P2X7 receptors (P2X7Rs) affect many epithelial cell functions including transcellular ion transport, secretion, and cell death. Here we used parotid acinar and duct cells to reveal the unique cell-specific assembly and gating of the P2X7R channels. Immunolocalization indicated expression of P2X7Rs in the luminal membrane of both cell types. Stimulation with 5 mM ATP raised [Ca\(^{2+}\)] levels in a cell-specific manner and activated multiple currents. The current mediated by P2X7R was isolated by infusing the cells with high [EGTA]. The initial activation of acinar cell P2X7Rs by ATP was slow requiring ~2.5 min. Subsequent removal and addition of ATP, however, resulted in rapid inhibition and activation (gating) of the P2X7Rs. By contrast, P2X7Rs in duct cells displayed only rapid gating by ATP. Activation of P2X7Rs in both cell types was verified by (a) low K\(_m\) for ATP, (b) sensitivity to external divalent ions, (c) lack of desensitization/inactivation, (d) permeability to Na\(^+\), and (e) inhibition by Brilliant Blue G, Cu\(^{2+}\), and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium. The slow P2X7R activation in acinar cells was not affected by manipulation of exo-/endocytosis. Rather, disassembly or solidification of the actin cytoskeleton prior to incubation with ATP prevented channel assembly. Remarkably, after completion of the slow activation, manipulation of the actin cytoskeleton no longer affected gating by ATP. Accordingly, manipulation of the actin cytoskeleton had no effect on P2X7R gating by ATP in duct cells. We concluded that P2X7Rs are not active in resting acinar cells. On exposure to ATP, P2X7Rs are assembled into functional channels with the aid of the actin cytoskeleton. Once assembled, P2X7Rs are subject to rapid gating by ATP. Duct cell P2X7Rs are preassembled and therefore continually subject to rapid gating by ATP. This cell-specific behavior may reflect the specific function of P2X7Rs in the two cell types.

Purinergic receptors (P2Rs)\(^1\) regulate numerous physiological and pathological functions in virtually all cell types through activation of metabotropic P2Y or ionotropic P2X receptors (1–4). P2YRs are G protein-coupled receptors that act by activation of phospholipase C\(\beta\) to evoke Ca\(^{2+}\) signals of specific patterns (3). To date, seven P2YR subtypes have been found (3, 4). P2XRs are nonspecific cation channels that can admit Ca\(^{2+}\) and, as a result, regulate cell function by increasing Ca\(^{2+}\) influx (1). Seven P2XRs have been identified (1).

All P2Rs are expressed in the brain and show region-specific expression (1–4). Multiple P2YRs and P2XRs are also expressed in epithelial tissues, including the respiratory epithelium, intestine, salivary glands, and the pancreas (5–7). An excellent recent survey of P2Rs in epithelia can be found in Ref. 2. The lack of specific agonists and antagonists for most P2Rs hampered early work attempting to identify and localize P2Rs. Recent reverse transcriptase (RT)-PCR analysis has revealed mRNA for P2Y1R, P2Y2R, P2Y4R, P2X4R, and P2X7R in pancreatic duct cells (5, 8) and P2Y1R, P2Y2R, P2X4R, and P2X7R in salivary gland cells.

Epithelial expression of P2Rs is unique in that it is membrane-specific, as indicated by functional studies suggesting expression of P2Y1R and P2Y2R at the basolateral membrane and of P2X7R at the luminal membrane of salivary and pancreatic ducts (5, 10). Because of overlap of stimulation by various P2R agonists, however, functional localization of P2Rs is not conclusive. A more definitive localization became possible with the availability of P2R-specific antibodies. The antibodies should be particularly useful for localization of P2Rs when access to the luminal membrane for functional studies is limited, as in secretory acinar cells. Although the antibodies have been applied extensively to localize P2Rs in neuronal and muscle tissues (for example, see Refs. 11 and 12), we found only one study in which antibodies were used to localize P2Y2R exclusively to the basolateral membrane and P2Y4R to the luminal membrane of strial marginal cells and vestibular dark cells (13). In the present work, we used antibodies to localize the P2X7R to the luminal membrane of salivary gland acinar and duct cells.

A particularly intriguing P2X receptor is the P2X7R, which functions as a nonselective Ca\(^{2+}\)-permeable cation channel (1). Although all P2XRs function as nonselcetive cation channels, only persistent activation of P2X7Rs induces a sustained rise in Ca\(^{2+}\) that ultimately leads to apoptosis (1). Furthermore, in several cells (1), including parotid acinar cells (14), P2X7Rs are associated with formation of large pores that admit molecules of \(M_r \approx 1000\). However, pore formation and generation of membrane blebs are not common to all cells (1). Little is known about the mechanism of pore formation, except that it requires the long cytoplasmic C-terminal domain of P2X7R (15). Recent work indicates that P2X7Rs interact with several cytoskeletal proteins in cellular microdomains (16) and that the C termini of P2X7Rs bind to the family of epithelial membrane proteins, which could mediate membrane blebbing (17). Interaction with specific membrane and cytoskeletal proteins may thus cause the cell-specific behavior of P2X7R. Here, we show the differ-

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**Cell-specific Behavior of P2X7 Receptors in Mouse Parotid Acinar and Duct Cells**

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\(1\) The abbreviations used are: P2R, purinergic receptor; P2XR, P2X receptor; P2YR, P2Y receptor; BBG, Brilliant Blue G; JP, jasplakinolide; BzATP, 3′,5′-O-(4-benzoyl)benzoyl ATP; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid.

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\(3\) The abbreviations used are: P2R, purinergic receptor; P2XR, P2X receptor; P2YR, P2Y receptor; BBG, Brilliant Blue G; JP, jasplakinolide; BzATP, 3′,5′-O-(4-benzoyl)benzoyl ATP; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium; RT, reverse transcriptase; ATP\(_S\), adenosine 5′-O-(3-thiotriphosphate; GTP\(_S\), guanosine 5′-O-(3-thiotriphosphate; GDP\(_S\), guanosine 5′-O-[(2-thio)diphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid.

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vential behavior of P2X7R in parotid gland acinar and duct cells. Activation of P2X7R in acinar cells involved slow assembly of the receptors and their subsequent rapid gating by ATP. This behavior could be attributed to the role of the cytoskeleton in channel assembly. By contrast, P2X7Rs were preassembled in duct cells and displayed only rapid gating by ATP and were not influenced by modification of the cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Materials and Solutions**—Anti-P2X7R antibodies were purchased from Alomone Laboratories (Jerusalem, Israel). All nucleotides and PPADS were from Sigma. Fura-2/AM was from Tef Laboratories (Australia). The bath solution (Solution A) contained (in mM) 140 KCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). This solution was supplemented with 10 mM sodium pyruvate, 0.02% soybean trypsin inhibitor, and 1 mg/ml bovine serum albumin to form the solution that was used for gland digestion. The standard solution was the bath solution during the recording of whole cell current. The pipette solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4 with NaOH), 1 MgCl2, 1 ATP, and the indicated concentration of EGTA. Na+–free solution was prepared by replacing NaCl with NMDG-Cl and titrating the pH with Tris base; Cl––free solution was prepared by replacing all Cl– salts with gluconate salts.

**Preparation of RNA and RT-PCR Analysis**—To prepare RNA, small segments of the main parotid duct were microdissected and carefully cleaned from adherent cells and connective tissue. The segments were cut, inspected for absence of acinar cells, blood capillaries, and cell debris, and transferred to clean Eppendorf tubes. The ducts were washed twice with Solution A, packed, and treated with Trizol to extract RNA. To prepare RNA from acinar cells, the glands were digested as described below, and the diluted digest was placed in a Petri dish. Small acinar clusters consisting of 3–5 cells were collected with a Pasteur pipette under microscopic examination to ensure lack of contamination with other cell types. The RNA was processed as above. RT-PCR was performed as detailed previously (5). Approximately 2 ng of RNA was used for each RT-PCR reaction. One set of primers was used to identify the P2Y receptors expressed in parotid cells, and two nonoverlapping primer sets were used for identification of the P2X7Rs. All primers and reaction conditions are available upon request. The identity of all amplified products was verified by sequencing.

**Immunocytochemistry**—Isolated cell clusters were allowed to attach to glass coverslips and then fixed and permeabilized with 0.5% of cold methanol or acetone for 10 min at −20 °C. The cells were washed with phosphate-buffered saline containing 50 mM glycine, and nonspecific sites were blocked by incubation with phosphate-buffered saline containing 5% goat serum, 1% bovine serum albumin, and 0.1% gelatin. The cells were then incubated overnight at 4 °C with a 1:100 dilution of anti-P2X7R antibodies or the same dilution of antibodies that were incubated with a blocking peptide (control). Primary antibodies were detected with a goat anti-rabbit or anti-mouse IgG tagged with fluorescein. Actin was detected with rhodamine-labeled phalloidin that was added together with the secondary antibodies. Images were collected with a confocal microscope (Bio-Rad model MRC 1024).

**Preparation of Single Parotid Acinar and Duct Cells**—Parotid acinar and duct cells were isolated by minor modifications of a standard digestion protocol (18). Mouse parotid glands were minced and treated with 0.025% trypsin for 5 min at 37 °C. After stopping the reaction with 1.5 mg/ml soybean trypsin inhibitor, the tissue was further digested for 20 min with 70 units/ml collagenase CLSFA. The cells were washed, suspended in solution A containing 1.5 mg/ml soybean trypsin inhibitor, and kept on ice until use.

**Measurement of [Ca2+]i.**—Cells in Solution A were incubated with 5 μM Fura-2/AM for 30 min at room temperature, washed once with Solution A and kept on ice until use. Samples of cells were plated on glass coverslips that formed the bottom of a perfusion chamber. After 2–3 min of incubation to allow cell attachment, the cells were continuously kept on ice for warmed (37 °C) solution A at a rate of 5 ml/min (30 chamber volumes/min). When Ca2+–free conditions were necessary, Ca2+ was omitted from solution A, and 0.2 mM EGTA was added. ATP was delivered to the cells by a custom-made rapid perfusion system. Fura-2/AM fluorescence was measured at excitation wavelengths of 340 and 380 nm using a PTI image acquisition and analysis system as described previously (19).

**Recording Current**—The current was recorded using the whole cell mode of the patch-clamp technique at the conditions described previously (20), except that, unless otherwise specified, EGTA concentration in the pipette solution was adjusted to 0.5 mM for recording from acinar cells and to 10 mM for recording from duct cells. Pipette resistance was 3–7 megohms and the access resistance was about 10 megohms. Tight seals of 5–8 gigaohms were formed on the cell surface, and the whole cell configuration was obtained by gentle suction. The current was recorded using the Axopatch 200B patch-clamp amplifier. Data was collected at 5 kHz and filtered at 1 kHz. During whole cell recording the membrane potential was held at −40 mV.

**Agonist Delivery System**—ATP and other agents were delivered to the cells by a custom-made rapid solution-exchange system. All agents were dissolved daily in external solution and delivered by gravity flow from a row of capillaries that were connected to a series of independent reservoirs placed within 100 μm of the cell examined. The rapid solution-exchange system was controlled by shifting the capillary array horizontally. The system included a bundle of 12 silicon capillaries of 200-μm inner diameter each that was attached to a joystick-controlled micromanipulator (Eppendorf). The reservoir of each capillary was controlled by an on-off valve. Continuous bath perfusion provided a counterflow to the solution containing the agonist so that stimulation could be rapidly terminated by setting the valve to the “off” position. Stimulation was initiated by advancing the required capillary to the cell and setting the valve to the “on” position. The proximity of the capillaries to the cell allowed stimulation within 1–2 s. The capillaries were filled with bath solution that contained the desired agonist concentration and a food coloring dye to allow visualization of solution application.

**RESULTS AND DISCUSSION**

**P2Rs in Parotid Gland Cells**—To analyze the P2XRs expressed in parotid gland cells, small acinar clusters and duct fragments were used to prepare mRNA. This avoided duct and acinar cell cross-contamination and contamination with nerve terminals and blood vessels. Fig. 1, A and B, shows that parotid acinar and duct cells express P2X4 and P2X7 receptors. Positive controls were obtained for all receptors analyzed in Fig. 1 using mRNA prepared from brain tissue (not shown), and similar results were found in submandibular gland acinar and duct cells.

The availability of anti-P2X7R antibodies allowed localization of the receptors at specific membrane domains (Fig. 1, C–J). This is particularly useful for acinar cells, in which the luminal membrane is not accessible for direct recording. P2X7Rs are expressed at only the luminal membrane of both duct cells and acinar cells (10). P2X7Rs interact with the cytoskeleton (16, 17), and modification of the actin cytoskeleton affects P2X7R function in acinar cells (see below). Therefore, it was of interest to test the effect of actin disassembly and actin solidification on the localization of P2X7Rs in acinar cells. Fig. 1, E–J, shows that P2X7Rs are localized at close proximity to the actin at the terminal web. However, neither dissociation (Fig. 1, G and H) nor solidification (Fig. 1, I and J) of the actin cytoskeleton had a noticeable effect on P2X7R localization at the resolution of the confocal microscope.

**Salivary gland cells express P2X7Rs in the basolateral and luminal membrane** (10). Expression is membrane-specific with P2Y1R in the basolateral membrane and P2X7R in the luminal membrane. This can be an ideal paracrine regulatory system that senses and responds to the energetic state of the cell and communicates with the nervous system to control glandular activity. ATP could be released from purinergic nerve terminals to the basolateral membrane to stimulate the P2X7R. At the same time, acinar cells could release ATP to the lumen to stimulate the P2X7R at the luminal membrane of acinar and duct cells. It has in fact been reported that cholinergic stimulation releases ATP stored in secretory granules to the lumen of pancreatic acinar cells (23). Hence, the more exocytosis, the more energy consumed and the more ATP released.

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X. Luo and S. Mualem, unpublished data.
leased to stimulate the P2Rs in both acinar and duct cells. P2Rs may thus serve to coordinate events at the basolateral and luminal membranes and tune the critical functions of these cells.

**Ca\textsuperscript{2+} Signaling by P2Rs**—All P2Rs increase \([\text{Ca}\textsuperscript{2+}]\textsubscript{i}\) (1–3, 21, 22), and activation of P2Rs increases \([\text{Ca}\textsuperscript{2+}]\textsubscript{i}\) in parotid acinar cells (7, 9, 24–26). We are interested in the possible physiological role of P2X7Rs in salivary glands and, therefore, compared the \([\text{Ca}\textsuperscript{2+}]\textsubscript{i}\) response of parotid acinar and duct cells to stimulation by ATP, which activates all P2Rs at different apparent affinity (1). Acinar and duct cells responded somewhat differently to ATP (Fig. 2). First, duct cells were more sensitive to ATP stimulation than acinar cells. Moreover, at 5 mM ATP, duct cells continued to display a peak plateau response, whereas \([\text{Ca}\textsuperscript{2+}]\textsubscript{i}\) in acinar cells continued to rise slowly after the initial peak. Because the slow rise in \([\text{Ca}\textsuperscript{2+}]\textsubscript{i}\) was observed only at 5 mM ATP, it likely reflects the activity of the P2X7Rs, which are activated at a higher ATP concentration than all other P2Rs. The differential response of duct and acinar cells provided the first clue that the P2X7Rs might behave differently in the two cell types. To examine this dissimilarity further, it was necessary to isolate activation of the P2X7Rs. To do so, we recorded the current mediated by P2X7Rs in the presence of divalent cations (Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), because the ligand for the receptors is ATP\textsuperscript{4–} (1). Therefore, to activate the P2X7Rs, the cells were stimulated with 5 mM ATP, which is sufficient to activate all P2Rs. Isolation of the current mediated by P2X7Rs was achieved by buffering the cytosol with EGTA.

![Image](https://via.placeholder.com/150)

**Fig. 1. RT-PCR analysis and immunolocalization of P2XRs in parotid acinar and duct cells.** Two sets of primers were used to probe for P2X7R mRNA and one set for all other P2XRs in acinar (A) and duct (B) cells. Anti-P2X7R antibodies were incubated with (D) or without (C) blocking peptides and used to localize the P2X7Rs in acini and ducts isolated from mouse parotid glands. The inset is a high magnification of the duct to show expression of P2X7Rs in the luminal, but not basolateral membrane. The basolateral membrane (BLM) is marked by dotted arrows. Solid arrows point to the luminal membrane (LM). Parotid acinar cells were treated with vehicle (E, F), cytochalasin D (G, H), or jasplakinolide (JP in I, J) for 20 min at 37 °C and fixed and stained for P2X7Rs (E, G, I) and actin (F, H, J). Similar results were obtained in two additional RT-PCR and 3–5 immunolocalization experiments.
The effect of EGTA on whole cell current in response to stimulation with 5 mM ATP is illustrated in Fig. 3. When the pipette solution was lightly buffered with 50 mM EGTA, stimulation of cells with 5 mM ATP held at ~40 mV resulted in a three-phase signal in both cell types: an initial inward current was followed by an outward current and then a sustained inward current (Fig. 3, A and C). Salivary gland cells have been shown to express several Ca\(^{2+}\)-activated Cl\(^{-}\) and K\(^{+}\) channels (27), which were probably responsible for part of the initial inward and outward currents that was activated by the [Ca\(^{2+}\)]\(_i\) increase (Fig. 2). This was confirmed by increasing the EGTA concentration in the pipette solution. Interestingly, whereas increasing the pipette EGTA concentration to 0.2 mM blocked the initial changes in current within acinar cells (Fig. 3B), it was necessary to increase pipette (and therefore cytosolic) EGTA concentration to 10 mM to eliminate the initial inward current (Fig. 3E). 0.5 mM EGTA was sufficient to block the outward, but not the initial inward, current in duct cells. This may reflect either differential sensitivity of the initial inward current to [Ca\(^{2+}\)]\(_i\) in acinar and duct cells or variable access of EGTA to the channels at plasma membrane microdomains. Irrespective of these experimental results, these experiments demonstrate that the inward current mediated by the P2X7Rs could be isolated by including a high concentration of EGTA in the pipette solution and stimulating the cells with 5 mM ATP. Therefore, unless indicated otherwise, in all subsequent experiments the pipette solution contained 0.5 or 10 mM EGTA during current recording in acinar and duct cells, respectively. Parotid cells express P2X4Rs and P2X7Rs. Activation of P2X4Rs saturates at 100 μM ATP irrespective of the concentration of divalent ions in the incubation medium (1). Importantly, under the conditions of Fig. 3, B and E, 0.2 mM ATP did not activate any current, providing the first evidence that most of the current under these conditions was mediated by the P2X7Rs. Because salivary gland and pancreatic cells do respond to ATP\(_{\gamma}\)S (a ligand for P2X4Rs but not P2X7Rs) by a transient [Ca\(^{2+}\)]\(_i\) increase (5, 6, 10), it is not clear at present why 0.2 mM ATP did not activate a cation current. It is possible that the P2X4Rs mediate only a small current or that they were rapidly desensitized under the experimental conditions employed to record the P2X7R current.

**Kinetics of P2X7R Activation in Acinar and Duct Cells**—It is evident that activation of P2X7Rs in parotid acinar and duct cells followed two very different time courses. This is further illustrated in Fig. 4. Maximal activation of P2X7Rs by the first application of ATP to acinar cells required about 2.5 min of continuous stimulation (2.33 ± 0.04 min, n = 52). Notably, removal of ATP resulted in a rapid termination of cell stimulation, and the subsequent application and removal of ATP was followed by rapid activation and inhibition of the current, respectively, even when the cells were incubated in the absence of ATP for as long as 10 min (Fig. 4A) or 25 min (not shown) min. By contrast, application and removal of ATP from duct cells, including the first application, always resulted in rapid activation and inactivation of the inward current (Fig. 4C).

**ATP Activates P2X7Rs in Both Acinar and Duct Cells**—The differential effect of ATP in the two cell types could be explained by activation of different P2Rs by 5 mM ATP. To test this possibility, we compared the properties of the current in both cell types. The substrate of P2X7Rs is believed to be ATP \(^{4-}\) (1). Accordingly, removal of Ca\(^{2+}\) and Mg\(^{2+}\) from the perfusate reduced the concentration of ATP needed for maximal activation of the current from 5 to ~1 mM for both cell types (n = 4, not shown). P2X7Rs activate a nonselective cation channel that conducts Na\(^{+}\) but not Cl\(^{-}\) (1). Removal of medium Cl\(^{-}\) had no effect on the inward current activated by ATP (not shown), whereas removal of medium Na\(^{+}\) abolished the current in both cell types (Fig. 4, B and C).
Brilliant Blue G (BBG) is a relatively specific P2X7R inhibitor (1, 35) that was shown to inhibit P2X7Rs in parotid acinar cells (24, 28). Fig. 5, A and C, shows that application of 10 μM BBG prior to stimulation with ATP completely inhibited the current in acinar and duct cells (n = 5). Approximately 50% inhibition of the current was observed at about 1 μM BBG. A novel and interesting finding is shown in Fig. 5, B and D. BBG, at concentrations as high as 5 mM, was without any effect when applied to cells incubated with ATP. Furthermore, relief of the inhibition by BBG was slow in both cell types. It thus appears that BBG cannot bind and inhibit P2X7Rs in the activated conformation and that, once bound, BBG stabilizes the P2X7Rs in the inactive state.

Another relatively selective and potent inhibitor of P2X7Rs is Cu_2^+ (1, 29). The effect of Cu_2^+ on the inward current is illustrated in Fig. 6, A–C. Cu_2^+ inhibited the current in acinar and duct cells similarly when added to cells incubated with ATP (Fig. 6, A and C) or prior to stimulation with ATP (Fig. 6B). Cu_2^+ inhibited 50% of the ATP-activated inward current in acinar and duct cells at 1.7 ± 0.2 and 1.6 ± 0.2 μM, n = 4, respectively. Finally, to further ensure that P2X4Rs did not contribute to the current in either cell type, we tested the effect of 10 μM PPADS. Although PPADS inhibits several P2XRs, it is the best inhibitor in discriminating between P2X4R and other P2XRs, because it virtually does not inhibit P2X4Rs (1). In various cells, PPADS inhibits P2X7Rs at concentrations between 10 and 50 μM depending on species and incubation time. Figs. 3D and 6E show that incubating acinar and duct cells, respectively, with 10 μM PPADS completely inhibited the current. These findings further indicate that most of the current activated by 5 mM ATP is mediated by P2X7Rs in both cell types.

We then tested activation of the inward current by the relatively selective P2X7R agonist, BzATP. Previous work by us (6, 10) and others (9, 14, 26, 28) has shown that BzATP is as effective as 1 mM ATP in increasing [Ca^{2+}]_i in salivary gland acinar and duct cells. In the present work, we found that 0.1 mM BzATP activated only a small inward current in acinar and duct cells, comparable with the current activated by 1 mM ATP, but much smaller than that activated by 5 mM ATP. In fact, even at concentrations as high as 2 mM, BzATP marginally activated the inward current in both acinar and duct cells (not shown). The reason for this aberrant response to BzATP is not clear at present. However, it is important to note that the same behavior was observed in acinar and duct cells.

Hence, based on the similarity of (a) the low apparent affinity for ATP, (b) the inhibitory effect of divalent ions, (c) the lack of effect of external Cl^- and elimination of the current by removal of external Na^+, (d) the mode of inhibition of the current by BBG, (e) inhibition of the current by Cu_2^+, and (f) inhibition of the current by PPADS, we conclude that 5 mM ATP activates the P2X7Rs in both parotid acinar and duct cells.

Mechanisms That Are Not Involved in the P2X7R Channel Activation in Acinar Cells—In the next stage of the studies, we considered potential mechanisms to explain the unusual kine-
obtained in duct cells because of the need to use 10 mM EGTA in the pipette solution of acinar cells. Similar response could not be obtained in duct cells because the 0.5 mM EGTA in the incubation medium was removed from the incubation medium to follow the time course of recovery of the current. Acinar (D) and duct (E) cells were incubated with 10 μM PPADS for ~10 min before stimulation with 5 mM ATP. Acinar cells were then stimulated with 100 μM carbachol. The response to carbachol was relatively small because of the 0.5 mM EGTA in the pipette solution of acinar cells. Similar response could not be obtained in duct cells because of the need to use 10 mM EGTA in the pipette solution of duct cells.

Fig. 6. Inhibition of P2X7Rs by Cu²⁺ and PPADS. Acinar (A, B) and duct (C) cells were stimulated with 5 mM ATP. The cells were then incubated with 1, 2.5, 5, 10, and 50 μM Cu²⁺ together with (B) or while being stimulated with ATP (A, C). Where indicated by the dotted arrows, Cu²⁺ was removed from the incubation medium to follow the time course of recovery of the current. Acinar (D) and duct (E) cells were incubated with 10 μM PPADS for ~10 min before stimulation with 5 mM ATP. Acinar cells were then stimulated with 100 μM carbachol. The response to carbachol was relatively small because of the 0.5 mM EGTA in the pipette solution of acinar cells. Similar response could not be obtained in duct cells because of the need to use 10 mM EGTA in the pipette solution of duct cells.

ics of P2X7R activation in acinar cells. One possibility is exocytotic insertion of the channel into the plasma membrane. This is a well established mechanism for other channels and transporters, such as the epithelial Na⁺ channel (30), the cystic fibrosis transmembrane conductance regulator (31), and the Na⁺/H⁺ exchanger (32). Exocytosis is tightly regulated by Ca²⁺- and GTP-binding proteins (33). Therefore, to examine this possibility, we tested the effect of clamping [Ca²⁺]i at below 1 nM with 10 mM BAPTA (Fig. 7A) and that of activating and inhibiting all heterotrimeric and small G proteins with GTP/βS (Fig. 7B) and GDP/βS (Fig. 7C), respectively, on development of the current. None of the treatments affected the kinetics of P2X7R activation. Furthermore, none of the treatments facilitated P2X7R inactivation upon removal of ATP or reversed the rapidly activated state even when the cells were incubated in the absence of ATP for a long period of time. We, therefore, conclude that activation of the P2X7Rs in parotid acinar cells is not regulated by exocytosis.

Involvement of the Cytoskeleton in P2X7R Activation in Acinar Cells—We reasoned that activation of P2X7Rs in acinar cells could occur in two steps: slow channel assembly and subsequent rapid channel gating, both of which require binding of ATP to the P2X7Rs. The slow initial activation would reflect slow and irreversible assembly of homomultimeric channels from monomeric P2X7R subunits. The subsequent rapid activation and inactivation upon addition and removal of ATP would reflect gating of the assembled channels by ATP. Channel assembly from subunits already present at the plasma membrane would probably be controlled by the cytoskeleton. In fact, it was shown recently that P2X7Rs exist in a complex with several cytoskeletal proteins (16). We, therefore, compared the role of the cytoskeleton in parotid acinar and duct cells on channel formation. The effect of actin disassembly is shown in Fig. 8. Preincubation of acinar cells for about 10 min with 10 μM cytochalasin D completely prevented assembly of the P2X7R channels (Fig. 8A). Remarkably, once the channel was assembled, cytochalasin D-induced dissociation of the actin cytoskeleton had no effect on channel activity in the presence (Fig. 8B) or absence (Fig. 8C) of ATP. Significantly, dissociation of the actin cytoskeleton had no effect on P2X7R channel activity in duct cells (Fig. 8D). Similar results were obtained in at least three experiments under each of the protocols in Fig. 8. This indicates a fundamental difference in the state of each of the P2X7R channels in acinar and duct cells; in acinar cells, the P2X7R channels need to be assembled, whereas in duct cells, they exist in a preassembled state.

To further probe the role of the cytoskeleton in P2X7R channel assembly, we tested the effect of solidifying the actin cytoskeleton on channel activity. Cells were exposed to jasplakinolide (JP), which slowly solidifies the actin cytoskeleton to form a thick ring at the inner plasma membrane surface (34). Staining with fluorescein isothiocyanate-phalloidin revealed noticeable actin solidification in parotid acinar and duct cells after 5 min of incubation and progressed further upon continued incubation with JP (see also Fig. 1). Actin solidification is expected to minimize diffusion of plasma membrane proteins that are anchored to the cytoskeleton. Accordingly, treatment of acinar cells with JP for ~10 min prevented activation of the
with 10 mM ATP concentrations used (1 and 5 mM) to activate the difference in apparent affinity can be explained by the different model system (36) but required 5–10 min of Cu$^{2+}$ washout of Cu$^{2+}$-P2X7Rs and binding of Cu$^{2+}$ to ATP. Note that after stimulation with ATP, cytochalasin D no longer inhibited the P2X7Rs.

P2X7Rs (Fig. 9A). On the other hand, after formation of the P2X7R channels, over 40 min of incubation with JP were necessary for channel inhibition (Fig. 9B). Furthermore, incubation of duct cells with JP for 45 min only partially inhibited the P2X7Rs (Fig. 9C). Thus, the results with JP reinforce the conclusion of a cytoskeleton role in the assembly of P2X7Rs in acinar cells.

Concluding Remarks—This work reveals several novel properties of P2X7Rs and demonstrates a prominent cell-specific behavior of the receptors in epithelial cells of the same tissue, which may have important physiological relevance. Potent inhibition of P2X7R current by Cu$^{2+}$ was reported before (29) and shown to be noncompetitive with ATP (36). However, we note two differences between the effect of Cu$^{2+}$ in model systems and native cells. Cu$^{2+}$ inhibited the current of mouse P2X7R in NG105–15 cells with an apparent affinity of 16 nM (36), whereas in native mouse parotid duct and acinar cells, inhibition by Cu$^{2+}$ had an apparent affinity of ~1.6 μM (Fig. 6). Washout of Cu$^{2+}$ inhibition was completed within 1 min in the model system (36) but required 5–10 min in native cells. The difference in apparent affinity can be explained by the different concentrations of ATP used (1 and 5 mM) to activate the P2X7Rs and binding of Cu$^{2+}$ to ATP. This implies that Cu$^{2+}$ is the inhibitory species rather than Cu$^{2+}$-ATP. This possibility is in line with the finding that inhibition of P2X7Rs by Cu$^{2+}$, like that by other divalent ions (29, 36), is noncompetitive with ATP or BzATP. At 5 mM ATP, 1 mM Ca$^{2+}$ and Mg$^{2+}$, 10 μM Cu$^{2+}$, and a dissociation constant for Cu$^{2+}$-ATP of 0.37 μM, the free [Cu$^{2+}$] is in the picomolar range. Inhibition with such a high affinity can explain the slow recovery from inhibition.

Another notable finding is the inability of BBG to inhibit ATP-bound P2X7Rs (Fig. 5). The fact that similar behavior is observed in duct and acinar cells indicates that this is a property of the assembled receptors. Inhibition by BBG is also noncompetitive with ATP (35), suggesting that BBG interacts with a site other than the ligand-binding site. Moreover, complete relief of BBG inhibition required at least 10 min of incubation with ATP in the absence of BBG (Fig. 5). Together, the data suggest that binding of ATP and BBG is mutually exclusive: ATP stabilizes the P2X7Rs in a conformation that cannot bind BBG, and BBG binding stabilizes the channel in a conformation that cannot bind ATP. These findings provide the first evidence that ATP-bound and ATP-free P2X7Rs are stabilized at a different conformation.

The most notable finding of the present work is that activation of P2X7Rs by ATP in parotid acinar cells occurs at two steps: slow assembly (which requires an intact cytoskeleton) and rapid gating (which does not). The P2X7Rs in the adjacent duct cells are preassembled and therefore subject only to rapid gating by ATP. What could the physiological significance of such an arrangement be? Acinar cells store a substantial amount of ATP in secretory granules, which is secreted upon stimulation of digestive enzyme secretion (23). Some ATP can also be released to the lumen of acinar cells with basal secretion during the resting stage. Because P2X7Rs are lytic in acinar cells (14), even basal secretion can induce cell damage. To guard against cell damage, P2X7Rs in acinar cells are kept in a disassembled inactive state. The ATP released during basal secretion is hydrolyzed by ecto-ATPases before it can activate the P2X7Rs. These receptors will be activated only during the period of stimulated exocytosis to aid in stimulus-secretion coupling. Once exocytosis stops, ATP secretion stops as well, and even if the P2X7Rs remain assembled, they will not cause cell damage in the absence of ligand. Thus, acinar...
cells need to be protected from themselves and may do so by keeping their P2X7Rs in an unassembled state. Duct cells, on the other hand, protect themselves by secreting HCO₃⁻ to the salivary or pancreatic fluids (27). In duct cells, ATP functions as a messenger to indicate ongoing acinar cell secretion. In this case, the duct may need to respond rapidly to ATP by augmenting HCO₃⁻ secretion. Duct cells can achieve this by maintaining their P2X7Rs in an unassembled state. Duct cells, on the other hand, may do so by secretory behavior.

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