Interaction of $\alpha_{1D}$-Adrenergic and P2X$_7$ Receptors in the Rat Lacrimal Gland and the Effect on Intracellular [Ca$^{2+}$] and Protein Secretion

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**Purpose.** To determine whether $\alpha_{1D}$-adrenergic receptors ($\alpha_{1D}-\text{AR}$) and P2X$_7$ receptors interact by determining their effect on ATP release, intracellular [Ca$^{2+}$] ([Ca$^{2+}$]), and protein secretion in rat lacrimal gland acini.

**Methods.** Exorbital lacrimal glands from male Sprague-Dawley rats were divided into pieces or digested with collagenase to form acini. With the use of an imaging system, [Ca$^{2+}$] was measured in acini loaded with fura-2. Adenosine triphosphate (ATP) release was determined using the luciferin-luciferase reaction. Peroxidase secretion, our index for protein secretion, was measured spectrophotometrically. Acini were stimulated with the P2X$_7$ receptor agonist, (benzoylbenzoyl)adenosine 5$'$ triphosphate (BzATP) or the $\alpha_{1D}$-AR agonist phenylephrine with or without antagonist preincubation.

**Results.** Phenylephrine increased ATP release from pieces in a time-dependent manner. The $\alpha_{1D}$-AR antagonist BMY7378 blocked the BzATP-stimulated increase in [Ca$^{2+}$], but not in peroxidase secretion. The P2X$_7$ antagonist A438079 blocked the phenylephrine-stimulated increase in [Ca$^{2+}$], but not peroxidase secretion. The increase in [Ca$^{2+}$], caused by phenylephrine and BzATP used simultaneously or sequentially was additive, as was the increase in peroxidase secretion. The inhibition of protein kinase C isoforms or calcium calmodulin kinase II did not alter the BzATP-induced increase in [Ca$^{2+}$].

**Conclusions.** The authors conclude that activation of $\alpha_{1D}$-AR releases ATP, which induces P2X$_7$ receptors to increase [Ca$^{2+}$], but not to stimulate protein secretion. P2X$_7$ receptors in turn activate $\alpha_{1D}$-AR to increase [Ca$^{2+}$], but not to stimulate protein secretion. Furthermore, $\alpha_{1D}$-AR compared with P2X$_7$ receptors use different cellular mechanisms to increase [Ca$^{2+}$] and cause protein secretion. (Invest Ophthalmol Vis Sci. 2011; 52:5720–5729) DOI:10.1167/iovs.11-7358

The lacrimal gland secretes proteins, electrolytes, and water into the tear film and helps maintain the health of the cornea and conjunctiva. When the volume or composition of secreted lacrimal gland fluid changes, the structure and function of the cornea and conjunctiva are altered, and dry eye results. Thus, identifying the agonists that stimulate lacrimal gland secretion and the intracellular signaling pathways used by these agonists is critical in describing the normal regulation of secretion. This knowledge forms the basis for determining dysfunction caused by lacrimal gland pathology in dry eye.

Nerves are the predominant stimuli of lacrimal gland secretion. Nerves are the predominant stimuli of lacrimal gland secretion. The lacrimal gland is innervated by effenter sympathetic and parasympathetic nerves that release the neurotransmitters norepinephrine (from sympathetic nerves) and acetylcholine (Ach) and VIP (from parasympathetic nerves). Norepinephrine and acetylcholine, and VIP are each potent and effective stimuli of lacrimal gland secretion, especially protein secretion, and each activates a separate, distinct signaling pathway. Norepinephrine activates $\alpha_{1D}$-adrenergic receptors ($\alpha_{1D}-\text{AR}$), which cause an increase in [Ca$^{2+}$], by a mechanism that is not yet determined but is not by production of inositol 1,3,5-trisphosphate (InsP$_3$). In addition, these receptors activate endothelial nitric oxide synthase to produce NO. The NO activates guanyl cyclase to increase cellular levels of cGMP, which phosphorylates specific substrates to stimulate protein secretion. Stimulation of $\alpha_{1D}$-AR, also using an unknown effector enzyme, produce diacylglycerol, which activates protein kinase C$_\epsilon$ (PKC$_\epsilon$) to stimulate secretion and PKC$\alpha$ and PKC$\delta$ to inhibit secretion. $\alpha_{1D}$-AR also transactivate the epidermal (EGF) receptor to increase extracellular-regulated kinase (ERK)1/2 activity, which attenuates secretion. Acetylcholine activates muscarinic type 3 acetylcholine receptors (M$_3$AchRs), which are coupled to phospholipase C$\beta$ (PLC$\beta$). PLC$\beta$ activation produces the PKC activator diacylglycerol and InsP$_3$. InsP$_3$ increases the [Ca$^{2+}$], that, along with the activation of PKC$\alpha$, $\delta$, and $\epsilon$, stimulates the secretion of protein stored in preformed secretory granules. M$_3$AchR also activate ERK 1/2 and phospholipase D, which attenuate secretion. VIP interacts with VIPAC1 to stimulate secretion by increasing cellular levels of cAMP and increasing [Ca$^{2+}$].

Most cell types can release ATP, which activates another type of receptor, purinergic receptors. P2 purinergic receptors are divided into two subtypes, P2Y and P2X. P2Y receptors are metabotropic, G protein–linked receptors that increase [Ca$^{2+}$], by activating PLC$\beta$ to produce InsP$_3$, as does the M$_3$AchR in the lacrimal gland. P2X receptors are ionotropic and nonselective ion channels that increase [Ca$^{2+}$], by inducing Ca$^{2+}$ influx. In lacrimal gland acini, ATP predominantly activates P2X rather than P2Y receptors. Even though all P2X receptors except P2X$_4$ are present in the lacrimal gland, only P2X$_2$ and P2X$_3$ appear to be functional because they increase [Ca$^{2+}$], and stimulate protein secretion.

We recently examined the interaction of M$_3$AchR- and P2X$_2$-induced responses. We found that M$_3$AchR activates P2X$_2$.
receptors by releasing ATP from cells other than acinar cells and by an intracellular interaction.\textsuperscript{13} M\textsubscript{3}AchR stimulation of P2X\textsubscript{7} receptors increases [Ca\textsuperscript{2+}]\textsubscript{i} and induces protein secretion. Compared with P2X\textsubscript{7}, receptors, M\textsubscript{3}AchR use additional cellular mechanisms to induce protein secretion.

In the present study we investigated the interaction between α\textsubscript{1D}-AR and P2X\textsubscript{7} receptors by determining their effect on ATP release, [Ca\textsuperscript{2+}]\textsubscript{i}, and protein secretion. We found that activation of α\textsubscript{1D}-AR releases ATP from acini, which induces P2X\textsubscript{7} receptors to increase [Ca\textsuperscript{2+}]\textsubscript{i} but not to stimulate protein secretion. P2X\textsubscript{7} receptors, in turn, activate α\textsubscript{1D}-AR to increase [Ca\textsuperscript{2+}]\textsubscript{i}, but not to stimulate protein secretion. Furthermore, α\textsubscript{1D}-AR and P2X\textsubscript{7} receptors use different cellular mechanisms to increase [Ca\textsuperscript{2+}]\textsubscript{i} and cause protein secretion. Interaction of P2X\textsubscript{7} receptors with α\textsubscript{1D}-AR is different from the interaction of P2X\textsubscript{7} receptors with M\textsubscript{3}AchR.

**Materials and Methods**

**Materials**

Fura-2 tetra-Acetoxyl-methyl ester (fura-2/AM) and reagent (Amplex Red) were purchased from Invitrogen (Carlsbad, CA). Collagenase (CLSIII) was from Worthington Biochemicals (Lakewood, NJ), whereas CSG 9343B was from Tocris Bioscience (Ellisville, MO). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Animals**

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Male Sprague-Dawley rats weighing 125 to 150 g were purchased from Taconic Farms (Germantown, NY). Rats were maintained in constant temperature rooms with fixed 12-hour light/12-hour dark intervals of 12 hours and were fed ad libitum. They were anesthetized for 1 minute in CO\textsubscript{2} and then decapitated. Both exorbital lacrimal glands were removed immediately.

**Preparation of Lacrimal Gland Acini**

Collagenase digestion was used to prepare acini. Lacrimal glands were fragmented before incubation at 37°C with collagenase (100 U/mL, CLSIII; Worthington Biochemicals) in Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES) (119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 10 mM HEPES, and 5.5 mM glucose, at pH 7.45) plus 0.5% BSA for 30 minutes. After incubation, fragments were triturated, filtered through a nylon mesh (150-μm pore size), and centrifuged at 50 g for 2 minutes. The pellet was washed twice through KRB-HEPES containing 4% BSA. The dispersed acini recovered for 60 minutes at 37°C before use.

**Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}**

Acini were incubated for 30 minutes at room temperature in the dark with KRB-HEPES containing 0.5% BSA, 0.5 μM fura 2/AM, 8 μM phloridzin acid F127, and 250 μM sulfipyrazone followed by washing in sulfipyrazone. Calcium measurements were made with a ratio imaging system (InCyt lm2; Intracellular Imaging, Cincinnati, OH) using excimer wavelengths of 540 and 380 nm and an emission wavelength of 505 nm. At least 10 clumps of acini were used for each condition. Inhibitors or antagonists were added 30 minutes before agonists (Table 1). CSG 9343B and calphostin C were dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.1%); all others were dissolved in dH\textsubscript{2}O. After the addition of antagonists, inhibitors, and agonists, data were collected in real time. Data are presented as the actual [Ca\textsuperscript{2+}]\textsubscript{i}, with time or as the change in peak [Ca\textsuperscript{2+}]\textsubscript{i}. Change in peak [Ca\textsuperscript{2+}]\textsubscript{i} was calculated by subtracting the average of the basal value (no added agonist) from the peak [Ca\textsuperscript{2+}]\textsubscript{i}. Although the data are not shown, the plateau [Ca\textsuperscript{2+}]\textsubscript{i}, was affected similarly to the peak [Ca\textsuperscript{2+}]\textsubscript{i}. All [Ca\textsuperscript{2+}]\textsubscript{i} measurements in the presence of BzATP were performed in the absence of extracellular Mg\textsuperscript{2+} to increase the P2X\textsubscript{7} receptor Ca\textsuperscript{2+} response.

**Measurement of ATP Release**

Lacrimal glands were removed and minced into small pieces. Pieces, or acini, were placed into cell strainers and preincubated in 0.5% BSA in KRB for 1 hour in a 12-well culture dish at 37°C. The strainers were moved to new wells containing fresh 0.5% BSA in KRB for an additional hour. Pieces were then placed in fresh 0.5% BSA in KRB containing 10\textsuperscript{-4} M ARL67516, α,β methylene adenosine diphosphate, and B,γ methylene ATP, which are inhibitors of ectonucleotide pyrophospha-

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**Table 1. Inhibitors and Their Target Molecules**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
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<tr>
<td>BMY 7378</td>
<td>α\textsubscript{1D}-Adrenergic receptors</td>
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<tr>
<td>A438079</td>
<td>P2X\textsubscript{7}, purinergic receptors</td>
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<tr>
<td>Apyrase</td>
<td>ATPases</td>
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<tr>
<td>Calphostin C</td>
<td>Protein kinase C (PKC)</td>
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<tr>
<td>CGS 9343B</td>
<td>Ca\textsuperscript{2+}/calmodulin-dependent kinase</td>
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tases and ectoATPases. Inhibitors were added 30 minutes before agonists (Table 1). Agonists were added for 0 to 10 minutes, and the lacrimal gland pieces were removed. The ATP concentrations in the supernatants were determined with the chemiluminescent luciferin-luciferase reaction using an ATP assay kit (FLAA; Sigma-Aldrich). The luciferin-luciferase working solution was 100 μL stock solution to 1 mL assay buffer, according to the manufacturer’s instructions. Twenty-five microliters of samples were added to a 96-well plate and placed in a luminometer (SynergyMx microplate reader; BioTek Instruments, Winooski, VT). The luciferin-luciferase working solution (15 μL) was injected into each well using the internal injector system. The emitted light was recorded with an integration time of 100 ms/ measurement every minute for 10 minutes. ATP levels were calculated by integrating under the luminescence curve. The luminescence was converted to ATP concentrations using an ATP standard curve. Data were expressed as fold increase above basal.

Measurement of Peroxidase Secretion

Acini were incubated for 40 minutes in KRB-HEPES containing 4% BSA at 37°C in the presence of agonists. Inhibitors or antagonists were added 30 minutes before agonists (Table 1). CSG 9343B and calphostin C were dissolved in DMSO (final concentration, 0.1%); all other compounds were dissolved in water. To terminate the incubation, acini were pelleted by centrifugation, and the supernatant was collected. The pellet was homogenized in 10 mM Tris-HCl (pH 7.5). Peroxidase activity, an index of protein secretion, was measured in duplicate in both the supernatant and the pellet. Peroxidase was measured using a reagent (Amplex Red; Invitrogen) that, when oxidized by peroxidase in the presence of hydrogen peroxide, produces a highly fluorescent molecule. The amount of fluorescence in the supernatant and pellet was quantified using a fluorescence microplate reader (model FL600; Bio-Tek Instruments) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Peroxidase was expressed as a percentage of peroxidase secreted into the media (supernatant) compared with the total peroxidase present in the cells before stimulation (pellet plus supernatant). Data were expressed as fold increase multiplied by basal, which was set to 1.

Statistical Analysis

Results were expressed as mean ± SEM. Data were analyzed by Student’s t test. P < 0.5 was considered statistically significant.

RESULTS

α1b-Adrenergic Agonists Increase [Ca2+]i in a Concentration-Dependent Manner

When the [Ca2+] was determined in a large population of acini suspended in a cuvette, the α1b-adrenergic agonist phenylephrine increased [Ca2+]i in a concentration-dependent manner. A maximum increase of 15 nM was induced by phenylephrine at 10⁻³ M. In the present experiments when

FIGURE 2. Effect of phenylephrine on ATP release from lacrimal gland pieces. Time-dependent release of ATP from lacrimal gland pieces stimulated with α₁b-adrenergic agonist phenylephrine (10⁻⁴ M) for 0 to 10 minutes (A). Pieces were preincubated with the α₁b-adrenergic receptor antagonist BMY 7378 (10⁻⁴ M) for 30 minutes before stimulation with Ph (10⁻⁴ M) for 30 seconds (B). Values are mean ± SEM of three independent experiments. *Significant difference from no additions. #Significant difference between agonist alone and agonist plus inhibitor.

FIGURE 3. Effect an inhibitor of ATPases, apyrase, on [Ca2⁺]i stimulated by phenylephrine in lacrimal gland acini. Time-dependent increase in [Ca2⁺]i induced by stimulation of lacrimal gland acini containing fura-2 with α₁b-adrenergic agonist phenylephrine (10⁻⁵ M) after 30-minute preincubation with apyrase (20 U/mL). Traces are mean of five experiments (A). Mean change in peak [Ca2⁺]i from five experiments (B). Values are mean ± SEM. Arrow: time of addition of phenylephrine. *Significant difference from no additions (0). #Significant difference between agonist alone and agonist plus inhibitor.
measuring \([\text{Ca}^{2+}]\), by fluorescence microscopy in approximately 10 to 20 clumps of acini, phenylephrine also caused an increase in \([\text{Ca}^{2+}]\) (Fig. 1A). A maximum increase in \([\text{Ca}^{2+}]\) of 128.6 ± 17.8 nM was obtained with 10⁻⁵ M phenylephrine. Using the microscopy measurement technique, the change in \([\text{Ca}^{2+}]\) caused by supramaximal concentrations of phenylephrine caused a decreased response, unlike the cuvette method, with which the response remained elevated (Fig. 1B).

α₁D-Adrenergic Agonists Release ATP from Lacrimal Gland Pieces

We sought to determine whether α₁D-AR agonists release ATP that could activate P2X₇ receptors. Pieces of lacrimal gland were stimulated with no agonists (basal) or the α₁D-adrenergic agonist phenylephrine (10⁻⁴ M) for 0 to 10 minutes. Basal ATP release was set to 1. Phenylephrine increased ATP release in a time-dependent manner with a significant increase in the release of ATP by 2.3 ± 0.04-fold at 0.5 minutes (Fig. 2A). Preincubation with BMY 7378, an α₁D-AR antagonist, completely blocked phenylephrine-stimulated ATP release (Fig. 2B). These data suggest that α₁D-adrenergic agonists can release ATP from lacrimal gland pieces.

A second method was used to confirm that α₁D-adrenergic agonists stimulated ATP release. Isolated acini were preincubated with apyrase, which breaks down extracellular ATP into adenosine monophosphate, which can be converted to adenosine and should thus prevent α₁D-AR agonist activation of P2X₇ receptors (Table 1). Apyrase completely blocked the phenylephrine-stimulated increase in \([\text{Ca}^{2+}]\) from acinar cells (Figs. 3A, 3B). We conclude that α₁D-adrenergic agonists release ATP from the lacrimal gland. The released ATP then would activate P2X₇ receptors to increase \([\text{Ca}^{2+}]\).

α₁D-Adrenergic and P2X₇ Receptor Antagonists Cross-Inhibit Each Other’s Intracellular Ca²⁺ Response in Lacrimal Gland Acinar Cells

Activation of α₁D-AR and P2X₇ receptors increases \([\text{Ca}^{2+}]\) in lacrimal gland acini. To determine whether these signaling pathways interact, we stimulated fura-2–loaded acini with the P2X₇ selective agonist BzATP after incubation with and without the α₁D-adrenergic receptor inhibitor BMY 7378 at 10⁻⁴ M (Table 1). BzATP at 10⁻⁴ M increased the \([\text{Ca}^{2+}]\) by 123.0 ± 32.5 nM (Figs. 4A, 4B). BMY 7378 significantly inhibited the response to 35.4 ± 12.0 nM. As a positive control, the α₁D-AR agonist phenylephrine increased \([\text{Ca}^{2+}]\) by 157.8 ± 26.0 nM. BMY 7378 at 10⁻⁴ M significantly reduced the phenylephrine response by 79%. These results suggest that the inhibition of α₁D-AR blocks P2X₇ receptor stimulation.

In separate experiments, cells were stimulated with the α₁D-adrenergic agonist phenylephrine (10⁻⁵ M) with and with-

**FIGURE 4.** Effect of α₁D-adrenergic agonist antagonist BMY 7378 and P2X₇ receptor antagonist A438079 on \([\text{Ca}^{2+}]\) in lacrimal gland acini. Time-dependent increase in \([\text{Ca}^{2+}]\), induced by stimulation of lacrimal gland acini containing fura-2 with BzATP (10⁻⁴ M) or α₁D-adrenergic agonist phenylephrine (10⁻⁵ M) after 30-minute preincubation with BMY 7378 (10⁻⁴ M). Traces are mean of five (phenylephrine) or six (BzATP) experiments (A). Mean change in peak \([\text{Ca}^{2+}]\) from five (phenylephrine) or six (BzATP) experiments (B). Time-dependent increase in \([\text{Ca}^{2+}]\), induced by stimulation of lacrimal gland acini containing fura-2 with α₁D-adrenergic agonist phenylephrine (10⁻⁵ M) after 30-minute preincubation with A438079 (10⁻⁴ M) (C). Trace is the mean of seven experiments. Mean change in peak \([\text{Ca}^{2+}]\), from seven experiments (D). Values are mean ± SEM. Arrows: time of addition of agonists. *Significant difference from no addition. #Significant difference between agonist alone and agonist plus antagonist.
out preincubation with the P2X<sub>7</sub> antagonist A438079 (10<sup>-4</sup> M; Table 1). Phenylephrine increased [Ca<sup>2+</sup>]<sub>i</sub> by 146.8 ± 28.5 nM (Figs. 4C, 4D). A438079 significantly decreased the response to 73.8 ± 17.0 nM. These data imply that the inhibition of P2X<sub>7</sub> receptors blocks the stimulation of α<sub>1D</sub>-AR.

Taken together these data suggest that P2X<sub>7</sub> and α<sub>1D</sub>-AR can activate each other to increase [Ca<sup>2+</sup>]<sub>i</sub>.

Activation of α<sub>1D</sub>-AR and P2X<sub>7</sub> Receptors Use Separate Cellular Signaling Pathways to Increase [Ca<sup>2+</sup>]<sub>i</sub>

[Ca<sup>2+</sup>]<sub>i</sub> was measured in fura-2–loaded acini stimulated simultaneously with phenylephrine and BzATP at maximal and submaximal concentrations for increasing [Ca<sup>2+</sup>]<sub>i</sub>. Phenylephrine (10<sup>-5</sup> M) and BzATP (10<sup>-4</sup> M), at maximal concentrations, each significantly increased [Ca<sup>2+</sup>]<sub>i</sub> to 161.7 ± 40.0 nM and 258.2 ± 29.1 nM, respectively (Figs. 5A, 5B). When the two were added at the same time, the increase in [Ca<sup>2+</sup>]<sub>i</sub> was 315.4 ± 67.6 nM, which was lower than, but not significantly different from, the calculated additivity. Similar results were obtained when a submaximal concentration of phenylephrine was used, but the difference in the calculated and experimentally obtained increase in [Ca<sup>2+</sup>]<sub>i</sub> was statistically significant (Figs. 5C, 5D).

A second experimental paradigm was used to further explore the signaling pathways used by α<sub>1D</sub>-AR and P2X<sub>7</sub> receptors to increase [Ca<sup>2+</sup>]<sub>i</sub>. Instead of adding the two agonists simultaneously, the addition of one agonist preceded the second. Maximal concentrations of phenylephrine (10<sup>-5</sup> M) and BzATP (10<sup>-4</sup> M) were used. When phenylephrine was used first, it increased the [Ca<sup>2+</sup>]<sub>i</sub>, by 170.4 ± 55.4 nM (Figs. 6A, 6B). When phenylephrine was used after BzATP, the response was not altered, and the increase in [Ca<sup>2+</sup>]<sub>i</sub> was 183.9 ± 82.7 nM. Use of BzATP first increased the [Ca<sup>2+</sup>]<sub>i</sub> to 232.5 ± 31.7 nM. When BzATP was used after phenylephrine, the response was not altered and the increase in [Ca<sup>2+</sup>]<sub>i</sub> was 276.0 ± 116.2 nM.
These results taken together suggest that when maximal concentrations of agonists were used P2X<sub>7</sub> and α<sub>1D</sub>-AR receptors used different cellular mechanisms to increase [Ca<sup>2+</sup>]<i>i</i>. When the α<sub>1D</sub>-adrenergic agonist was decreased, the pathways were no longer additive. This could have reflected the use of an overlapping pathway when the agonist concentration was altered.

α<sub>1D</sub>-Adrenergic but Not P2X<sub>7</sub>, Receptors Use PKC to Increase [Ca<sup>2+</sup>]<i>i</i>

Acini containing fura-2 were stimulated with phenylephrine (10<sup>-5</sup> M) or BzATP (10<sup>-4</sup> M) that had been preincubated with and without the PKC inhibitor calphostin C (Table 1). Phenylephrine increased [Ca<sup>2+</sup>]<i>i</i> to 241 ± 61.2 nM (Figs. 7A, 7C). Preincubation with calphostin C at 10<sup>-9</sup> and 10<sup>-7</sup> M significantly decreased [Ca<sup>2+</sup>]<i>i</i> to 88.5 ± 22.1 nM and 65.8 ± 24.1 nM, respectively. BzATP (10<sup>-4</sup>) increased the intracellular [Ca<sup>2+</sup>]<i>i</i> by 119.0 ± 30.8 nM (Figs. 7B, 7C). Preincubation with calphostin C at either concentration did not alter the BzATP-induced Ca<sup>2+</sup> response. Thus, stimulation of α<sub>1D</sub>-adrenergic, but not of P2X<sub>7</sub>, receptors activates PKC isoforms to alter the [Ca<sup>2+</sup>]<i>i</i>. Differential activation of PKC isoforms by α<sub>1D</sub>-adrenergic and P2X<sub>7</sub> agonists is consistent with the use of separate signaling pathways by these two agonists to increase [Ca<sup>2+</sup>]<i>i</i>.

Activation of Ca<sup>2+</sup>/Calmodulin-Dependent Protein Kinase Does Not Alter the P2X<sub>7</sub>, Receptor-Induced Increase in [Ca<sup>2+</sup>]<i>i</i> in Lacrimal Gland Acini

Because P2X<sub>7</sub>-receptors have been reported to activate Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMK) to increase pore formation, and we next investigated whether stimulation of the P2X<sub>7</sub>-receptor activates CaMK to increase [Ca<sup>2+</sup>]<i>i</i> in rat lacrimal gland. Acini were preincubated with or without the CaMKII inhibitor CSG 9343B (10<sup>-5</sup> and 10<sup>-4</sup> M) and then were stimulated with phenylephrine (Table 1). Phenylephrine significantly increased [Ca<sup>2+</sup>]<i>i</i> by 198.0 ± 53.2 nM. Preincubation with CSG 9343B (10<sup>-4</sup>) significantly decreased the phenylephrine-induced response to 61.8 ± 19.9 nM (Figs. 8A, 8B). BzATP significantly increased [Ca<sup>2+</sup>]<i>i</i> by 233.6 ± 40.7 nM (Figs. 8C, 8D). Inhibition of CaMKII with CSG, used at 10<sup>-6</sup> to 10<sup>-4</sup> M, did not alter the BzATP-induced increase in [Ca<sup>2+</sup>]<i>i</i> at any concentration. Thus, P2X<sub>7</sub>-receptors do not appear to activate CaMKII to increase [Ca<sup>2+</sup>]<i>i</i>.

Differential activation of CaMKII by α<sub>1D</sub>-adrenergic and P2X<sub>7</sub> agonists is consistent with the use of separate signaling pathways by these two agonists to increase [Ca<sup>2+</sup>]<i>i</i>.

α<sub>1D</sub>-Adrenergic and P2X<sub>7</sub>, Receptor Inhibitors Do Not Cross-Inhibit Each Other’s Secretory Response in Lacrimal Gland Acinar Cells

Because α<sub>1D</sub>-adrenergic agonists release ATP to activate P2X<sub>7</sub>-receptors and increase the [Ca<sup>2+</sup>]<i>i</i>, we determined whether this effect was extended to the entire signaling pathway, resulting in protein secretion. Lacrimal gland acini were stimulated with BzATP at 10<sup>-4</sup> M, and protein secretion was measured. BzATP significantly stimulated protein secretion by 1.5 ± 0.1-fold (Fig. 9A). Preincubation with BMY 7378 (10<sup>-4</sup>) did not affect BzATP-stimulated secretion. In analogous experiments, acini were stimulated with the α<sub>1D</sub>-adrenergic agonist phenylephrine at 10<sup>-4</sup> M, which increased secretion by 2.7 ± 0.5-fold (Fig. 9B). Preincubation with the P2X<sub>7</sub>-inhibitor A438079 (10<sup>-4</sup>) M did not alter phenylephrine-stimulated secretion. In contrast to the increase in [Ca<sup>2+</sup>]<i>i</i> and α<sub>1D</sub>-AR or P2X<sub>7</sub>-receptor inhibitors did not block protein secretion stimulated by the other agonist.

α<sub>1D</sub>-Adrenergic and P2X<sub>7</sub>, Receptor Agonists Use Separate Cellular Pathways to Stimulate Protein Secretion from Lacrimal Gland Acini

Protein secretion was measured from acini stimulated simultaneously with phenylephrine (10<sup>-5</sup> M) and BzATP (10<sup>-4</sup> M) at concentrations maximal for secretion. Phenylephrine and BzATP each significantly increased protein secretion by 1.7 ± 0.3- and 1.2 ± 0.1-fold respectively (Fig. 10A). When the two agonists were added simultaneously, the increase in protein secretion was not significantly different from the calculated additive response. Similar results were obtained when this experiment was performed in the absence of Mg<sup>2+</sup> to increase the P2X<sub>7</sub>-receptor response (Fig. 10B). These results suggest that, similar to the increase in [Ca<sup>2+</sup>]<i>i</i>, α<sub>1D</sub>-AR and P2X<sub>7</sub>-receptors use different cellular pathways to stimulate protein secretion.
In the present study we found that activation of α1D-AR releases ATP from acini and stimulates P2X7 receptor activity to increase \([\text{Ca}^{2+}]_i\), but not to stimulate protein secretion. Similarly, P2X7 receptors stimulate α1D-AR stimulation of P2X7 receptors. Third, blocking α1D-AR receptors with the α1D-AR antagonist prevents the P2X7 receptor from increasing \([\text{Ca}^{2+}]_i\), and, conversely, inhibiting P2X7 receptors with a P2X7 receptor antagonist blocks α1D-AR from increasing \([\text{Ca}^{2+}]_i\). Interestingly, this cross-stimulation does not extend to protein secretion. The interaction of the two receptors to increase \([\text{Ca}^{2+}]_i\) could be a direct interaction, suggesting that the two receptors are part of a signaling complex such as a signalosome. In support of the possible direct interaction of receptors, in myenteric neurons, nicotinic acetylcholine and P2X7, P2X4, and P2X7 receptors are close enough so that a conformational change in one induced by activation can inhibit the other receptor.\(^{15,16}\) In the lacrimal gland, it is possible that α1D-AR and P2X7 receptors are colocalized in a complex so that inhibition of P2X7 and α1D-AR could affect the activation of the other. It is also possible that the two receptors regulate one another’s signaling components within the signalosome.\(^{17}\) In addition, Michel et al.\(^{18}\) showed that different inhibitors of the P2X7 receptor have negative and positive allosteric effects on these receptors and, thus, could change the P2X7 receptor conformation so that it could alter the α1D-AR.

Both α1D-AR and P2X7 receptors have extracellular and intracellular domains that can be modified to change the way the receptor increases \([\text{Ca}^{2+}]_i\).\(^{19}\) For example, the intracellular tail of the P2X7 receptor contains consensus sites for PKC phosphorylation. In addition the rat, but not the human, P2X7 receptor has a calmodulin-binding domain in its C-terminal\(^{20}\) that prevents its desensitization with repeated agonist application. P2X7 receptors also have a Src homology 3 death domain in the C terminus,\(^{21}\) which is involved in P2X7 receptor activation of pannexin1, resulting in pore formation. Findings from a previous study\(^{15}\) along with those in the present study indicate that neither stimulation nor inhibition of PKC activity appears to alter the P2X7 receptor increase in \([\text{Ca}^{2+}]_i\).\(^{19}\) In the design of the present study, the activation of CaMKII did not alter the single addition of BzATP to increase in \([\text{Ca}^{2+}]_i\). Finally, we did not test the role of Src in the present study. Thus, to date, the mechanism used by α1D-AR to stimulate P2X7 receptors to increase in \([\text{Ca}^{2+}]_i\), and vice versa has not been identified.

Given that P2X7 and α1D-AR stimulate each other, it might be expected that these two receptors activate a common signaling pathway to increase \([\text{Ca}^{2+}]_i\), and to stimulate secretion. Thus, with the sequential addition of P2X7 and α1D-AR agonists, the agonist added first would decrease the response of the second. This was not the case in the lacrimal gland. Both simultaneous and sequential addition of P2X7- and α1D-AR agonists caused additive \([\text{Ca}^{2+}]_i\) responses, and simultaneous addition of both agonists caused additive secretory responses. Furthermore, inhibitors of P2X7 receptors and α1D-AR did not block the secretion stimulated by the other agonist. The results indicate that P2X7 and α1D-AR use different cellular mechanisms to increase \([\text{Ca}^{2+}]_i\), and to stimulate secretion. P2X7 receptors are nonselective ion channels that increase \([\text{Ca}^{2+}]_i\). The mechanism that the activation of α1D-AR uses to increase \([\text{Ca}^{2+}]_i\) in rat lacrimal gland acini is unknown. It is known that α1D-AR does not induce the production of InsP3 in rats, though α1D-AR uses this compound to increase \([\text{Ca}^{2+}]_i\), in mouse lacrimal gland acini.

**DISCUSSION**

**FIGURE 7.** Effect of inhibition of PKC on phenylephrine- and BzATP-stimulated \([\text{Ca}^{2+}]_i\) in lacrimal gland acini. Time-dependent increase in \([\text{Ca}^{2+}]_i\), induced by stimulation of lacrimal gland acini containing fura-2 with α1D-adrenergic agonist phenylephrine (10^{-5} M) or BzATP (10^{-4} M) after 30-minute preincubation with calphostin C (CalC). Traces are the mean of seven experiments for phenylephrine (A), and traces are the mean of four experiments for BzATP (B). Mean change in peak \([\text{Ca}^{2+}]_i\), from seven experiments (phenylephrine) and four experiments (BzATP) (C). Values are mean ± SEM. Arrows: addition of agonists.

*Significant difference from no additions (0). #Significant difference between agonist alone and agonist plus inhibitor.
Inhibition of PKC and CaMK in turn inhibited 1D-AR, but not P2X7, receptor-stimulated increases in \([\text{Ca}^{2+}]_i\), indicating that P2X7 and 1D-AR use separate mechanisms to increase intracellular calcium, thus accounting for the additivity of the increase in \([\text{Ca}^{2+}]_i\) when both receptors are activated.

In the present study, P2X7 and 1D-AR antagonists cross-inhibited the increase in \([\text{Ca}^{2+}]_i\) caused by the other receptor but did not block protein secretion. These differential effects on \([\text{Ca}^{2+}]_i\), compared with secretion, suggest that increasing \([\text{Ca}^{2+}]_i\) is not the major mechanism by which 1D-AR stimulates protein secretion. The results also imply that the 1D-AR and P2X7 receptor-mediated increase in \([\text{Ca}^{2+}]_i\) is regulated by different components of the signalosome than are the components that cause secretion.

Results of the present study showed that protein secretion, similar to \([\text{Ca}^{2+}]_i\), increased by the simultaneous addition of P2X7 and 1D-AR agonists was additive, suggesting that in the lacrimal gland \(\alpha_{1D}\)-AR and P2X7 receptors activate different signaling pathways to stimulate protein secretion. In lacrimal gland acini, \(\alpha_{1D}\)-AR activate eNOS/cGMP, increase PKCε activity, and raise the \([\text{Ca}^{2+}]_i\), by an unidentified mechanism to stimulate secretion. To attenuate secretion \(\alpha_{1D}\)-AR agonists transactivate the EGF receptor to induce the ERK1/2 cascade and activate PKC and PKCδ. Signaling pathways used by P2X7 receptors in lacrimal acini have been less well studied. A previous publication showed that P2X7 receptors increase the \([\text{Ca}^{2+}]_i\) using the ionotropic receptor itself to stimulate secretion and activate ERK1/2, which could attenuate secretion. In a previous study, as in the present study, an effect of P2X7 receptors on PKC isoforms and CaMKII was ruled out. To date, the signaling pathways used by 1D-AR and P2X7 receptors differ in their use of PKC isoforms. Further research is warranted on the signaling mechanisms used by 1D-AR and P2X7 receptors.

In the lacrimal gland, M3AchR and 1D-AR use separate different mechanisms to stimulate protein secretion. The interaction of the M3AchR and 1D-AR with P2X7 receptors supports this concept. Compared with the signaling pathways used by 1D-AR, described in the previous paragraph, to stimulate the secretion, M3AchR activates PLCβ, producing InsP3 that increases the \([\text{Ca}^{2+}]_i\), and producing

**FIGURE 8.** Effect of inhibition of calcium/calmodulin kinase II (CamKII) on phentolamine- and BzATP-stimulated \([\text{Ca}^{2+}]_i\) in lacrimal gland acini. Time-dependent increase in \([\text{Ca}^{2+}]_i\) induced by stimulation of lacrimal gland acini containing fura-2 with 1D-adrenergic agonist phentolamine (10⁻⁵ M; A) or BzATP (10⁻⁴ M, C) after 30-minute preincubation with the CamKII inhibitor CSG 9343B. Traces are the mean of four experiments. Mean change in peak \([\text{Ca}^{2+}]_i\), from Ph (B) and BzATP (D) are from four experiments. Values are mean ± SEM. Arrows: addition of agonists. \(*\) Significant difference from agonist alone.
diacylglycerol that activates PKCa, PKCe, and PKCδ. To attenuate secretion, M3AchR induces PLD that, using Rho and ROCK, stimulates ERK1/2, and it induces the nonreceptor tyrosine kinases Pyk2 and Src to transactivate the EGFR, thereby activating the ERK1/2 cascade.5 Even though there is some overlap in the signaling components, distinct signaling pathways are activated by the two types of receptors. In the same type of paradigm, M3AchR and 1D-AR interact differently with P2X7 receptors. M3AchR activates P2X7 receptors to increase [Ca2+]i and to stimulate secretion. M3AchR releases ATP from nonacinar cells that could activate P2X7 receptors and probably use a direct intracellular pathway to stimulate the activity of P2X7 receptors.13 In contrast, 1D-AR release ATP from acini to activate P2X7 receptors and, in turn, the activation of P2X7 receptors stimulates 1D-AR to increase [Ca2+]i. The signaling mechanisms used by 1D-AR and P2X7 receptors to increase [Ca2+]i appear to be different, but those used by the M3AchR and P2X7 receptors overlap. However, the signaling mechanisms used by 1D-AR, M3AchR, and P2X7 receptors to stimulate protein secretion are different. Thus, to date, the cellular mechanisms used by M3AchR and 1D-AR to induce P2X7 receptors activity appear to be different.

In one other tissue, the interaction of αAR and P2X7 receptors has been investigated. In hypothalamo-neurohypophysial explants, the α1D-AR agonist causes oxytocin and vasopressin secretion, and co-exposure of α1-AR agonists with ATP prolongs and potentiates the secretory response.23,24 Gomes et al.24 found that ATP activates P2X7 receptors that recruit P2X7 and P2X8 receptors, which are responsible and desensitization resistant and thereby prolong secretion.24 In contrast, in the present study, P2X7-receptors and α1D-AR do not appear to potentiate the secretory response.

We conclude that in the lacrimal gland, the activation of α1D-AR releases ATP that induces P2X7 receptors to increase [Ca2+]i, but not to stimulate protein secretion. P2X7 receptors, in turn, activate α1D-AR to increase [Ca2+]i, but not to stimulate protein secretion. Furthermore, α1D-AR compared with P2X7 receptors use different cellular mechanisms to increase [Ca2+]i, and to cause protein secretion. The interaction of P2X7 receptors with α1D-AR is different from the interaction of P2X7 receptors with M3AchR.

**FIGURE 9.** Effect of α1D-adrenergic and P2X7 receptor antagonists on protein secretion stimulated by phenylephrine or BzATP from lacrimal gland acini. Peroxidase secretion, our index of protein secretion, was measured from lacrimal gland acini preincubated with the α1D-adrenergic receptor antagonist BMY 7378 (10^-4 M) for 30 minutes and then stimulated with BzATP (10^-4 M) for 40 minutes (A) or preincubated with the P2X7 receptor antagonist A438079 (10^-4 M) for 30 minutes and then stimulated with phenylephrine (phenylephrine) (10^-4 M) for 40 minutes (B). Values are the mean ± SEM of four independent experiments. *Significant difference from no additions.

**FIGURE 10.** Effect of simultaneous addition of phenylephrine and BzATP on protein secretion from lacrimal gland acini. Peroxidase secretion, our index of protein secretion, was measured from lacrimal gland acini stimulated for 40 minutes with phenylephrine (10^-4 M), BzATP (10^-4 M), or the two agonists added simultaneously. The calculated amount of secretion from the two agonists added simultaneously is also plotted. Results obtained in the presence (A) and the absence (B) of extracellular Mg2+. Values are the mean ± SEM of four independent experiments. *Significant difference from no addition. n.s., no significant difference.
References