanced the peak amplitude of the CCh-evoked [Ca\(^{2+}\)] signal by 146 ± 17% and 135 ± 13%, respectively, over the control response; p = 0.01. These data are consistent with studies in mouse parotid in which forskolin enhanced the CCh-evoked [Ca\(^{2+}\)] response by ~150% (18). The cAMP-induced potentiation of CCh-stimulated Ca\(^{2+}\) signaling was blocked by prior incubation with H89 (second response reduced to 94 ± 3% of the first response) (Fig. 1D) suggesting that PKA activity was responsible for the effect. These data in human acinar cells are entirely consistent with studies from mouse and presumably reflect a similar mechanism; i.e. potentiation of Ca\(^{2+}\) release via phosphorylation of InsP\(_3\)R.

Extracellular ATP Stimulates an Increase in [Ca\(^{2+}\)] in Human Parotid Acinar Cells—Purinergic receptor stimulation has been reported to regulate secretory function in mouse and rat salivary glands by increasing [Ca\(^{2+}\)] (15). We therefore evaluated the effects of ATP in stimulating Ca\(^{2+}\)-signaling events in human parotid acinar cells. A minority of cells responded following exposure to 1–50 μM ATP. 100 μM ATP however, consistently elicited an increase in [Ca\(^{2+}\)] (Fig. 2A), although

![Fig. 1. CCh-evoked Ca\(^{2+}\) signals can be potentiated by cAMP raising agonists. A, Ca\(^{2+}\) oscillations were generated at low concentrations of CCh (100–300 nM), whereas higher concentrations (5 μM) elicited a sustained [Ca\(^{2+}\)], peak and plateau-type response. Ca\(^{2+}\) oscillations were observed in all 15 experiments. B, forskolin treatment increased the peak [Ca\(^{2+}\)] response to 100 nM CCh (147 ± 17% of control response, n = 5, p = 0.01). C, the β-adrenergic agonist isoprenaline also enhanced the peak [Ca\(^{2+}\)] response to CCh (135 ± 13% of control response, n = 6, p = 0.01). D, treatment of the cells with H89 blocked the potentiation caused by forskolin (94 ± 3% of control response, n = 3, p = 0.85). Each trace is representative of three or more experiments.](http://www.jbc.org/content/394/8/39487/F1.large.jpg)
in modest increases in [Ca]\textsuperscript{2+} purinergic receptor, stimulation of these receptors results only (data not shown). Thus, although these observations suggest responses were not induced by higher concentrations of ATP ical muscarinic receptor stimulation. Significantly larger re-
cellular medium resulted in a significant increase in [Ca\textsuperscript{2+}]. These data provide an initial suggestion that P2XR are the predominant mediator of purinergic receptor Ca\textsuperscript{2+} release through P2XR. In Ca\textsuperscript{2+}-free solution. However, addition of Ca\textsuperscript{2+} to the extracellular medium resulted in a significant increase in [Ca\textsuperscript{2+}]. This suggests that ATP is acting on P2XR. Each trace is representative of four or more experiments.

markedly smaller than Ca\textsuperscript{2+} elevations initiated by physiological muscarinic receptor stimulation. Significantly larger re-
ponses were not induced by higher concentrations of ATP (data not shown). Thus, although these observations suggest that human parotid acinar cells express at least one type of purinergic receptor, stimulation of these receptors results only in modest increases in [Ca\textsuperscript{2+}], in contrast to muscarinic stimulation.

Experiments were next performed to define the class of purinergic receptor responsible for the ATP-stimulated increase in [Ca\textsuperscript{2+}], Although ATP is an agonist at both P2YR and P2XR, UTP is considered to be a relatively selective activator of P2YR (24, 41). Stimulation with 100 M\textsubscript{U} UTP did not cause a detectable [Ca\textsuperscript{2+}], response in the vast majority of human parotid acinar cells (Fig. 2B) (54 of 59 cells). In the four cells where an increase was detected, UTP stimulation resulted in a change of 0.018 ± 0.005 ratio units. In contrast, stimulation with 100 M\textsubscript{U} ATP in the same cells resulted in a significantly larger Ca\textsuperscript{2+} transient of 0.088 ± 0.021 units; p = 0.04 (Fig. 2B). These data provide an initial suggestion that P2XR are the predominant mediator of purinergic receptor Ca\textsuperscript{2+} signaling in human parotid acinar cells.

Conceptually, a further paradigm to identify any contribution of P2YRs is to isolate Ca\textsuperscript{2+} release either by removing extracellular Ca\textsuperscript{2+} or alternatively by blocking Ca\textsuperscript{2+} influx through P2XR. In Ca\textsuperscript{2+}-free external solution, 100 M\textsubscript{U} ATP failed to increase [Ca\textsuperscript{2+}], indicating that Ca\textsuperscript{2+} release (presumably through P2YR) was not occurring to a significant degree. Following re-addition of extracellular Ca\textsuperscript{2+}, a rapid increase in [Ca\textsuperscript{2+}], was always observed, indicating that the ATP-stimulated elevation in [Ca\textsuperscript{2+}], was dependent on Ca\textsuperscript{2+} influx (Fig. 2C). Similarly, no increase in Ca\textsuperscript{2+} following ATP stimulation was observed in cells incubated with LaCl\textsubscript{3}, a broad spectrum antagonist of Ca\textsuperscript{2+} influx (see Fig. 4B). These findings provide further evidence that Ca\textsuperscript{2+} entry through P2XRs are primarily responsible for the ATP-induced [Ca\textsuperscript{2+}], signal, and consistent with rat parotid studies where the effect of ATP was found to be primarily due to Ca\textsuperscript{2+} influx across the plasma membrane (22, 23).

cAMP-raising Agents Potentiate ATP-mediated Ca\textsuperscript{2+} Signals in Human Parotid Acinar Cells—Raising cAMP significantly enhances the peak amplitude of the CCh-evoked Ca\textsuperscript{2+} signals in human parotid acinar cells, an effect consistent with enhanced Ca\textsuperscript{2+} release. We next evaluated potential effects of raising cAMP on purinergic Ca\textsuperscript{2+} signaling. Cells were stimulated with 100 M\textsubscript{U} ATP in the absence and then in the presence of either forskolin or isoprenaline. Prior incubation with either forskolin (Fig. 3A) or isoprenaline (Fig. 3B) resulted in a significant increase in the peak amplitude of the Ca\textsuperscript{2+} response to ATP. Experiments were also performed in naïve cells comparing initial ATP-stimulated increases in [Ca\textsuperscript{2+}], in the absence or absence of forskolin. In these unpaired experiments, forskolin significantly potentiated the peak [Ca\textsuperscript{2+}], response to ATP by ~5-fold (Fig. 3C, control 0.06 ± 0.01 Δ ratio units versus forskolin 0.30 ± 0.06 Δ ratio units, n = 3, p = 0.01).

Enhanced Ca\textsuperscript{2+} signaling following elevation of cAMP would not be particularly surprising if this [Ca\textsuperscript{2+}], increase was mediated via activation of P2YR, because phosphorylation of InsP\textsubscript{3}R would provide a common module in the transduction pathways activated by both muscarinic and purinergic receptors. The data to this point, however, suggest that a novel mechanism involving P2XR is responsible for enhanced purinergic Ca\textsuperscript{2+} signaling in human parotid acinar cells. To further support this contention, experiments were next performed to confirm that the mechanism responsible for potentiated purinergic signaling was not dependent on sensitizing Ca\textsuperscript{2+} release and that extracellular Ca\textsuperscript{2+} was required and thus likely a result of a mechanism involving P2XR.

We first addressed whether elevating cAMP resulted in enhanced InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release, through sensitization of InsP\textsubscript{3}R to levels, which were previously at sub-threshold. To explore this, experiments were performed to isolate Ca\textsuperscript{2+} release through P2YR. First, no potentiation of ATP-evoked Ca\textsuperscript{2+} signals by forskolin treatment was evoked in Ca\textsuperscript{2+}-free solution (Fig. 4A) or in the presence of LaCl\textsubscript{3}, (Fig. 4B), although following removal of LaCl\textsubscript{3} an immediate, robust increase in [Ca\textsuperscript{2+}], was observed. Second, forskolin treatment did not enhance [Ca\textsuperscript{2+}], signals to the selective P2YR agonist UTP (100 M\textsubscript{U}), whereas the same cells responded robustly to ATP (100 M\textsubscript{U}) (Fig. 4C). These data suggest that the potentiation of Ca\textsuperscript{2+} signaling is unlikely to be mediated via P2YR and sensitization of Ca\textsuperscript{2+} release through InsP\textsubscript{3}R.

These data do not however, exclude the possibility that Ca\textsuperscript{2+} release through RyR could be involved in the enhanced P2XR Ca\textsuperscript{2+}-signaling events. RyR have been shown to be expressed in salivary acinar cells and are subject to phosphoregulation by PKA (42–44). If RyRs are localized adjacent to P2XR, then a significant proportion of the cAMP-enhanced ATP-stimulated [Ca\textsuperscript{2+}], signal might conceivably be the result of calcium-in-
duced calcium release via RyR. Phosphorylation of RyR under these conditions could result in enhanced calcium-induced calcium release. This possibility was tested by blocking RyR with a high concentration of ryanodine. Because blockade of RyR by ryanodine is reported to be use-dependent, cells were first stimulated with CCh in the presence of 100 M\textsubscript{U} ryanodine prior to the experimental paradigm to assess potentiation of puri-
nergic signaling. As shown in Fig. 4D, ryanodine did not significantly alter the cAMP-mediated potentiation of purinergic Ca$^{2+}$ signaling, and thus RyR are unlikely to be involved in the cAMP-mediated potentiation of ATP-evoked Ca$^{2+}$-signaling events. Taken together these data are consistent with members of the P2XR family being the principle receptors involved in both purinergic receptor signaling in general and involved in the mechanism underlying cAMP-enhanced purinergic Ca$^{2+}$ signaling in human parotid acinar cells.

**Identification of P2XR in Human Parotid Acinar Cells**—We next performed experiments to identify which P2XR were expressed in human parotid tissue and were thus candidates for regulation by elevating cAMP. Initially RT-PCR was performed using total RNA prepared from human parotid glands. Positive cDNA controls for all P2XR were transcribed from commercially available total RNA from either human brain (P2X5R), testis (P2X2R, P2X3R, P2X4R, and P2X6R), or spleen (P2X1R and P2X7R). Each primer set amplified appropriate-sized PCR fragments (see Table I) for each P2XR (Fig. 5A). PCR amplification of human parotid cDNA resulted in correct PCR products for only P2X4R and P2X7R (Fig. 5B; 294 and 410 bp, respectively). This result is consistent with data published from mouse and rat parotid and from other salivary tissues (24, 26, 32, 45). Analysis of three independent human parotid samples revealed identical results (Fig. 5C). As a positive control for the correct product size, testis (P2X4R) and spleen (P2X7R) samples were included (lanes 5 and 9). All PCR products were extracted from the gel and sequenced to positively confirm their identification.

Next, experiments were performed to confirm protein expression of the P2XR identified in human parotid acinar cells at the message level. The whole parotid tissue used for the RNA preparations is contaminated with stromal cells such as ducts, nerves, and blood vessels. We therefore utilized Western analysis to confirm protein expression of P2X4R and P2X7R in preparations of human parotid acinar cells enriched signifi-
confirmed that these cells were responsive. These results strongly suggest an anomalous electrophoretic migration of P2X4R, possibly due to however, larger than the predicted size (43 kDa in human). The specific antibody. The sizes of the immunoreactive bands were, D for the potentiation. Ca2+

acinar cells, two distinct bands were identified by a P2X4R-
classically in parenchymal cells. In lysates prepared from parotid acinar cells, two distinct bands were identified by a P2X4R-specific antibody. The sizes of the immunoreactive bands were, however, larger than the predicted size (43 kDa in human). The anomalous electrophoretic migration of P2X4R, possibly due to glycosylation of the protein, has been reported previously (45). Regardless of the reason for the larger than predicted band sizes, preincubation of the primary antibody with the antigenic peptide blocked recognition of both these bands (Fig. 6A). In a similar fashion, a specific antibody raised against P2X7R recognized a single band from human parotid lysates of predicted size (~68 kDa), which was not present following preincubation of the antibody with the antigenic peptide (Fig. 6B).

As further evidence for the functional expression of P2X7R in human parotid acinar cells, experiments were performed comparing [Ca2+]i signals stimulated by ATP in the presence or absence of ivermectin (10 μM). Because ivermectin specifically potentiates currents mediated through P2X7R with no effect on other P2XR (31, 46, 47), this agent is often used as a diagnostic tool to confirm the expression of P2X7R. As shown in the representative trace in Fig. 6C, ivermectin treatment enhanced the initial peak [Ca2+]i, stimulated by ATP ~5-fold (∆ ratio units 0.06 ± 0.02 in the absence and 0.30 ± 0.07 in the presence of ivermectin, p = 0.018). A similar functional diagnostic tool for the expression of P2X4R is the sensitivity to the selective agonist Bz-ATP (31, 32). As shown in the representative [Ca2+]i trace in Fig. 6D, human parotid acinar cells respond robustly to stimulation with 100 μM Bz-ATP. Taken together these data confirm the functional expression of P2X7R and P2X4R in human parotid acinar cells.

**Forskolin Mediates Enhanced Current through P2X4R but Not P2X7R**—Determining the contribution of each receptor to the enhanced Ca2+ signal following elevation of cAMP in human acinar cells is complicated by the expression of multiple P2XR. We therefore decided to study the regulation of each receptor by cAMP in isolation following expression in HEK-293 cells, which do not express endogenous P2X7R. Many types of cells demonstrate a reduced response to ATP in the absence of forskolin was considered, this potentiation reached 4.5-fold when compared with the initial stimulation in the absence of forskolin. When desensitization of the second ATP-induced current in the absence of forskolin was considered, this potentiation reached ~6-fold (Fig. 7D, P2X7R control; second response 76 ± 10% of initial stimulation) versus Fig. 7E, P2X7R forskolin treatment; second response 45 ± 101% second response of initial stimulation, p = 0.004). These data clearly demonstrate that P2X7R, but not P2X4R, are subject to regulation following elevation of cAMP. These findings are consistent with P2X7R mediating the enhanced purinergic [Ca2+]i signaling in human parotid acinar cells.

**DISCUSSION**

Mouse parotid acinar cells are routinely used as a model system to study Ca2+ signaling and the mechanisms under-
ing fluid secretion in salivary glands. The initial goal of this study was to confirm that Ca\textsuperscript{2+}/H\textsubscript{11001}-signaling events in human parotid tissue are similar to this model system and thus reinforce the relevance of its use. In a manner consistent with reports from mouse acinar cells, CCh stimulation resulted in the characteristic concentration-dependent profile of Ca\textsuperscript{2+}/H\textsubscript{11001} signaling; sinusoidal Ca\textsuperscript{2+}/H\textsubscript{11001} oscillations at low, presumably physiological agonist concentrations and “peak and plateau” type responses at maximal agonist concentrations. Importantly, the initial peak in [Ca\textsuperscript{2+}/H\textsubscript{11001}], indicative of Ca\textsuperscript{2+} release, was markedly enhanced by raising cAMP. This observation is entirely consistent with a mechanism involving phosphoregulation of InsP\textsubscript{3}R as reported recently for mouse parotid acinar cells (18).

It would be predicted that Ca\textsuperscript{2+} release stimulated by any InsP\textsubscript{3}-producing agonist would be potentiated via this mechanism. When the effects of elevating cAMP on purinergic receptor signaling were evaluated, ATP-mediated Ca\textsuperscript{2+} signaling was also found to be markedly increased by raising cAMP; however, this appeared to be exclusively the result of increasing Ca\textsuperscript{2+} influx. This conclusion was reached because little evidence for any contribution from Ca\textsuperscript{2+} release via G\textsubscript{q}-coupled P2YR was obtained either under basal conditions or when increased cAMP levels were present. These observations suggest that the functionally significant purinoreceptor in human parotid acinar cells, in terms of Ca\textsuperscript{2+} signaling, belongs to the P2XR class. Indeed, our data clearly indicate the presence of P2X\textsubscript{4} and P2X\textsubscript{7} purinoceptors both at the mRNA and the protein level. These results in human parotid acinar cells are consistent with the complement of P2X purinoreceptor expression reported in mouse and rat parotid acinar cells (24, 32, 45).

To test the hypothesis that cAMP-raising agents can mediate enhanced cation entry through P2XR, we studied these receptors in isolation using the whole cell patch clamp technique in a heterologous expression system. Of the two P2XR found in human parotid, P2X\textsubscript{4}R displayed almost a 5-fold enhanced maximum inward current in the presence of forskolin. This result strengthens the view that the potentiated ATP response shown in Fig. 3A is most likely the result of enhanced Ca\textsuperscript{2+} entry through the P2X\textsubscript{4}R.

The present study suggests a previously unreported mechanism whereby direct regulation of P2X\textsubscript{4}R, or a closely associated regulatory protein, is responsible for the enhanced Ca\textsuperscript{2+} signaling observed. A straightforward explanation for the enhanced purinergic Ca\textsuperscript{2+} signaling is that the P2X\textsubscript{4}R is phosphorylated by PKA. This phosphorylation event could modulate the activity of the P2X\textsubscript{4}R in a number of ways, for example, by increasing the affinity of the receptor for ATP. However, the observation that the Ca\textsuperscript{2+} responses at very high ATP concen-

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**Fig. 5.** PCR analysis identifies P2X\textsubscript{4}R and P2X\textsubscript{7}R mRNA in human parotid acini. A, PCR products of appropriate size were produced for each primer set for all P2XR (see Table I). Lane M is a 100-bp DNA ladder. B, only correct PCR products for P2X\textsubscript{4}R and P2X\textsubscript{7}R were amplified from human parotid acinar samples. Ninth lane (−) no cDNA. C, both P2X\textsubscript{4}R and P2X\textsubscript{7}R were identified in three different human parotid samples, samples from testis (P2X\textsubscript{4}R) and spleen (P2X\textsubscript{7}R) were included to confirm product size (294 bp and 410 bp, respectively).
trations did not reach the magnitude of responses to low ATP concentrations in the presence of cAMP argue against an effect to “tune” the sensitivity of the receptor. Alternatively, phospho-regulation by PKA could alter the gating characteristics of the channel or possibly modulate the trafficking or assembly of the receptor (32). Whatever the mechanism underlying this phenomenon, it would appear that, physiologically, purinergic stimulation of human parotid acinar cells might only be functionally relevant under conditions where cAMP is elevated.

In terms of the molecular mechanism involved, PKA-mediated phosphorylation of P2X4R appears a plausible explanation, because the receptor sequence does contain PKA consensus sequences. A comparison of rat P2X4R and human P2X4R reveals that both contain RAAS motifs at amino acids 265–268 as well as RLDT motifs at amino acids 278–281. To our knowledge no direct evidence has been reported regarding PKA modulation of P2X4R. Indirect evidence, however, suggests that phosphorylation can modulate the activity of the channel, because relatively nonspecific kinase inhibitors were reported to enhance the activity of P2X4R. Nonetheless, these data would, in contrast to the present study, suggest that dephosphorylation of P2X4R favors the most active state of the receptor (45, 54). A further possibility, not addressed by this study is that the potentiation of purinergic signaling following elevation of cAMP is independent of PKA activity. For example, there is emerging evidence that cAMP can activate guanine exchange factors (exchange proteins directly activated by cAMP) leading to activation of the monomeric G protein Rap1. GTP-bound Rap1 has been shown to lead to downstream events, including secretion, adhesion, and activation of other novel targets (55, 56). The effectors of Epac-mediated signal transduction are not fully understood, and it is therefore possible that P2X4R represent a novel effector of this pathway.

The robust expression of P2X4R mRNA and protein, coupled with the functional effects, suggests that ATP-gated P2X4R channels are important in human parotid acinar cells, especially under physiological conditions of concurrent parasympathetic and sympathetic stimulation of the gland. Although parasympathetic nerves releasing ACh are thought to be the major pathway of fluid production, it is interesting to note that parasympathetic denervation increased the number of cells with P2X4R mRNA levels (45), again supporting a physiological role of these receptors.

The source of ATP that activates the acinar cell P2X4R is not
clear. Likely it is co-released from parasympathetic nerve terminals with ACh. In support of this idea, it is known that ATP is stored at high concentrations in nerve endings (57). Alternatively, some evidence exists for the release of ATP from cells directly (33, 35). This has been suggested to occur via gap junction hemi-channels, anion transporters, and during the discharge of secretory granule content (33, 35, 58). ATP release from pancreatic acini during stimulation with carbachol has been suggested as a source of ATP to activate downstream duct cells (33, 34). A similar mechanism is possible in parotid, whereby ATP release acts in an autocrine or paracrine manner to fine-tune Ca^{2+}-activated fluid secretion in human parotid acinar cells.

In summary, cAMP-mediated modulation of the P2X_R is a novel mechanism for the potentiation of Ca^{2+} signaling in human parotid acinar cells. This new finding contributes to our understanding of the synergistic relationship between cAMP-raising agonists and Ca^{2+} mobilizing agonists in the parotid gland. These results support the emerging consensus that cAMP-raising pathways can regulate diverse Ca^{2+}-signaling pathways in parotid acinar cells.

**Fig. 7.** Whole cell patch clamp analysis shows forskolin enhances current through P2X_R but not P2X_7R. Mock-, P2X_R-, or P2X_7R-transfected HEK-293 cells were whole cell patch-clamped at a holding potential of −30 mV. A, mock-transfected HEK-293 cells did not elicit any current upon ATP stimulation. Forskolin treatment also did not cause any change in the current in mock-transfected cells. B, P2X_R-transfected HEK-293 cells elicit inward current when exposed to 100 μM ATP. C, treatment with forskolin of P2X_R-transfected HEK-293 cells did not affect the inward current (control 90 ± 3% of initial response versus forskolin 94 ± 3% of initial response, n = 3 and n = 7, respectively, p = 0.42). D, 25 μM ATP stimulates an inward current in P2X_R-transfected HEK-293 cells. E, forskolin treatment of P2X_R-transfected HEK-293 cells resulted in a 4.5-fold enhanced inward current when cells were re-exposed to ATP (control 76 ± 10% of initial response versus forskolin 451 ± 101% of initial response, n = 8 and n = 9, respectively, p = 0.004). F, whole cell patch clamp from paired experiments, where normalized maximum inward current relative to the baseline for both the first and second application of ATP. Forskolin enhanced inward current by 4.5-fold in P2X_R-expressing HEK-293 cells but had no effect on P2X_R-expressing HEK-293 cells. The data are presented as the mean ± S.E. (*, p < 0.05).
pathways (InsP$_3$R, RyR, Ca$^{2+}$-ATPases, and P2XR (59)). This pathway is likely an important mechanism for the "shaping" of Ca$^{2+}$ signals and thus has broad implications for the fidelity of Ca$^{2+}$-mediated processes.

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