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

Darlene A. Dartt and Robin R. Hodges

Purpose. To determine whether α_{1D}-adrenergic receptors (α_{1D}-AR) and P2X$_7$ receptors interact by determining their effect on ATP release, intracellular [Ca^{2+}] ([Ca^{2+}]$_i$), 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+}]$_i$ 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 α_{1D}-AR agonist phenylephrine with or without antagonist preincubation.

Results. Phenylephrine increased ATP release from pieces in a time-dependent manner. The α_{1D}-AR antagonist BMY7378 blocked the BzATP-stimulated increase in [Ca^{2+}]$_i$ but not in peroxidase secretion. The P2X$_7$ antagonist A438079 blocked the phenylephrine-stimulated increase in [Ca^{2+}]$_i$ but not peroxidase secretion. The increase in [Ca^{2+}]$_i$ 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+}]$_i$.

Conclusions. The authors conclude that activation of α_{1D}-AR releases ATP, which induces P2X$_7$ receptors to increase [Ca^{2+}]$_i$ but not to stimulate protein secretion. P2X$_7$ receptors in turn activate α_{1D}-AR to increase [Ca^{2+}]$_i$ but not to stimulate protein secretion. Furthermore, α_{1D}-AR compared with P2X$_7$ receptors use different cellular mechanisms to increase [Ca^{2+}]$_i$ 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.[1] The lacrimal gland is innervated by efferent sympathetic and parasympathetic nerves that release the neurotransmitters norepinephrine (from sympathetic nerves) and acetylcholine (Ach) and VIP (from parasympathetic nerves). Norepinephrine, acetylcholine, and VIP are each potent and effective stimuli of lacrimal gland secretion, especially protein secretion, and each activates a separate, distinct signaling pathway.[2–5] Norepinephrine activates α_{1D}-adrenergic receptors (α_{1D}-AR), which cause an increase in [Ca^{2+}]$_i$ by a mechanism that is not yet determined but is not by production of inositol 1,3,5-trisphosphate ($InsP_3$).[4] In addition, these receptors activate endothelial nitric oxide synthase to produce NO.[6] The NO activates guanylyl cyclase to increase cellular levels of cGMP, which phosphorylates specific substrates to stimulate protein secretion.[6] Stimulation of α_{1D}-AR, also using an unknown effector enzyme, produce diacylglycerol, which activates protein kinase Cε (PKCε) to stimulate secretion and PKCα and PKCδ to inhibit secretion.[5] α_{1D}-AR also transactivate the epidermal (EGF) receptor to increase extracellular-regulated kinase (ERK)1/2 activity, which attenuates secretion.[7,8] Acetylcholine activates muscarinic type 3 acetylcholine receptors (M_3AchRs), which are coupled to phospholipase Cβ (PLCβ). PLCβ activation produces the PKC activator diacylglycerol and $InsP_3$.[3] $InsP_3$ increases the [Ca^{2+}]$_i$ that, along with the activation of PKCα, -δ, and -ε, stimulates the secretion of protein stored in preformed secretory granules.[3,5] M_3AchR also activate ERK 1/2 and phospholipase D, which attenuate secretion.[9,10] VIP interacts with VIPAC1 to stimulate secretion by increasing cellular levels of cAMP and increasing [Ca^{2+}]$_i$.[11] Even though norepinephrine, Ach, and VIP activate distinct signaling pathways, the neurotransmitters can be released together and can interact, causing a different secretory response than that activated by each agonist alone. For example, phenylephrine and VIP added together potentiate secretion,[2] whereas phenylephrine and carbachol (an Ach analog) added at the same time cause additive secretion.[4]

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+}]$_i$ by activating PLCβ to produce $InsP_3$, as does the M_3AchR in the lacrimal gland. P2X receptors are ionotropic and nonselective ion channels that increase [Ca^{2+}]$_i$ by inducing Ca^{2+} influx. In lacrimal gland acini, ATP predominantly activates P2X rather than P2Y receptors. Even though all P2X receptors except P2X$_5$ are present in the lacrimal gland, only P2X$_3$ and P2X$_7$ appear to be functional because they increase [Ca^{2+}]$_i$ and stimulate protein secretion.[12]

We recently examined the interaction of M_3AchR- and P2X$_7$-induced responses. We found that M_3AchR activates P2X$_7$

From the Department of Ophthalmology, Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts.

Supported by National Institutes of Health Grant EY06177.

Submitted for publication February 9, 2011; revised May 14, 2011; accepted June 8, 2011.

Disclosure: **D.A. Dartt**, None; **R.R. Hodges**, None

Corresponding author: Darlene A. Dartt, Department of Ophthalmology, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114; darlene.dartt@schepens.harvard.edu.

Table 1. Inhibitors and Their Target Molecules

Inhibitor	Target
BMY 7378	α_{1D}-Adrenergic receptors
A438079	P2X$_7$ purinergic receptors
Apyrase	ATPases
Calphostin C	Protein kinase C (PKC)
CGS 9343B	Ca^{2+}/calmodulin-dependent kinase

receptors by releasing ATP from cells other than acinar cells and by an intracellular interaction.[13] M_3AchR stimulation of P2X$_7$ receptors increases $[Ca^{2+}]_i$ and induces protein secretion. Compared with P2X$_7$ receptors, M_3AchR use additional cellular mechanisms to induce protein secretion.

In the present study we investigated the interaction between α_{1D}-AR and P2X$_7$ receptors by determining their effect on ATP release, $[Ca^{2+}]_i$, and protein secretion. We found that activation of α_{1D}-AR releases ATP from acini, which induces P2X$_7$ receptors to increase $[Ca^{2+}]_i$ but not to stimulate protein secretion. P2X$_7$ receptors, in turn, activate α_{1D}-AR to increase $[Ca^{2+}]_i$ but not to stimulate protein secretion. Furthermore, α_{1D}-AR and P2X$_7$ receptors use different cellular mechanisms to increase $[Ca^{2+}]_i$ and cause protein secretion. Interaction of P2X$_7$ receptors with α_{1D}-AR is different from the interaction of P2X$_7$ receptors with M_3AchR.

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_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 $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 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 $[Ca^{2+}]_i$

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 pluronic acid F127, and 250 μM sulfinpyrazone followed by washing in sulfinpyrazone. Calcium measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using excimer wavelengths of 340 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_2O. After the addition of antagonists, inhibitors, and agonists, data were collected in real time. Data are presented as the actual $[Ca^{2+}]_i$ with time or as the change in peak $[Ca^{2+}]_i$. Change in peak $[Ca^{2+}]_I$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[Ca^{2+}]_i$. Although the data are not shown, the plateau $[Ca^{2+}]_i$ was affected similarly to the peak $[Ca^{2+}]_i$. All $[Ca^{2+}]_i$ measurements in the presence of BzATP were performed in the absence of extracellular Mg^{2+} to increase the P2X$_7$ receptor Ca^{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^{-4} M ARL-67516, α,β methylene adenosine diphosphate, and β,γ methylene ATP, which are inhibitors of ectonucleotide pyrophospha-

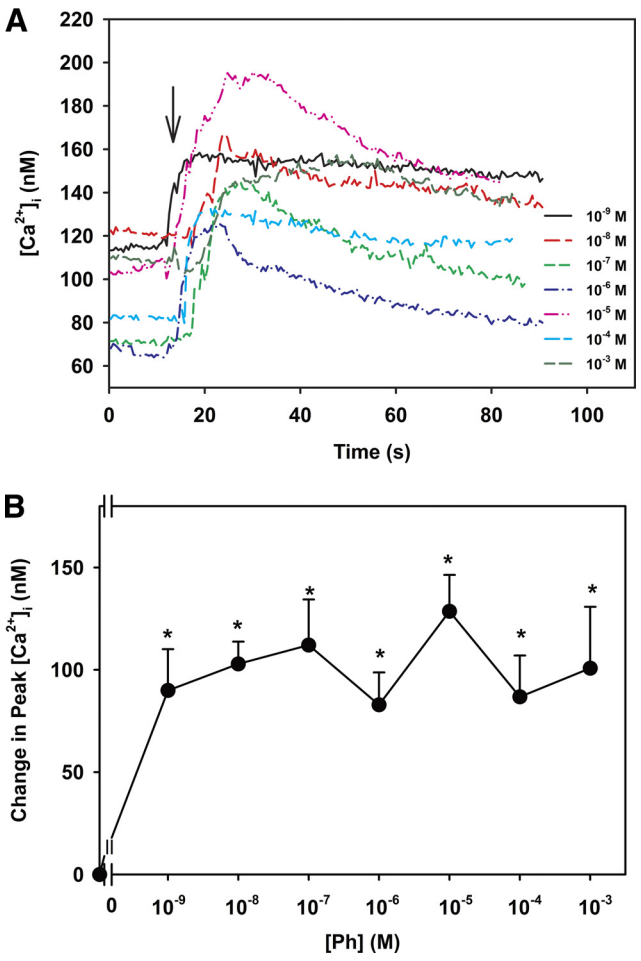

Figure 1. Effect of phenylephrine concentration on $[Ca^{2+}]_i$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing Fura-2 with increasing concentrations of the α_{1D}-adrenergic agonist phenylephrine (phenylephrine) added after 15 seconds of incubation. The trace shown (**A**) is the mean of four experiments. Mean change in peak $[Ca^{2+}]_i$ from four experiments (**B**). Values are mean ± SEM. *Arrow*: time of phenylephrine addition. *Significant difference from no addition (0).

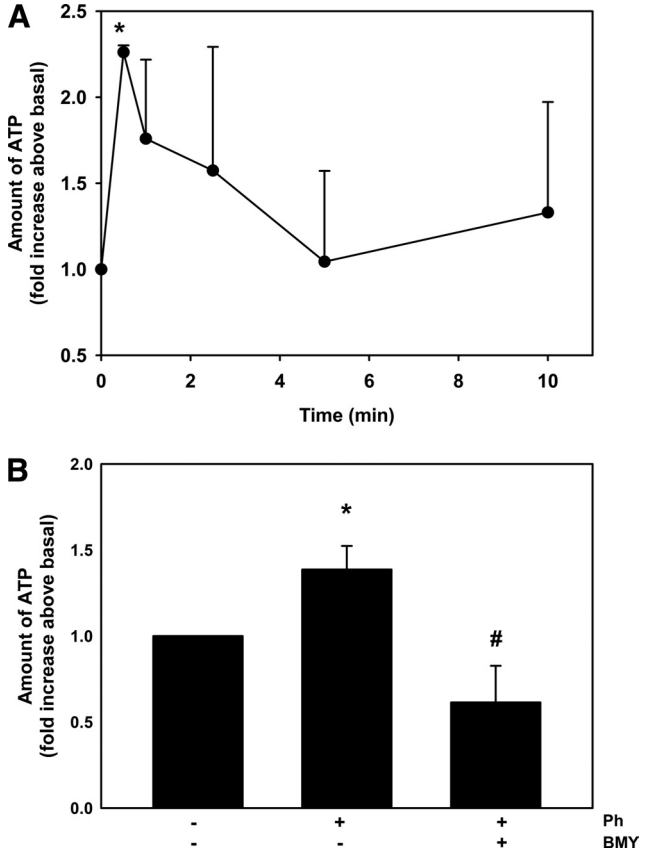

FIGURE 2. Effect of phenylephrine on ATP release from lacrimal gland pieces. Time-dependent release of ATP from lacrimal gland pieces stimulated with α_{1D}-adrenergic agonist phenylephrine (10^{-4} M) for 0 to 10 minutes (**A**). Pieces were preincubated with the α_{1D}-adrenergic receptor antagonist 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.

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 (SynergMx 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

α_{1D}-Adrenergic Agonists Increase $[Ca^{2+}]_i$ in a Concentration-Dependent Manner

When the $[Ca^{2+}]_i$ was determined in a large population of acini suspended in a cuvette, the α_{1D}-AR agonist phenylephrine increased $[Ca^{2+}]_i$ in a concentration-dependent manner.[4] A maximum increase of 15 nM was induced by phenylephrine at 10^{-3} M[4]. In the present experiments when

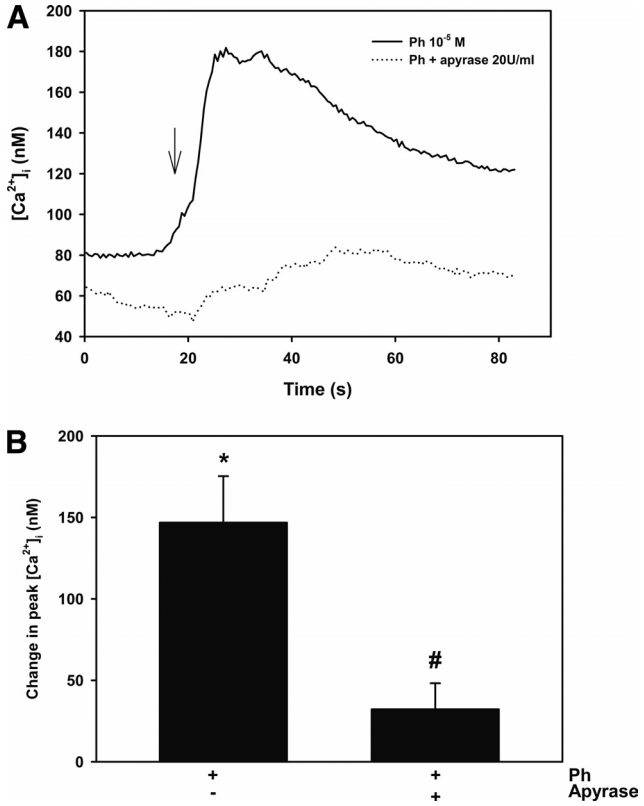

FIGURE 3. Effect an inhibitor of ATPases, apyrase, on $[Ca^{2+}]_i$ stimulated by phenylephrine in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with α_{1D}-adrenergic agonist phenylephrine (10^{-5} M) after 30-minute preincubation with apyrase (20 U/mL). Traces are mean of five experiments (**A**). Mean change in peak $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ by fluorescence microscopy in approximately 10 to 20 clumps of acini, phenylephrine also caused an increase in $[Ca^{2+}]_i$ (Fig. 1A). A maximum increase in $[Ca^{2+}]_i$ of 128.6 ± 17.8 nM was obtained with 10^{-5} M phenylephrine. Using the microscopy measurement technique, the change in $[Ca^{2+}]_i$ caused by supramaximal concentrations of phenylephrine caused a decreased response, unlike the cuvette method, with which the response remained elevated (Fig. 1B).

α_{1D}-Adrenergic Agonists Release ATP from Lacrimal Gland Pieces

We sought to determine whether α_{1D}-AR agonists release ATP that could activate P2X$_7$ receptors. Pieces of lacrimal gland were stimulated with no agonists (basal) or the α_{1D}-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 α_{1D}-AR antagonist, completely blocked phenylephrine-stimulated ATP release (Fig. 2B). These data suggest that α_{1D}-adrenergic agonists can release ATP from lacrimal gland pieces.

A second method was used to confirm that α_{1D}-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 α_{1D}-AR agonist activation of P2X$_7$ receptors (Table 1). Apyrase completely blocked the phenylephrine-stimulated increase in $[Ca^{2+}]_i$ from acinar cells (Figs. 3A, 3B). We conclude that α_{1D}-adrenergic agonists release ATP from the lacrimal gland. The released ATP then would activate P2X$_7$ receptors to increase $[Ca^{2+}]_i$.

α_{1D}-Adrenergic and P2X$_7$ Receptor Antagonists Cross-Inhibit Each Other's Intracellular Ca^{2+} Response in Lacrimal Gland Acinar Cells

Activation of α_{1D}-AR and P2X$_7$ receptors increases $[Ca^{2+}]_i$ in lacrimal gland acini. To determine whether these signaling pathways interact, we stimulated fura-2–loaded acini with the P2X$_7$ selective agonist BzATP after incubation with and without the α_{1D}-adrenergic receptor inhibitor BMY 7378 at 10^{-4} M (Table 1). BzATP at 10^{-4} M increased the $[Ca^{2+}]_i$ 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 α_{1D}-AR agonist phenylephrine increased $[Ca^{2+}]_i$ 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 α_{1D}-AR blocks P2X$_7$ receptor stimulation.

In separate experiments, cells were stimulated with the α_{1D}-adrenergic agonist phenylephrine (10^{-5} M) with and with-

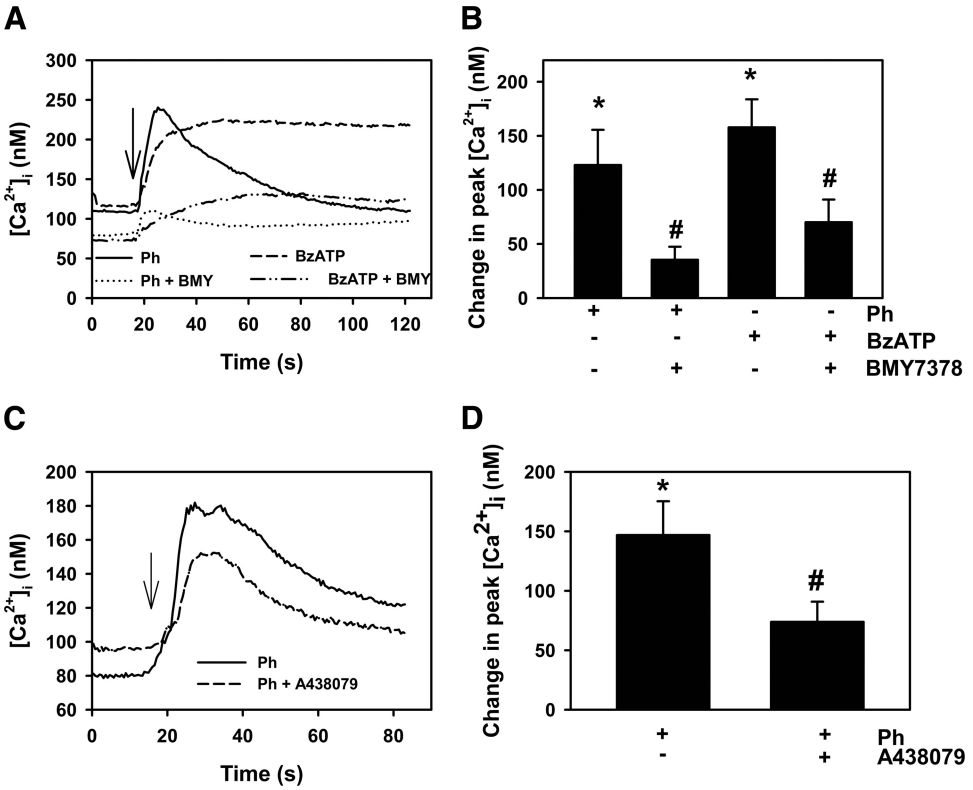

Figure 4. Effect of α_{1D}-adrenergic agonist antagonist BMY 7378 and P2X$_7$ receptor antagonist A438079 on $[Ca^{2+}]_i$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with BzATP (10^{-4} M) or α_{1D}-adrenergic agonist 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 $[Ca^{2+}]_i$ from five (phenylephrine) or six (BzATP) experiments (**B**). Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with α_{1D}-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 $[Ca^{2+}]_i$ 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 $P2X_7$ antagonist A438079 (10^{-4} M; Table 1). Phenylephrine increased $[Ca^{2+}]_i$ 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 $P2X_7$ receptors blocks the stimulation of α_{1D}-AR.

Taken together these data suggest that $P2X_7$ and α_{1D}-AR can activate each other to increase $[Ca^{2+}]_i$.

Activation of α_{1D}-AR and $P2X_7$ Receptors Use Separate Cellular Signaling Pathways to Increase $[Ca^{2+}]_i$ in Lacrimal Gland Acini

$[Ca^{2+}]_i$ was measured in fura-2–loaded acini stimulated simultaneously with phenylephrine and BzATP at maximal and submaximal concentrations for increasing $[Ca^{2+}]_i$. Phenylephrine (10^{-5} M) and BzATP (10^{-4} M), at maximal concentrations, each significantly increased $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ was statistically significant (Figs. 5C, 5D).

A second experimental paradigm was used to further explore the signaling pathways used by α_{1D}-AR and $P2X_7$ receptors to increase $[Ca^{2+}]_i$. Instead of adding the two agonists simultaneously, the addition of one agonist preceded the second. Maximal concentrations of phenylephrine (10^{-5} M) and BzATP (10^{-4} M) were used. When phenylephrine was used first, it increased the $[Ca^{2+}]_i$ by 170.4 ± 55.4 nM (Figs. 6A, 6B). When phenylephrine was used after BzATP, the response was not altered, and the increase in $[Ca^{2+}]_i$ was 183.9 ± 82.7 nM. Use of BzATP first increased the $[Ca^{2+}]_i$ to 232.5 ± 31.7 nM. When BzATP was used after phenylephrine, the response was not altered and the increase in $[Ca^{2+}]_i$ was 276.0 ± 116.2 nM.

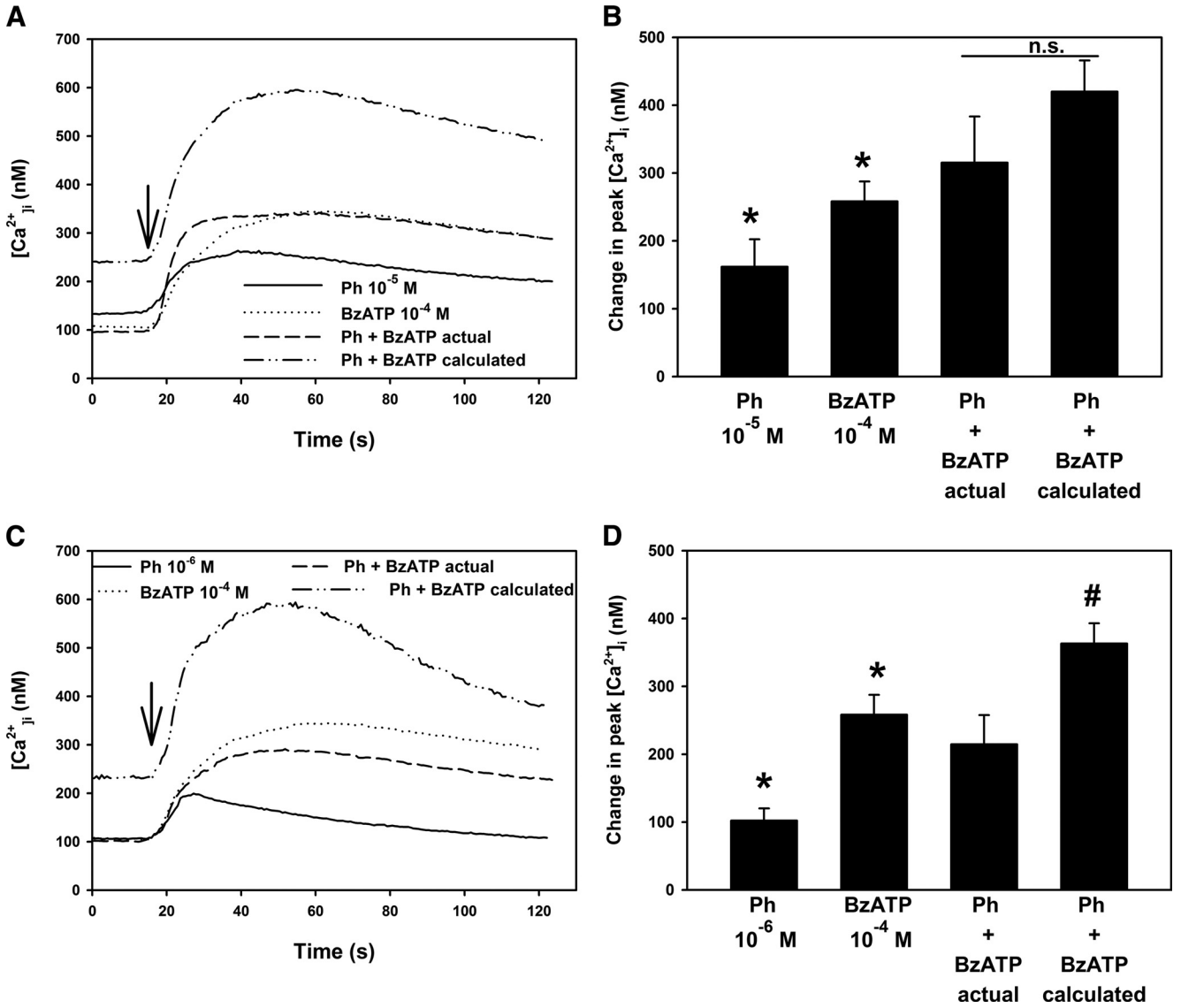

FIGURE 5. Effect of simultaneous addition of phenylephrine and BzATP on $[Ca^{2+}]_i$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by the stimulation of lacrimal gland acini containing fura-2 with α_{1D}-adrenergic agonist phenylephrine (10^{-5} M or 10^{-6} M) or BzATP (10^{-4} M) or the two added simultaneously. Traces are the mean of seven experiments (**A**, **C**). Mean change in peak $[Ca^{2+}]_i$ from seven experiments (**B**, **D**). Values are mean ± SEM. (*arrows*) Addition of agonists. *Significant difference from no additions (0). #Significant difference of experimental additivity from calculated additivity. n.s., no significant difference.

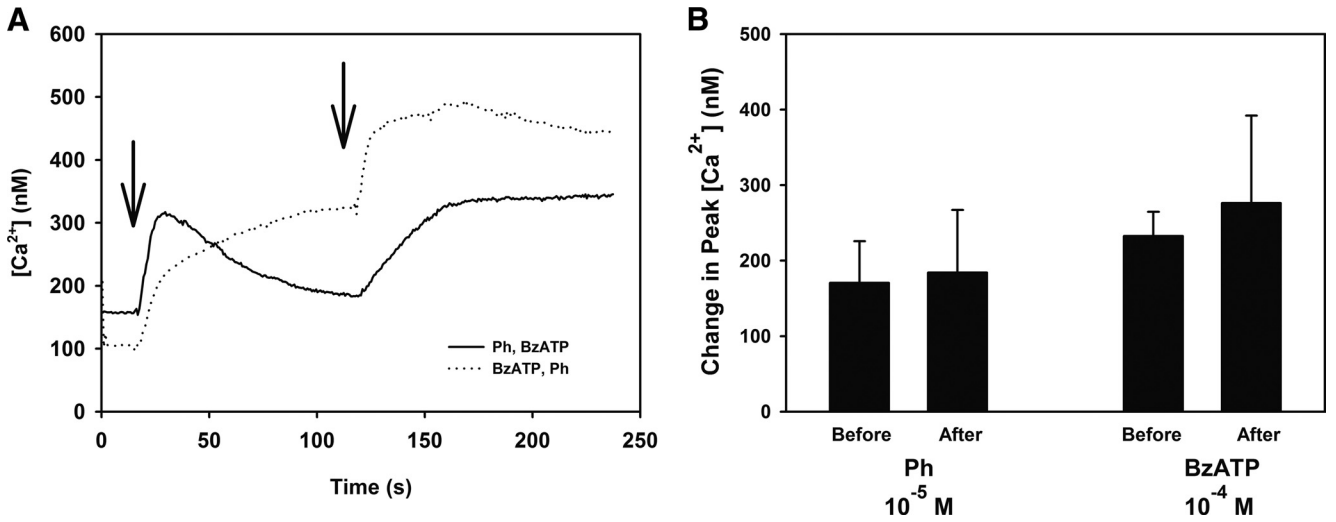

FIGURE 6. Effect of sequential addition of phenylephrine and BzATP on $[Ca^{2+}]_i$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with α_{1D}-adrenergic agonist phenylephrine (10^{-5} M; *first arrow*) followed by BzATP (10^{-4} M; *second arrow*) or the reverse. Traces are mean from four experiments (**A**). Mean change in peak $[Ca^{2+}]_i$ from four experiments (**B**). Values are mean ± SEM.

These results taken together suggest that when maximal concentrations of agonists were used P2X$_7$ and α_{1D}-AR receptors used different cellular mechanisms to increase $[Ca^{2+}]_i$. When the α_{1D}-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_{1D}-Adrenergic, but Not P2X$_7$, Receptors Use PKC to Increase $[Ca^{2+}]_i$

Acini containing fura-2 were stimulated with phenylephrine (10^{-5} M) or BzATP (10^{-4} M) that had been preincubated with and without the PKC inhibitor calphostin C (Table 1). Phenylephrine increased $[Ca^{2+}]_i$ to 241 ± 61.2 nM (Figs. 7A, 7C). Preincubation with calphostin C at 10^{-8} and 10^{-7} M significantly decreased $[Ca^{2+}]_i$ to 88.3 ± 22.1 nM and 65.8 ± 24.1 nM, respectively. BzATP (10^{-4} M) increased the intracellular $[Ca^{2+}]$ by 119.0 ± 30.8 nM (Figs. 7B, 7C). Preincubation with calphostin C at either concentration did not alter the BzATP-induced Ca^{2+} response. Thus, stimulation of α_{1D}-adrenergic, but not of P2X$_7$, receptors activates PKC isoforms to alter the $[Ca^{2+}]_i$. Differential activation of PKC isoforms by α_{1D}-adrenergic and P2X$_7$ agonists is consistent with the use of separate signaling pathways by these two agonists to increase $[Ca^{2+}]_i$.

Activation of Ca^{2+}/Calmodulin-Dependent Protein Kinase Does Not Alter the P2X$_7$ Receptor-Induced Increase in $[Ca^{2+}]_i$ in Lacrimal Gland Acini

Because P2X$_7$ receptors have been reported to activate Ca^{2+}/calmodulin-dependent protein kinase (CaMK) to increase pore formation,[14] we next investigated whether stimulation of the P2X$_7$ receptor activates CaMK to increase $[Ca^{2+}]_i$ in rat lacrimal gland. Acini were preincubated with or without the CaMKII inhibitor CSG 9343B (10^{-5} and 10^{-4} M) and then were stimulated with phenylephrine (Table 1). Phenylephrine significantly increased $[Ca^{2+}]_i$ by 198.0 ± 53.2 nM. Preincubation with CSG 9343B (10^{-4} M) significantly decreased the phenylephrine-induced response to 61.8 ± 19.9 nM (Figs. 8A, 8B). BzATP significantly increased $[Ca^{2+}]_i$ by 233.6 ± 40.7 nM (Figs. 8C, 8D). Inhibition of CaMKII with CSG, used at 10^{-6} to 10^{-4} M, did not alter the BzATP-induced increase in $[Ca^{2+}]_i$ at any concentration. Thus, P2X$_7$ receptors do not appear to activate CaMKII to increase $[Ca^{2+}]_i$.

Differential activation of CaMKII by α_{1D}-adrenergic and P2X$_7$ agonists is consistent with the use of separate signaling pathways by these two agonists to increase $[Ca^{2+}]_i$.

α_{1D}-Adrenergic and P2X$_7$ Receptor Inhibitors Do Not Cross-Inhibit Each Other's Secretory Response in Lacrimal Gland Acinar Cells

Because α_{1D}-adrenergic agonists release ATP to activate P2X$_7$ receptors and increase the $[Ca^{2+}]_i$, 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^{-4} M, and protein secretion was measured. BzATP significantly stimulated protein secretion by 1.5 ± 0.1-fold (Fig. 9A). Preincubation with BMY 7378 (10^{-4} M) did not affect BzATP-stimulated secretion. In analogous experiments, acini were stimulated with the α_{1D}-adrenergic agonist phenylephrine at 10^{-4} M, which increased secretion by 2.7 ± 0.5-fold (Fig. 9B). Preincubation with the P2X$_7$ inhibitor A438079 (10^{-4} M) did not alter phenylephrine-stimulated secretion. In contrast to the increase in $[Ca^{2+}]_i$, α_{1D}-AR or P2X$_7$ receptor inhibitors did not block protein secretion stimulated by the other agonist.

α_{1D}-Adrenergic and P2X$_7$ 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^{-4} M) and BzATP (10^{-4} 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^{2+} to increase the P2X$_7$ receptor response (Fig. 10B). These results suggest that, similar to the increase in $[Ca^{2+}]_i$, α_{1D}-AR and P2X$_7$ receptors use different cellular pathways to stimulate protein secretion.

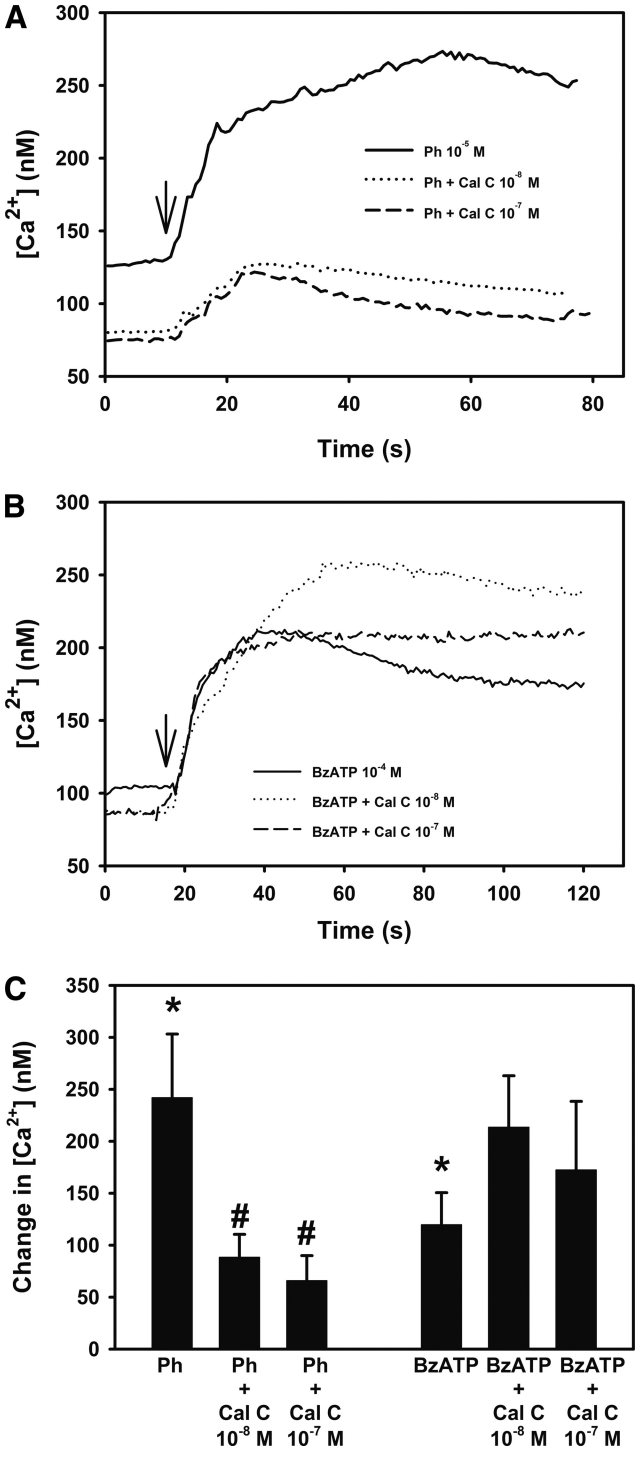

FIGURE 7. Effect of inhibition of PKC on phenylephrine- and BzATP-stimulated $[Ca^{2+}]_i$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with α_{1D}-adrenergic agonist phenylephrine (10^{-5} M) or BzATP (10^{-4} M) after 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 $[Ca^{2+}]_i$ 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.

DISCUSSION

In the present study we found that activation of α_{1D}-AR releases ATP from acini and stimulates $P2X_7$ receptor activity to increase $[Ca^{2+}]_i$, but not to stimulate protein secretion. Similarly, $P2X_7$ receptors stimulate α_{1D}-AR activity to increase $[Ca^{2+}]_i$ but not to stimulate protein secretion. These results are supported by three different types of experiments. First, α_{1D}-AR releases ATP from pieces. Second, the removal of extracellular ATP with apyrase blocks α_{1D}-AR stimulation of $P2X_7$ receptors. Third, blocking α_{1D}-AR receptors with the α_{1D}-AR antagonist prevents the $P2X_7$ receptor from increasing $[Ca^{2+}]_i$ and, conversely, inhibiting $P2X_7$ receptors with a $P2X_7$ receptor antagonist blocks α_{1D}-AR from increasing $[Ca^{2+}]_i$. Interestingly, this cross-stimulation does not extend to protein secretion. The interaction of the two receptors to increase $[Ca^{2+}]_i$ 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 $P2X_2$, $P2X_3$, and $P2X_4$ 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 α_{1D}-AR and $P2X_7$ receptors are colocalized in a complex so that inhibition of $P2X_7$ and α_{1D}-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 $P2X_7$ receptor have negative and positive allosteric effects on these receptors and, thus, could change the $P2X_7$ receptor conformation so that it could alter the α_{1D}-AR.

Both α_{1D}-AR and $P2X_7$ receptors have extracellular and intracellular domains that can be modified to change the way the receptor increases $[Ca^{2+}]_i$.[19] For example, the intracellular tail of the $P2X_7$ receptor contains consensus sites for PKC phosphorylation. In addition the rat, but not the human, $P2X_7$ receptor has a calmodulin-binding domain in its C-terminal[20] that prevents its desensitization with repeated agonist applications. $P2X_7$ receptors also have a Src homology 3 death domain in the C terminus,[21] which is involved in $P2X_7$ receptor activation of pannexin1, resulting in pore formation. Findings from a previous study[13] along with those in the present study indicate that neither stimulation nor inhibition of PKC activity appears to alter the $P2X_7$ receptor increase in $[Ca^{2+}]_i$.[19] In the design of the present study, the activation of CaMKII did not alter the single addition of BzATP to increase in $[Ca^{2+}]_i$. Finally, we did not test the role of Src in the present study. Thus, to date, the mechanism used by α_{1D}-AR to stimulate $P2X_7$ receptors to increase in $[Ca^{2+}]_i$ and vice versa has not been identified.

Given that $P2X_7$ and α_{1D}-AR stimulate each other, it might be expected that these two receptors activate a common signaling pathway to increase $[Ca^{2+}]_i$ and to stimulate secretion. Thus, with the sequential addition of $P2X_7$ and α_{1D}-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 $P2X_7$ and α_{1D}-AR agonists caused additive $[Ca^{2+}]_i$ responses, and simultaneous addition of both agonists caused additive secretory responses. Furthermore, inhibitors of $P2X_7$ receptors and α_{1D}-AR did not block the secretion stimulated by the other agonist. The results indicate that $P2X_7$ and α_{1D}-AR use different cellular mechanisms to increase $[Ca^{2+}]_i$ and to stimulate secretion. $P2X_7$ receptors are nonselective ion channels that increase $[Ca^{2+}]_i$. The mechanism that the activation of α_{1D}-AR uses to increase $[Ca^{2+}]_i$ in rat lacrimal gland acini is unknown. It is known that α_{1D}-AR does not induce the production of $InsP_3$ in rats, though α_{1D}-AR uses this compound to increase $[Ca^{2+}]_i$ in mouse lac-

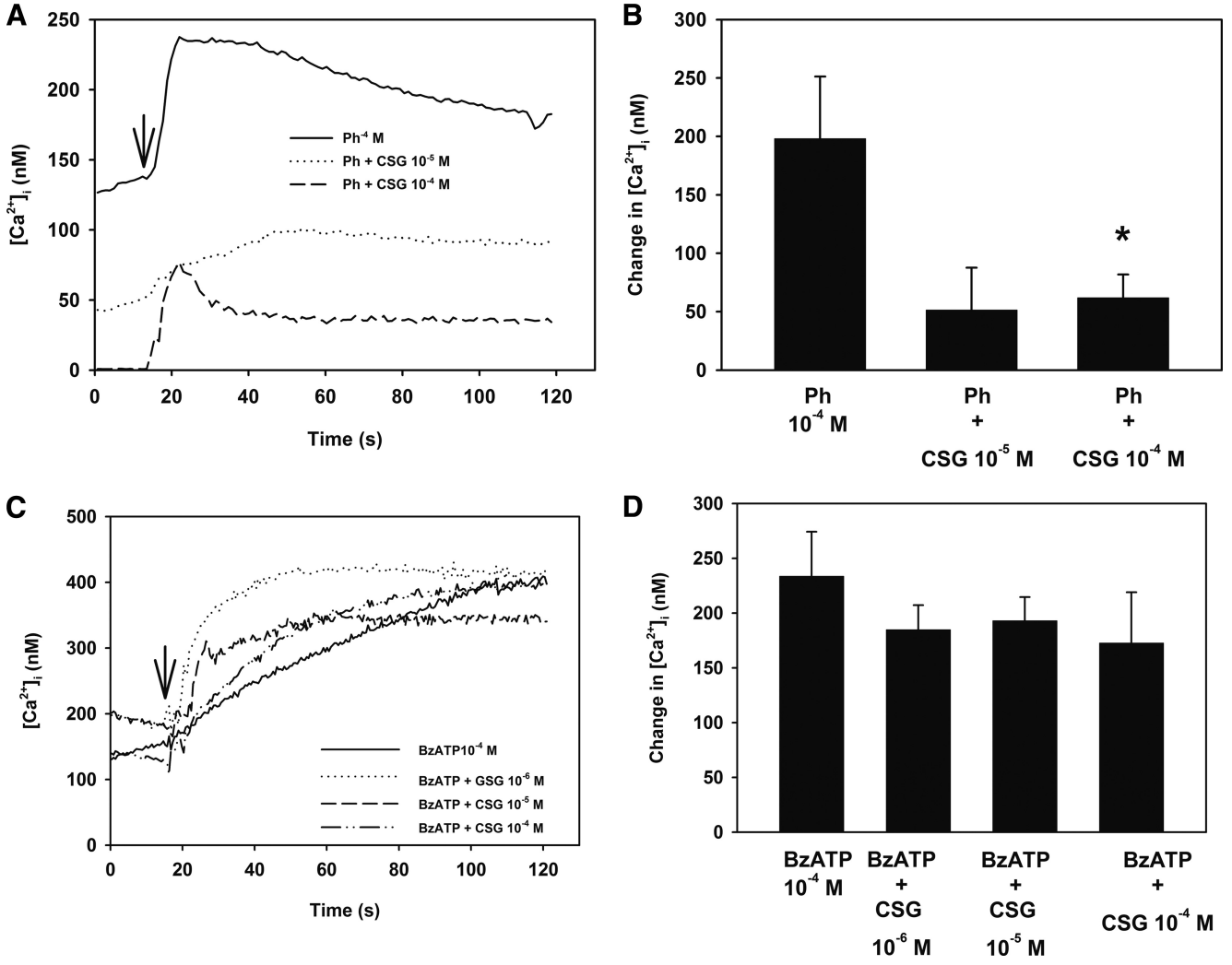

FIGURE 8. Effect of inhibition of calcium/calmodulin kinase II (CamKII) on phenylephrine- and BzATP-stimulated $[Ca^{2+}]$ in lacrimal gland acini. Time-dependent increase in $[Ca^{2+}]_i$ induced by stimulation of lacrimal gland acini containing fura-2 with α_{1D}-adrenergic agonist phenylephrine (10^{-5} 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 $[Ca^{2+}]_i$ from Ph (**B**) and BzATP (**D**) are from four experiments. Values are mean ± SEM. *Arrows*: addition of agonists. *Significant difference from agonist alone.

rimal gland acini.[3,22] Inhibition of PKC and CaMK in turn inhibited α_{1D}-AR, but not P2X$_7$, receptor-stimulated increases in $[Ca^{2+}]_i$, indicating that P2X$_7$ and α_{1D}-AR use separate mechanisms to increase intracellular calcium, thus accounting for the additivity of the increase in $[Ca^{2+}]_i$ when both receptors are activated.

In the present study, P2X$_7$ and α_{1D}-AR antagonists cross-inhibited the increase in $[Ca^{2+}]_i$ caused by the other receptor but did not block protein secretion. These differential effects on $[Ca^{2+}]_i$ compared with secretion suggest that increasing $[Ca^{2+}]_i$ is not the major mechanism by which α_{1D}-AR stimulates protein secretion. The results also imply that the α_{1D}-AR and P2X$_7$ receptor-mediated increase in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, increased by the simultaneous addition of P2X$_7$ and α_{1D}-AR agonists was additive, suggesting that in the lacrimal gland α_{1D}-AR and P2X$_7$ receptors activate different signaling pathways to stimulate protein secretion. In lacrimal gland acini, α_{1D}-AR activate eNOS/cGMP, increase PKCε activity, and raise the $[Ca^{2+}]_i$ by an unidentified mechanism to stimulate secretion.[4–6] To attenuate secretion α_{1D}-AR agonists transactivate the EGF receptor to induce the ERK1/2 cascade and activate PKCα and PKCδ.[5,7] Signaling pathways used by P2X$_7$ receptors in lacrimal acini have been less well studied. A previous publication showed that P2X$_7$ receptors increase the $[Ca^{2+}]_i$ using the ionotropic receptor itself to stimulate secretion and activate ERK1/2, which could attenuate secretion.[12] In a previous study,[13] as in the present study, an effect of P2X$_7$ receptors on PKC isoforms and CaMKII was ruled out. To date, the signaling pathways used by α_{1D}-AR and P2X$_7$ receptors differ in their use of PKC isoforms. Further research is warranted on the signaling mechanisms used by P2X$_7$ receptors to stimulate lacrimal gland secretion to identify the different signaling mechanisms used by α_{1D}-AR and P2X$_7$ receptors.

In the lacrimal gland, M_3AchR and α_{1D}-AR use separate different mechanisms to stimulate protein secretion.[4,5,8] The interaction of the M_3AchR and α_{1D}-AR with P2X$_7$ receptors supports this concept. Compared with the signaling pathways used by α_{1D}-AR, described in the previous paragraph, to stimulate the secretion, M_3AchR activates PLCβ, producing $InsP_3$ that increases the $[Ca^{2+}]_i$ and producing

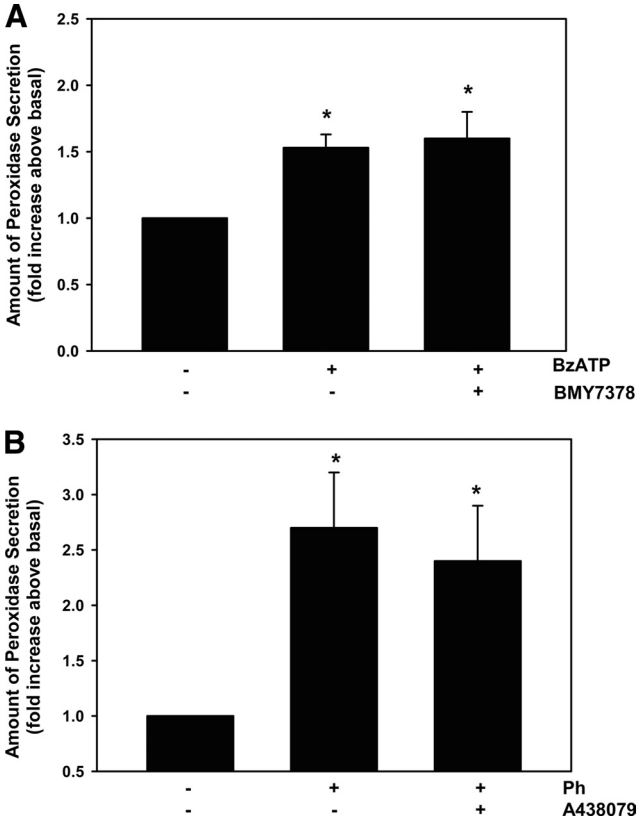

FIGURE 9. Effect of α_{1D}-adrenergic and $P2X_7$ receptor antagonists on protein secretion stimulated by phenylephrine or BzATP from lacrimal gland acini. Peroxidase secretion, our index of protein secretion, was measured from lacrimal gland acini preincubated with the α_{1D}-adrenergic receptor antagonist BMY 7378 (10^{-4} M) for 30 minutes and then stimulated with BzATP (10^{-4} M) for 40 minutes (**A**) or preincubated with the $P2X_7$ 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.

diacylglycerol that activates PKCα, PKCδ, and PKCε.[3,5] To attenuate secretion, M_3AchR 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.[9] 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, M_3AchR and α_{1D}-AR interact differently with $P2X_7$ receptors. M_3AchR activates $P2X_7$ receptors to increase the $[Ca^{2+}]_i$ and to stimulate secretion. M_3AchR releases ATP from nonacinar cells that could activate $P2X_7$ receptors and probably use a direct intracellular pathway to stimulate the activity of $P2X_7$ receptors.[13] In contrast, α_{1D}-AR release ATP from acini to activate $P2X_7$ receptors and, in turn, the activation of $P2X_7$ receptors stimulates α_{1D}-AR to increase $[Ca^{2+}]_i$. The signaling mechanisms used by α_{1D}-AR and $P2X_7$ receptors to increase the $[Ca^{2+}]_i$ appear to be different, but those used by the M_3AchR and $P2X_7$ receptors overlap. However, the signaling mechanisms used by α_{1D}-AR, M_3AchR, and $P2X_7$ receptors to stimulate protein secretion are different. Thus, to date, the cellular mechanisms used by M_3AchR and α_{1D}-AR to induce $P2X_7$ receptors activity appear to be different.

In one other tissue, the interaction of αAR and $P2X_7$ receptors has been investigated. In hypothalamo-neurohypophyseal explants, the α_{1D}-AR agonist causes oxytocin and vasopressin secretion, and co-exposure of α_1-AR agonists with ATP prolongs and potentiates the secretory response.[23,24] Gomes et al.[24] found that ATP activates $P2X_2$ receptors that recruit $P2X_3$ and $P2X_7$ receptors, which are responsible and desensitization resistant and thereby prolong secretion.[24] In contrast, in the present study, $P2X_7$ receptors and α_{1D}-AR do not appear to potentiate the secretory response.

We conclude that in the lacrimal gland, the activation of α_{1D}-AR releases ATP that induces $P2X_7$ receptors to increase $[Ca^{2+}]_i$, but not to stimulate protein secretion. $P2X_7$ receptors, in turn, activate α_{1D}-AR to increase $[Ca^{2+}]_i$, but not to stimulate protein secretion. Furthermore, α_{1D}-AR compared with $P2X_7$ receptors use different cellular mechanisms to increase $[Ca^{2+}]_i$ and to cause protein secretion. The interaction of $P2X_7$ receptors with α_{1D}-AR is different from the interaction of $P2X_7$ receptors with M_3AchR.

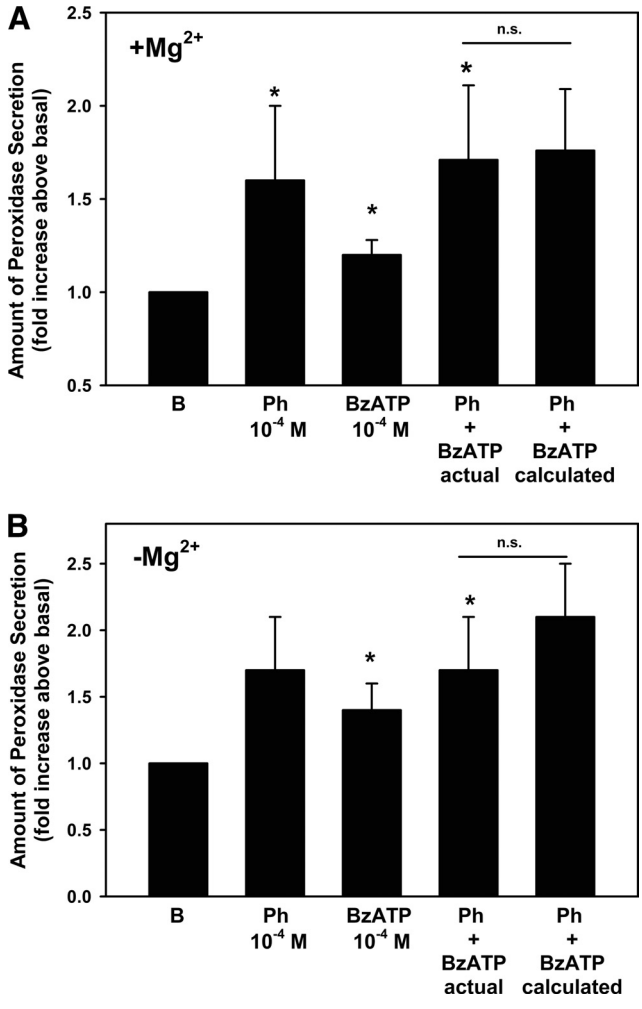

FIGURE 10. Effect of simultaneous addition of phenylephrine and BzATP on protein secretion from lacrimal gland acini. Peroxidase secretion, our index of protein secretion, was measured from lacrimal gland acini stimulated for 40 minutes with phenylephrine (10^{-4} M), BzATP (10^{-4} M), or the two agonists 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 Mg^{2+}. Values are the mean ± SEM of four independent experiments. *Significant difference from no addition. n.s., no significant difference.

References

1. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res*. 2009;28:155–177.
2. Dartt DA, Baker AK, Vaillant C, Rose PE. Vasoactive intestinal polypeptide stimulation of protein secretion from rat lacrimal gland acini. *Am J Physiol*. 1984;247(pt 1):G502–G509.
3. Dartt DA, Dicker DM, Ronco LV, Kjeldsen IM, Hodges RR, Murphy SA. Lacrimal gland inositol trisphosphate isomer and inositol tetrakisphosphate production. *Am J Physiol*. 1990;259(pt 1):G274–G281.
4. Hodges RR, Dicker DM, Rose PE, Dartt DA. Alpha 1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. *Am J Physiol*. 1992;262(pt 1):G1087–G1096.
5. Zoukhri D, Hodges RR, Sergheraert C, Toker A, Dartt DA. Lacrimal gland PKC isoforms are differentially involved in agonist-induced protein secretion. *Am J Physiol*. 1997;272(pt 1):C263–C269.
6. Hodges RR, Shatos MA, Tarko RS, Vrouvlianis J, Gu J, Dartt DA. Nitric oxide and cGMP mediate alpha1D-adrenergic receptor-stimulated protein secretion and p42/p44 MAPK activation in rat lacrimal gland. *Invest Ophthalmol Vis Sci*. 2005;46:2781–2789.
7. Chen L, Hodges RR, Funaki C, et al. Effects of alpha1D-adrenergic receptors on shedding of biologically active EGF in freshly isolated lacrimal gland epithelial cells. *Am J Physiol Cell Physiol*. 2006;291:C946–C956.
8. Ota I, Zoukhri D, Hodges RR, et al. Alpha 1-adrenergic and cholinergic agonists activate MAPK by separate mechanisms to inhibit secretion in lacrimal gland. *Am J Physiol Cell Physiol*. 2003;284:C168–C178.
9. Hodges RR, Guilbert E, Shatos MA, Natarajan V, Dartt DA. Phospholipase D1, but not D2, regulates protein secretion via Rho/ROCK in a Ras/ Raf-independent, MEK-dependent manner in rat lacrimal gland. *Invest Ophthalmol Vis Sci.* 2011;52:2199–2210.
10. Zoukhri D, Dartt DA. Cholinergic activation of phospholipase D in lacrimal gland acini is independent of protein kinase C and calcium. *Am J Physiol*. 1995;268(pt 1):C713–C720.
11. Hodges RR, Zoukhri D, Sergheraert C, Zieske JD, Dartt DA. Identification of vasoactive intestinal peptide receptor subtypes in the lacrimal gland and their signal-transducing components. *Invest Ophthalmol Vis Sci*. 1997;38:610–619.
12. Hodges RR, Vrouvlianis J, Shatos MA, Dartt DA. Characterization of P2X7 purinergic receptors and their function in rat lacrimal gland. *Invest Ophthalmol Vis Sci*. 2009;50:5681–5689.
13. Dartt DA, Hodges RR. Cholinergic agonists activate P2X7 receptors to stimulate protein secretion by the rat lacrimal gland. *Invest Ophthalmol Vis Sci*. 2011;52:3381–3390.
14. Faria RX, Reis RA, Casabulho CM, et al. Pharmacological properties of a pore induced by raising intracellular Ca^{2+}. *Am J Physiol Cell Physiol.* 2009;297:C28–C42.
15. Decker DA, Galligan JJ. Cross-inhibition between nicotinic acetylcholine receptors and P2X receptors in myenteric neurons and HEK-293 cells. *Am J Physiol Gastrointest Liver Physiol*. 2009;296:G1267–G1276.
16. Barajas-Lopez C, Espinosa-Luna R, Zhu Y. Functional interactions between nicotinic and P2X channels in short-term cultures of guinea-pig submucosal neurons. *J Physiol*. 1998;513(pt 3):671–683.
17. Kim M, Jiang LH, Wilson HL, North RA, Surprenant A. Proteomic and functional evidence for a P2X7 receptor signalling complex. *EMBO J*. 2001;20:6347–6358.
18. Michel AD, Chambers LJ, Walter DS. Negative and positive allosteric modulators of the P2X(7) receptor. *Br J Pharmacol*. 2008;153:737–750.
19. Brown DA, Yule DI. Protein kinase C regulation of P2X3 receptors is unlikely to involve direct receptor phosphorylation. *Biochim Biophys Acta*. 2007;1773:166–175.
20. Roger S, Gillet L, Baroja-Mazo A, Surprenant A, Pelegrin P. C-terminal calmodulin-binding motif differentially controls human and rat P2X7 receptor current facilitation. *J Biol Chem*. 2010;285:17514–17524.
21. Iglesias R, Locovei S, Roque A, et al. P2X7 receptor-Pannexin1 complex: pharmacology and signaling. *Am J Physiol Cell Physiol*. 2008;295:C752–C760.
22. Bird GS, Putney JW Jr. Effect of inositol 1,3,4,5-tetrakisphosphate on inositol trisphosphate-activated Ca^{2+} signaling in mouse lacrimal acinar cells. *J Biol Chem.* 1996;271:6766–6770.
23. Sladek CD, Song Z. Regulation of vasopressin release by co-released neurotransmitters: mechanisms of purinergic and adrenergic synergism. *Prog Brain Res*. 2008;170:93–107.
24. Gomes DA, Song Z, Stevens W, Sladek CD. Sustained stimulation of vasopressin and oxytocin release by ATP and phenylephrine requires recruitment of desensitization-resistant P2X purinergic receptors. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R940–R949.