Increase of Intracellular Ca^{2+} by Purinergic Receptors in Cultured Rat Lacrimal Gland Myoepithelial Cells

Kaori Ohtomo, Marie A. Shatos, Joanna Vrouvlianis, Dayu Li, Robin R. Hodges, and Darlene A. Dartt

PURPOSE. To isolate and characterize cultured myoepithelial cells (MECs) from rat lacrimal gland and determine which purinergic receptor subtypes are present and functional in MECs.

METHODS. Rat lacrimal glands were subjected to collagenase digestion, and MECs were grown. RT-PCR was performed for the purinergic receptors P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ on RNA isolated from the MECs. Immunofluorescence experiments were performed with antibodies against MEC markers and P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ purinergic receptors. Proteins from MECs were separated using Western blot analysis techniques. In addition, cells were incubated with Fura 2 tetra acetoxymethyl ester, and intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) was determined in response to P2 purinergic agonists.

RESULTS. MECs expressed the MEC proteins α -smooth muscle actin, vimentin, α -actinin, and adenylyl cyclase II. RT-PCR, Western blot, and immunofluorescence techniques demonstrated the presence of the purinergic receptors P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃. The purinergic agonists ATP, benzoyl-benzoyl ATP (BzATP), α,β methylene ATP, UTP, 2-methylthio-ATP (MeSATP), and ATP γ S increased $[\text{Ca}^{2+}]_i$. As BzATP binds to the P2X₇ receptor, specific characteristics of this receptor were investigated. Neither inhibitors of P2X₇ receptors nor removal of extracellular Mg^{2+} or Ca^{2+} had an effect on the BzATP-stimulated increase in $[\text{Ca}^{2+}]_i$. Repeated applications of BzATP desensitized this response. Inhibitors for P2Y₁, P2Y₁₁, and P2Y₁₃ each decreased the BzATP-stimulated increase in $[\text{Ca}^{2+}]_i$ with the P2Y₁ inhibitor most effective.

CONCLUSIONS. MECs can be isolated from rat lacrimal glands, and they express P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ purinergic receptors. Surprisingly, BzATP binds the P2Y₁ receptor, which is primarily responsible for the BzATP-stimulated increase in $[\text{Ca}^{2+}]_i$. (*Invest Ophthalmol Vis Sci.* 2011;52:9503-9515) DOI: 10.1167/iops.11-7809

The lacrimal gland is the major contributor to the tear film and as such is vital to maintaining the health of the cornea and conjunctiva.¹ A dysfunction in the lacrimal gland results in altered tear secretion, leading to the development of dry eye syndrome. The lacrimal gland is largely composed of three major cell types: acinar, myoepithelial (MEC), and ductal cells. Acinar cells, which compose approximately 80% of the gland, synthesize and secrete proteins, water, and electrolytes in response to cholinergic ago-

nists released from parasympathetic nerves and α_1 -adrenergic agonists released from sympathetic nerves. Ductal cells secrete mainly water and electrolytes and some proteins, whereas the role of MECs has never been substantiated.¹

MECs have been described in a variety of exocrine organs, including salivary, mammary, sweat, and lacrimal glands.²⁻⁵ Although the exact origin of MECs has not yet been unequivocally identified, MECs morphologically resemble smooth muscle cells, as they express α -smooth muscle actin as well as proteins typical of epithelial cells.³

MECs have been implicated in a variety of different functions within the glands. These cells possess a characteristic shape that is typically stellate, consisting of a central cell body and thin branching cellular processes⁶ that surround the basolateral membranes of the acinar cells. One function involves contraction of the MECs, squeezing the acinar cell and thereby expelling the secretory products into the duct system.^{6,7} It has been shown in the mammary gland that MECs also function by secreting basement membrane proteins, which results in the formation of polarized epithelia and the elongation of ducts.^{8,9} In addition, MECs have been implicated in tumor suppression as they can alter matrix metalloproteinases in breast tumors and the surrounding cells.^{8,9}

In the lacrimal gland, little is known about MECs. Similar to lacrimal gland acinar cells, MECs express receptors to muscarinic and vasoactive intestinal peptide receptors^{10,11} and cholinergic, but not adrenergic, agonists induce contraction.¹² Because these cells express receptors for agonists that are major stimuli of protein secretion, it is likely that MECs play an active role in lacrimal gland function. It has also been observed that, in the wounded lacrimal gland, MECs express the stem cell marker nestin, indicating a possible stem cell niche.¹³ Therefore, MECs must be instrumental in lacrimal gland physiology during health and possibly in disease.

The purinergic P2 receptor family comprises ionotropic P2X and G-protein-coupled P2Y receptors, and its members are activated by extracellular ATP. Seven P2X receptors (P2X₁₋₇) and eight P2Y receptors (P2Y_{1,2,4,6,11-13,14}) have been cloned and are widely distributed in different cell types.¹⁴ Activation of both subfamilies of P2 receptors with purines causes an increase in $[\text{Ca}^{2+}]_i$. P2Y receptors are divided pharmacologically into three groups according to their activation by endogenous adenine and uracil nucleotides.¹⁵ Group I receptors (P2Y_{1,11,12,13}) are activated by ATP and ADP, group II (P2Y₆) are stimulated by UTP and UDP, and group III (P2Y_{2,4}) respond to both adenine and uracil nucleotides.¹⁶

Recent studies in rat lacrimal gland acini have shown that stimulation of P2X₇ receptors leads to an increase in intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$), protein secretion, and extracellular regulated kinase (ERK) 1/2 activation.¹⁷ In addition, M₃ muscarinic receptors stimulate P2X₇ receptors to increase $[\text{Ca}^{2+}]_i$ and protein secretion.¹⁸ These studies were performed with a cellular preparation that selects for a predominance of acinar cells, although MECs are not completely eliminated from this

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

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Corresponding author: Robin R. Hodges, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114; robin.hodges@schepens.harvard.edu.

TABLE 1. Primers for RT-PCR

Receptor	Primer Sequences 3'-5'	Annealing Temp (°C)	Fragment Size (bp)	Accession No.*
P2X ₇	GTGCCATTCTGACCAGGGTTGTATAAA GCCACCTCTGTAAAGTTCTCTCCGAT	54	354	X95882
P2Y ₁	GGAAACAGTACAATCGCC ACCACAATGAGCCATACC	60	464	NM_012800
P2Y ₁₁	CTGGTGGTTGAGTTCCTGGT GTGCAGGTGAAGAGGAAGC	60	234	Q96G91
P2Y ₁₃	GGCATCAACCGTGAAGAAAT GGGCAAAGCAGACAAAGAAG	59	245	NM_001002 853
β -Actin	ATGGATGACGATATCGCTG ATGAGGTAGTCTGTCAGGT	59	568	X03765

GenBank, National Institutes of Health, Bethesda, MD; <http://www.ncbi.nlm.nih.gov/genbank>.

preparation. Thus, the relative importance of MECs in lacrimal gland function is unknown.

The purpose of this study was to isolate and characterize MECs of the rat lacrimal gland and to determine which purinergic receptor subtypes are present and functional in these cells. In this study, we successfully isolate and culture MECs from the lacrimal gland and show that P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ purinergic receptors are functionally expressed in MECs.

MATERIALS AND METHODS

RPMI 1640, L-glutamine and penicillin-streptomycin were from Lonza (Walkersville, MD). The P2X₇, P2Y₁, and P2Y₁₁ antibodies and the competing peptides for each antibody were from Alomone Laboratories (Jerusalem, Israel). The P2Y₁₃ antibody was purchased from Millipore (Billerica, MA). Extraction reagent (TRIzol), Fura-2 tetra-acetoxy-methyl ester (Fura-2/AM), and an RT-PCR kit (Super-

FIGURE 1. Light micrographs of the growth of cultured rat MECs. Lacrimal glands were digested with collagenase, and the liberated cells were plated. Cell growth was observed and photographed after 7 (**A**), 14 (**B**), 18 (**C, D**), 21 (**E**), and 28 (**F**) days. (**B**, arrow) Nodules from which MECs emerge. Magnification, $\times 200$.

FIGURE 2. Characterization of cultured rat MECs. Immunofluorescence micrographs of MEC markers α -SMA in cultured MECs (*green*, **A**) and rat lacrimal gland (*green*, **B**); α -actinin in cultured MECs (*red*, **C**) and rat lacrimal gland (*red*, **D**); vimentin in cultured MECs (*red*, **E**) and rat lacrimal gland (*red*, **F**); and adenylyl cyclase II in cultured MECs (*red*, **G**) and rat lacrimal gland (*green*, **H**). (**I**, **J**) Rabbit IgG control antibodies; (**K**, **L**) mouse control IgG antibodies. Cell nuclei are labeled with DAPI (*blue*). Magnification, $\times 200$.

script) were purchased from Invitrogen Corporation (Carlsbad, CA). KN62 was obtained from EMD Biosciences (Gibbstown, NJ), and NF157, MRS 2211, and MRS 2279 were from Tocris Biosciences (Ellisville, MO). Adenylyl cyclase II, vimentin, α -actinin, and isotype control IgG antibodies were purchased from Santa Cruz Biotechnol-

ogy (Santa Cruz, CA) and the antibody against α -smooth muscle actin (α -SMA) was from Sigma-Aldrich (St. Louis, MO). Glass bottomed 35-mm culture dishes were purchased from MatTek Corp. (Ashland, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

FIGURE 3. Effect of purinergic agonists on $[Ca^{2+}]_i$ from cultured rat MECs. Cultured MECs loaded with Fura 2 were stimulated with increasing concentrations of the P2X agonists benzoylbenzoylATP (BzATP), ATP, and α, β methylene ATP (A) and the P2Y agonists UTP, methylthioATP (MeSATP), and ATP γ S (B). The change in peak $[Ca^{2+}]_i$ was calculated; the data shown are the mean $\pm$ SEM of results in four independent experiments. *Significant difference from basal.

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). The rats were maintained in constant-temperature rooms with fixed light-dark intervals of 12 hours and were fed ad libitum. For euthanasia, they were anesthetized for 1 minute in CO₂ and then decapitated. Both exorbital lacrimal glands were removed immediately.

Isolation of MECs from Rat Lacrimal Gland

Rat lacrimal glands were minced and dissociated by three repetitive 20-minute cycles of incubation in collagenase type I (Sigma-Aldrich). After each digestion, liberated cells were collected and forced through a 70- μ m mesh strainer. The cells were then centrifuged at 500g and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μ g/mL penicillin-streptomycin. They were fed every other day until the MECs appeared, usually after 4 weeks in culture, during which time the acinar cells died and only the MECs remained. The cells were routinely evaluated for expression of the MEC markers α -SMA, α -actinin, vimentin, and adenylyl cyclase II. All experiments were performed with first- or second-passage cells.

Reverse Transcription-Polymerase Chain Reaction

Cultured MECs were homogenized in extraction reagent (TRIzol; Invitrogen), and total RNA was isolated according to the manufacturer's instructions. RNA was also isolated from rat brain to use as a positive control. One microgram of purified total RNA was used for complementary (c)DNA synthesis (Jumpstart Reaction Mix; Sigma-Aldrich). The cDNA was amplified by the polymerase chain reaction (PCR), with primers specific to the P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ receptors, in a thermal cycler (Master Cycler; Eppendorf, Hauppauge, NY). The primers used for purinergic receptors were derived from previously published sequences^{19–22} and listed in Table 1. The conditions were as follows: 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 90 seconds at annealing temperature listed in Table 1, and 90 seconds at 72°C with a final hold at 72°C for 10 minutes. Samples with no cDNA served as the negative control, while the presence of β -actin was the positive control. Amplification products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Western Blot Analysis

The presence of purinergic receptors in cultured MECs was also determined by Western blot analysis. The cells were scraped on ice in RIPA buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA containing protease inhibitors (phenylmethylsulfonyl fluoride 100 μ L/mL, aprotinin 30 μ L/mL, and sodium orthovanadate 100 nM). Homogenized cells were sonicated and centrifuged at 2000g for 15 minutes at 4°C. Proteins in the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked overnight at 4°C in 5% nonfat dried milk in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20, and then incubated with primary antibody at 1:500 dilution for 1 hour at room temperature or overnight at 4°C followed by incubation with HRP-conjugated secondary antibody (1:2000 dilution for anti-mouse IgG conjugated to HRP and 1:5000 for anti-rabbit IgG conjugated to HRP). Immunoreactive bands were detected by the enhanced chemiluminescence method.

Immunohistochemistry

Cultured MECs were grown on coverslips and fixed in 4% formaldehyde diluted in phosphate-buffered saline (PBS; 145 mM NaCl, 7.3 mM Na₂HPO₄, and 2.7 mM NaH₂PO₄ [pH 7.2]) for 4 hours at 4°C. The cells were rinsed for 5 minutes in PBS, and nonspecific sites were blocked by incubation with 10% normal goat serum, 1% bovine serum albumin, and 0.2% Triton X-100 in PBS for 45 minutes at room temperature. The MECs were then incubated with the primary antibody for 2 hours at either 1:50 (vimentin, α -actinin) or 1:100 (α -SMA, adenylyl cyclase II and purinergic receptors) dilution at room temperature or overnight at 4°C in a humidified chamber. The secondary antibody conjugated to either Cy2 or Cy3 (1:300 dilution) was applied for 1 hour at room temperature. Coverslips were mounted on slides with a medium consisting of glycerol and paraphenylenediamine. Sections incubated in the presence of isotype controls served as the negative control. For the purinergic receptors, the primary antibodies were also incubated overnight in the presence of 10-fold excess peptide, against which the antibody was made to ensure the specificity of the antibodies. The cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan), and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc., Sterling Heights, MI) or viewed by confocal microscopy (TCS-SP2, Leica Microsystems, Bannockburn, IL).

Measurement of $[Ca^{2+}]_i$

First- or second-passage cultured MECs were grown overnight in 35-mm glass-bottomed culture dishes. The cells were then incubated in

FIGURE 4. Presence and localization of P2X₇ receptors in cultured rat MECs. RT-PCR was performed on RNA from brain and cultured MECs. (A) A representative blot of three separate animals. (B) The MECs were homogenized and analyzed by Western blot for the presence of P2X₇. Each lane indicates a different animal. MECs were double-labeled with P2X₇ and α -SMA (α -sm mus actin) to determine localization of P2X₇ receptors and ensure that the cultured cells are MECs (C). Peptide control as well as rabbit and mouse isotype-negative controls are also shown in (C). MWM, molecular weight markers; Br, brain; LG, lacrimal gland; NC, negative control. Magnification, $\times 200$.

the dark for 1 hour at room temperature in buffer containing 119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, and 5.5 mM glucose (KRB-HEPES) containing 0.5% BSA, 8 μ M pluronic acid F127, 250 μ M sulfinpyrazone, and 0.5 μ M of the calcium indicator fluorescent molecule Fura-2/AM. The cells were washed in KRB-HEPES with sulfinpyrazone. The dishes were placed on the stage of the microscope of a Ca²⁺ imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) that measures the ratio of Fura 2 using excimer wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells per dish were selected to measure for each condition. The inhibitors were added 30 minutes before the agonists. U73122 and KN62 were dissolved in DMSO (final concentration, 0.1%), all others were dissolved in dH₂O. Data were collected in real time, every 0.75 seconds. After a basal reading was obtained for 15 seconds, the agonists were added. [Ca²⁺]_i concentrations were determined by using a standard curve from 0 to 602 nM Ca²⁺. Data are presented as the actual [Ca²⁺]_i with time or as the change in peak [Ca²⁺]_i. The change in peak [Ca²⁺]_i was calculated by subtracting the average of the basal value (no added agonist) from the peak [Ca²⁺]_i. The plateau [Ca²⁺]_i was calculated by integrating the area under the curve from 40 to 90 seconds. The negative control was the addition of buffer alone and was performed with each experiment.

Data Presentation and Statistical Analysis

Data are expressed as the fold increase over the basal value, which was standardized to 1.0. Results are expressed as the mean $\pm$ SEM. Data were analyzed by Student's *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Growth of Cultured MECs

As MECs have never been isolated or grown in culture from the lacrimal gland, we developed a method to achieve this goal. To determine how MECs grow in culture, we incubated the lacrimal gland with repeated cycles of collagenase

digestion, as described in the Methods section. Liberated cells were plated and allowed to grow, and micrographs were taken by bright-field microscopy. Initially, a mixture of cells grew to confluence (Fig. 1A). Small nodules of immature cells appeared shortly afterward (Fig. 1B). Single cells then dispersed from the nodules (Figs. 1C, 1D, 1E) gradually the immature cells differentiated to cells with MEC-like morphology (Fig. 1F).

Identification of MECs in Culture

To ensure that the cells were indeed MECs, the cells were grown on glass coverslips and analyzed using markers known to be present in lacrimal gland and mammary MECs and compared them with MECs in sections from intact lacrimal gland.^{23,24} These markers include α -SMA, α -actinin, vimentin, and adenylyl cyclase II. As shown in Figure 2A, α -SMA in cultured MECs was clearly present as fibers within the cells, as expected for actin. In the intact lacrimal gland, this protein was present in the MECs surrounding the acini (Fig. 2B). The stellate morphology of the MECs in situ is visible. The cultured MECs also expressed the MEC markers α -actinin, vimentin, and adenylyl cyclase II on fibers throughout the cell (Figs. 2C, 2E, 2G). The lacrimal gland also expressed these markers in a pattern similar to that of α -SMA (Figs. 2D, 2F, 2H). Thus, the cells in culture were MECs.

Effect of Purinergic Agonists on [Ca²⁺]_i in Cultured MECs

Lacrimal gland acinar cells express multiple P2X receptors, the activation of which increases intracellular [Ca²⁺]_i and stimulates protein secretion.^{17,25} To determine whether cultured MECs express active purinergic receptors, the cells were loaded with Fura-2 and stimulated with increasing concentrations of the purinergic agonist ATP, which activates multiple purinergic receptors with different affinities.²⁶ ATP induced a robust increase in [Ca²⁺]_i in a concentration-dependent man-

ner. A maximum increase of 360.2 ± 32.1 nM occurred at 10^{-3} M ATP (Fig. 3A). Benzoylbenzoyl ATP (BzATP), which shows preferential binding for P2X₇ receptors, and α,β methylene ATP, which shows preferential binding to P2X₃ receptors, were also used, as P2X₃ and P2X₇ receptors had been identified in lacrimal gland acini.^{27,28} BzATP increased $[Ca^{2+}]_i$ in a concentration-dependent manner, with a maximum increase of 297.3 ± 57.3 nM at 10^{-4} M (Fig. 3A), before declining slightly to 279.2 ± 50.5 nM at 10^{-3} M BzATP (Fig. 3A). α,β -Methylene ATP also increased $[Ca^{2+}]_i$, although the response was not as great as either ATP or BzATP. A maximum of 161.5 ± 23.2 nM occurred at 10^{-3} M α,β methylene ATP, the only concentration that was significantly increased above basal. The rank order of agonists is ATP > BzATP >> α,β methylene ATP. These data indicate that cultured MECs contain functional P2X purinergic receptors.

Effect of P2Y Agonists on $[Ca^{2+}]_i$ in Cultured MECs

To determine whether cultured MECs have functional P2Y receptors, three different P2Y agonists were used to stimulate

cultured MECs, and the increase in $[Ca^{2+}]_i$ was measured. The agonists used were UTP, which binds preferentially to P2Y₂ and P2Y₄; ATP γ S, which binds to the P2Y₁₁ receptor; and MeSATP, which binds to the P2Y₁ and P2Y₁₁ receptors.^{15,16} As shown in Figure 3B, all agonists increased $[Ca^{2+}]_i$ in a concentration-dependent manner. UTP was the most potent agonist and increased $[Ca^{2+}]_i$ a maximum of 351.3 ± 3.8 nM at 10^{-4} M. ATP γ S was also very effective in increasing $[Ca^{2+}]_i$, and a maximum of 260.4 ± 38.9 nM was measured at 10^{-4} M. MeSATP was the least effective, with a maximum increase in $[Ca^{2+}]_i$ of 231.2 ± 33.2 nM at 10^{-3} M. Based on these results, multiple types of P2Y receptors appear to be active in cultured MEC receptors.

Identification and Localization of P2X₇ Receptors in Cultured MECs

As P2X₇ receptors are known to play an important role in protein secretion in lacrimal gland acinar cells¹⁷ and BzATP stimulates an increase in $[Ca^{2+}]_i$ in cultured MECs, we investigated the presence and localization of P2X₇ receptors in these cells. RT-PCR was performed on RNA isolated from cultured

FIGURE 5. Effect of removal of extracellular Mg²⁺ or Ca²⁺ and repeated applications of BzATP on $[Ca^{2+}]_i$ in cultured rat MECs. Cultured MECs loaded with Fura 2 were stimulated with BzATP (10^{-4} M) in the presence and absence of Mg²⁺. (A) The mean of 10 cells from a single animal, representative of seven individual experiments. (B) The mean $\pm$ SEM of change in peak $[Ca^{2+}]_i$ from seven independent experiments. Cultured MECs were loaded with Fura 2 and stimulated with BzATP (10^{-4} M) in the presence and absence of extracellular Ca²⁺. (C) The mean of at least 10 cells from a single animal, representative of three independent experiments. (D) The mean $\pm$ SEM of change in peak $[Ca^{2+}]_i$ from three independent experiments. Cultured MECs were loaded with Fura 2 and stimulated with repeated applications of BzATP (10^{-4} M) at 15 and 120 seconds. (E) The mean of 10 cells from a single animal and is representative of three independent experiments. (F) The mean $\pm$ SEM of change in peak $[Ca^{2+}]_i$ from three independent experiments. (A, C, E, arrows) addition of BzATP. *Significant difference from the peak $[Ca^{2+}]_i$ obtained with the first addition of BzATP.

FIGURE 6. Effect of inhibition of $P2X_7$ receptors on BzATP-stimulated $[Ca^{2+}]_i$ in cultured rat MECs. Cultured MECs were loaded with Fura 2 and preincubated for 30 minutes with brilliant blue G (10^{-4} M, BBG), KN62 (10^{-4} M), or A438079 (10^{-4} M). (A) The mean of 10 cells from a single animal and are representative of three individual experiments. (B) The change in peak $[Ca^{2+}]_i$ was calculated. Data are the mean $\pm$ SEM from three (BBG and KN62) and five (A438079) independent experiments. Arrow: addition of BzATP. *Significant difference from BzATP alone.

MECs using primers specific to rat $P2X_7$. As shown in Figure 4A, a single band at the expected size of 354 bp was obtained in both brain (positive control) and MECs, whereas no band was detected in the negative control. One band of 568 bp was obtained using primers specific to β -actin (data not shown).

To ensure that the RNA is expressed as protein, MECs were homogenized and subjected to Western blot analysis with a $P2X_7$ antibody. One band was seen at 69 kDa, the expected molecular mass for $P2X_7$ receptors, similar to that demonstrated in the lacrimal gland (Fig. 4B).¹⁷

In a previous study, $P2X_7$ receptors were present in the lacrimal gland acinar cell plasma membranes as well as in MECs.¹⁷ To determine the cellular localization of $P2X_7$ receptors in MECs, cells were grown on coverslips and subjected to immunofluorescence experiments with the same antibody as that used in the Western blot analysis. As shown in Figure 4C, $P2X_7$ receptors were localized in a perinuclear pattern in the cultured MECs. This binding pattern was inhibited by preincubation with the peptide against which the antibody was raised (Fig. 4C). In addition, to confirm that the cells used were MECs, the cells were double labeled with α -SMA, which is known to be present in MECs of the lacrimal and mammary glands. All

cells in the culture expressed α -SMA, indicating that they were MECs (Fig. 4C).

Effect of Mg^{2+} on BzATP-Stimulated $[Ca^{2+}]_i$ Increase

To ensure that the Ca^{2+} response seen with BzATP occurs through activation of the $P2X_7$ receptors in cultured MECs, we studied several unique characteristics of the $P2X_7$ receptor. The first characteristic tested was whether the activity of these receptors is potentiated in the absence of Mg^{2+} . Cultured MECs loaded with Fura-2 were stimulated with BzATP (10^{-4} M) in the presence and absence of Mg^{2+} . As shown in Figure 5A, the BzATP Ca^{2+} response was unchanged in the presence or absence of Mg^{2+} . When peak $[Ca^{2+}]_i$ was calculated, the BzATP response in the presence of Mg^{2+} increased $[Ca^{2+}]_i$ by 304.5 ± 31.1 nM. This result is not significantly different from the response obtained in the absence of Mg^{2+} (279.7 ± 44.0 nM; Fig. 5B).

Because $P2X_7$ receptors are ion channels, the increase in intracellular Ca^{2+} seen with these receptors is dependent on extracellular Ca^{2+} entering through the channel. Thus, removal of extracellular Ca^{2+} should abolish a BzATP-stimulated increase in Ca^{2+} . When cultured MECs were stimulated with BzATP (10^{-4} M) in the absence of extracellular Ca^{2+} , the

FIGURE 7. Effect of inhibition of PLC on BzATP-stimulated $[Ca^{2+}]_i$ in cultured rat MECs. Cultured MECs were loaded with Fura 2 and preincubated for 30 minutes with U73122 (10^{-4} M). (A) The mean of at least 10 cells from a single animal, representative of three individual experiments. (B) The change in peak $[Ca^{2+}]_i$ was calculated. Data are the mean $\pm$ SEM from three independent experiments. Arrow: addition of BzATP. *Significant difference from BzATP alone.

BzATP response was unchanged from that obtained in the presence of extracellular Ca^{2+} (Fig. 5C). When peak $[\text{Ca}^{2+}]_i$ was calculated, the BzATP response in the presence of Ca^{2+} increased $[\text{Ca}^{2+}]_i$ by 339.9 ± 35.1 nM, which is not significantly different from the response obtained in the absence of Ca^{2+} (330.9 ± 117.4 nM; Fig. 5D).

The third characteristic tested was that P2X_7 receptors do not desensitize with repeated applications of agonist. Fura 2-loaded MECs cells were stimulated with BzATP (10^{-4} M) 30 and 120 seconds after the start of the experiment. As shown in Figure 5E, the first addition of BzATP significantly decreased the $[\text{Ca}^{2+}]_i$ response obtained with the second addition (Fig. 5F). The peak $[\text{Ca}^{2+}]_i$ for the first application of BzATP was 237.6 ± 47.3 nM. The $[\text{Ca}^{2+}]_i$ obtained with the second application was significantly decreased from the first and was 18.3 ± 16.1 nM (Fig. 5F).

The fourth characteristic tested was the ability of the P2X_7 inhibitors A438079, brilliant blue G (BBG), and KN62 to decrease the BzATP-stimulated increase in $[\text{Ca}^{2+}]_i$. In Fura

2-loaded MECs preincubated for 30 minutes with A438079, the BzATP-induced increase in $[\text{Ca}^{2+}]_i$ was decreased by $10.7\% \pm 25.2\%$, $16.1\% \pm 19.1\%$, $18.8\% \pm 16.2\%$, and $52.1\% \pm 15.8\%$ at 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M, respectively (Fig. 6). Only the highest concentration of A438079 significantly decreased the peak $[\text{Ca}^{2+}]_i$ from that stimulated by BzATP alone. Similar results were obtained when the plateau Ca^{2+} level was calculated by measuring the area under the curve from 40 to 90 seconds, with a decrease of $19.0\% \pm 20.4\%$, $31.9\% \pm 16.4\%$, $39.2\% \pm 11.1\%$, and $59.2\% \pm 15.6\%$ at 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M, respectively (data not shown). Again, only the highest concentration of A438079 was significantly different from no addition.

When MECs were preincubated for 30 minutes with BBG, the BzATP-induced increase in $[\text{Ca}^{2+}]_i$ was decreased by $32.28\% \pm 11.7\%$, $0.7\% \pm 10.2\%$, $0\% \pm 7.6\%$, and $36.4\% \pm 8.4\%$ at 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M, respectively (Fig. 6). Again, only the highest concentration of BBG significantly decreased the peak $[\text{Ca}^{2+}]_i$ from that stimulated by BzATP alone. When

FIGURE 8. Effect of inhibition of P2Y_1 , P2Y_{11} , and P2Y_{13} receptors on BzATP-stimulated $[\text{Ca}^{2+}]_i$ in cultured rat MECs. Cultured MECs were loaded with Fura 2 and preincubated for 30 minutes with the P2Y_1 receptor inhibitor MRS 2279 (10^{-7} – 10^{-5} M), the P2Y_{11} inhibitor NF157 (10^{-7} – 10^{-4}), or the P2Y_{13} inhibitor MRS 2211 (10^{-7} – 10^{-4}). (A, C) The mean of 10 cells from a single animal, representative of three individual experiments. (B, D) Change in peak $[\text{Ca}^{2+}]_i$. Data are the mean $\pm$ SEM from three independent experiments. Arrow: addition of BzATP. *Significant difference from BzATP alone.

FIGURE 9. Presence and localization of P2Y₁ receptors in cultured rat MECs. RT-PCR was performed on RNA from brain and cultured MECs. **(A)** A representative blot of three separate animals. **(B)** The presence of P2Y₁ receptors were determined by Western blot analysis in cultured MECs and lacrimal gland. Each lane (1–3) represents an individual animal. The localization of P2Y₁ receptors in the lacrimal gland were determined by confocal microscopy **(C)**; *red*: P2Y₁ receptor, *green*: α-SMA (α-SMA). *Arrows*: co-localization of P2Y₁ and α-SMA. **(D)** Localization of P2Y₁ receptors (*red*) and co-localization with α-SMA (*green*). **(C, D)** Peptide control for P2Y₁ receptors and rabbit and mouse isotype negative controls. NC, negative control; Br, brain; MWM, molecular weight markers; LG, lacrimal gland.

the area under the curve was determined, 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M decreased the BzATP response by $2.9\% \pm 32.5\%$, $0\% \pm 15.3\%$, $0\% \pm 19.2\%$, and $24.3\% \pm 3.1\%$ (data not shown). Only the highest concentration of BBG was significantly different from BzATP alone.

KN62 decreased BzATP-stimulated $[Ca^{2+}]_i$ by $21.5\% \pm 15.7\%$, $9.8\% \pm 9.5\%$, $19.1\% \pm 34.0\%$, and $42.7\% \pm 13.9\%$ at 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M, respectively (Fig. 6). Again, only the highest concentration of KN62 significantly decreased the peak $[Ca^{2+}]_i$ from that stimulated by BzATP alone. Similar results were obtained when the area under the curve was determined. KN62 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M decreased the BzATP response by $21.7\% \pm 13.3\%$, $8.0\% \pm 8.0\%$, $28.0\% \pm$

28.0% , and $52.0\% \pm 14.1\%$ (data not shown). Only the highest concentration of KN62 was significantly different from BzATP alone. In each case, the inhibitor alone had no effect on the basal $[Ca^{2+}]_i$ (i.e., before the addition of BzATP).

Taken together, these data indicate that, although P2X₇ receptors are present in cultured MECs, they, surprisingly, are not the predominant receptor activated by BzATP.

Effect of Inhibition of Phospholipase C on BzATP-Stimulated $[Ca^{2+}]_i$

Although BzATP has a high affinity for P2X₇ receptors, it has also been shown to activate selected P2Y receptors.^{29–31} Since

MECs responded to P2Y agonists (Fig. 3B), we investigated whether BzATP binds to and activates P2Y receptors. Because many P2Y receptors are G-protein-coupled receptors that activate phospholipase C (PLC),³² we wanted to determine whether P2Y receptors are activated by BzATP in MECs. MECs were loaded with Fura-2 and preincubated with the PLC inhibitor U73122 (Fig. 7A). BzATP increased $[Ca^{2+}]_i$ by 351.5 ± 55.3 nM (data not shown). U73122 decreased this response by $45.7\% \pm 21.3\%$ and $80.7\% \pm 11.3\%$ at 10^{-6} and 10^{-5} M, respectively. The BzATP-induced response in the presence of 10^{-5} M U73122 was significantly decreased from BzATP alone (Fig. 7B). U73122 had no effect on the basal $[Ca^{2+}]_i$. These results indicate that BzATP activates P2Y receptors in cultured MECs.

Effect of Inhibition of P2Y₁, P2Y₁₁, and P2Y₁₃ Receptors on the BzATP-Stimulated Increase in $[Ca^{2+}]_i$

To determine whether BzATP activates the P2Y₁, P2Y₁₁, or P2Y₁₃ receptors in cultured MECs, cells were loaded with Fura-2 and preincubated with the P2Y₁ inhibitor MRS 2279 (10^{-7} – 10^{-5} M), the P2Y₁₁ receptor inhibitor NF157 (10^{-7} – 10^{-4} M), or the P2Y₁₃ receptor inhibitor MRS 2211 (10^{-7} – 10^{-4} M) for 30 minutes. The cells were then stimulated with BzATP (10^{-4} M), and $[Ca^{2+}]_i$ was measured. In the first set of experiments, BzATP (10^{-4} M) significantly increased $[Ca^{2+}]_i$ (167.2 ± 30.0 nM; Fig. 8A). MRS 2279 significantly decreased this response ($53.7\% \pm 13.9\%$, $74.7\% \pm 11.9\%$, and $70.0\% \pm 13.0\%$) at 10^{-7} , 10^{-6} , and 10^{-5} M, respectively (Fig. 8B). The P2Y₁ receptor inhibitor MRS 2279 did not have a significant effect on basal $[Ca^{2+}]_i$.

In a second set of experiments, BzATP significantly increased $[Ca^{2+}]_i$ by 200.2 ± 83.5 nM (Fig. 8D). The BzATP response was unchanged by the P2Y₁₁ receptor inhibitor NF157 at 10^{-7} to 10^{-5} M, but was significantly decreased at 10^{-4} M ($56\% \pm 11\%$; Figs. 8C, 8D). Similarly, the BzATP response was also unchanged by the P2Y₁₃ receptor inhibitor MRS 2211 at 10^{-7} – 10^{-5} M but was significantly decreased ($25.0\% \pm 8.7\%$) at MRS 2211 10^{-4} M (Figs. 8C, 8D). Neither NF157 nor MRS 2211 had an effect on basal $[Ca^{2+}]_i$.

These data indicate that BzATP binds predominantly to the P2Y₁ receptor, whereas P2Y₁₁ and P2Y₁₃ receptors play a small role in this response.

Identification and Localization of P2Y₁, P2Y₁₁, and P2Y₁₃ Receptors in Cultured MECs

To examine the presence and localization of the P2Y₁ receptors in MECs, RT-PCR was performed with RNA isolated from rat brain and cultured MEC using primers specific to rat P2Y₁ receptors. One band was obtained in both brain and MECs at the expected size of 464 bp. (Fig. 9A). One band of 568 bp was obtained using primers specific to β -actin (data not shown).

To confirm that the P2Y₁ protein is expressed in cultured MECs, Western blot analysis was performed with MEC homogenate. As shown in Figure 9B, P2Y₁ was present as major three bands of approximately 100, 76, and 35 kDa in MECs, which has been reported.^{33,34} It is well established that purinergic

receptors are differentially glycosylated depending on tissue and cell type, accounting for the presence of multiple bands in Western blot analysis.³⁵ Multiple bands were also detected in the lacrimal gland (Fig. 9B).

The localization of P2Y₁ receptors in cultured MECs was determined by immunofluorescence techniques using the same antibody as was used in the Western blot analysis. To ensure that P2Y₁ receptors are present in the lacrimal gland and do not appear in cultured MECs as a result of the culturing techniques, the localization of the P2Y₁ receptor in the lacrimal gland was also determined. In the intact gland, P2Y₁ receptors were localized on the basolateral membranes in the acinar cells of the lacrimal gland and clearly outlined the individual cells within the acinus (Fig. 9C). In addition, these receptors colocalized with α -SMA, the marker for MECs (Fig. 9C). In cultured MECs, P2Y₁ receptors were localized in a punctate pattern throughout the cytoplasm, on fibers, and in the plasma membranes (Fig. 9D). Because of the multiple bands present in MEC lysate by Western blot analysis, we determined the effects of the competing peptide on the immunofluorescence binding of the P2Y₁ antibody. The binding of the P2Y₁ antibody was abolished after incubation of the antibody with the peptide against which it was raised in both the lacrimal gland and MECs (Fig. 9C, 9D). To confirm that the cells are indeed MECs, the cells were double labeled with α -SMA. All cells expressed both P2Y₁ receptors and α -SMA (Fig. 9D).

P2Y₁₁ and P2Y₁₃ were also present in cultured MECs as determined by RT-PCR and Western blot analysis. As the rat P2Y₁₁ receptor has not yet been cloned, we used primers specific to the human P2Y₁₁ receptor. As shown in Figure 10A, multiple bands were obtained in both brain and cultured MECs, one of which was at the expected size of 234 bp. (Fig. 10A). One band of 568 bp was obtained using primers specific to β -actin (data not shown). By Western blot analysis, three bands were detected using the P2Y₁₁ antibody in cultured MECs and one band in brain (Fig. 10A). When RT-PCR was performed using primers specific to rat P2Y₁₃ receptors, a single band of 245 bp was obtained in both lacrimal gland and cultured MECs (Fig. 10B). One band of 568 bp was obtained using primers specific to β -actin (data not shown). By Western blot analysis, two major bands were detected using the P2Y₁₃ antibody in cultured MECs, which also appeared in the lacrimal gland (Fig. 10B).

To ensure that P2Y₁₁ and P2Y₁₃ receptors were present in the lacrimal gland and did not appear in cultured MECs as a result of the culturing techniques, sections of lacrimal gland were incubated with P2Y₁₁ and P2Y₁₃ antibodies, and confocal microscopy was performed (Fig. 10C). P2Y₁₁ and P2Y₁₃ were both present in MECs in the lacrimal gland, as demonstrated by significant co-localization with α -SMA (Fig. 10C).

To determine the localization of P2Y₁₁ and P2Y₁₃ receptors in cultured MECs, MECs were grown on coverslips, and immunofluorescence experiments were performed. P2Y₁₁ receptors were localized in a perinuclear pattern (Fig. 10D). P2Y₁₃ receptors were localized in a similar pattern (Fig. 10D). To ensure that the cells used were MECs, they were double labeled with α -SMA. As shown in Figure 10D, all cells expressed

FIGURE 10. Presence and localization of P2Y₁₁ and P2Y₁₃ receptors in cultured rat MECs. RT-PCR using RNA from brain and cultured MECs was performed with primers specific for P2Y₁₁. (A) A representative blot of three separate animals. The presence of P2Y₁₁ was also determined by Western blot analysis in cultured MECs and lacrimal gland or brain. Using RNA from brain and cultured MECs, RT-PCR was performed using primers specific for P2Y₁₃ receptors. (B) A representative blot of three separate animals. The presence of P2Y₁₁ was also determined by Western blot analysis in cultured MECs and lacrimal gland or brain. (C) The localization of P2Y₁₁ and P2Y₁₃ were determined by confocal microscopy of lacrimal gland; P2Y receptors (red), α -SMA (green). (D) Localization of P2Y₁₁ and P2Y₁₃ receptors (red) and co-localization with α -SMA (green). (C, E) Peptide controls for P2Y₁₁ and P2Y₁₃ receptors and rabbit and mouse isotype negative controls. NC, negative control, Br, brain; LG, lacrimal gland, MWM, molecular weight markers. Magnification, $\times 200$.

α -SMA and either P2Y₁₁ or P2Y₁₃. Incubation of the antibodies with the peptides against which the antibodies were raised completely abolished the binding indicating that the antibodies are specific to the P2Y₁₁ and P2Y₁₃ receptors (Fig. 10E).

DISCUSSION

MECs in the lacrimal gland comprise a small percentage of the total number of cells. Their low number has hampered investigation into the roles these cells play in lacrimal gland function. In this study, we have developed a simple method of growing MECs from the lacrimal gland in culture. These cultured cells express α -SMA, α -actinin, vimentin, and adenylyl cyclase II, markers of MECs in the lacrimal gland as well as MECs from mammary glands.¹⁹

The function of the MECs in the lacrimal gland has not been extensively studied. In situ, these cells express the M₃ muscarinic, VIP, and P2X₇ receptors, all of which are known to be involved in secretion from the lacrimal gland acinar cells.^{10,11,17} It has also been shown that cholinergic agonists cause contraction of the MECs in the intact lacrimal gland, indicating that they contract to help expel the secretory products from the acinar cells.¹² In addition, it has been reported that MECs express stem cell markers after wounding of the lacrimal gland, implying that they play a role in wound repair.¹³ With the development of a pure population of MECs, the roles that these cells play in lacrimal gland functions can now be elucidated.

Cultured MECs contain functional P2X and P2Y receptors as demonstrated by the abilities of agonists to these receptors to stimulate an increase in $[Ca^{2+}]_i$. ATP, which binds to multiple purinergic receptors (including P2Y₁₃), increases $[Ca^{2+}]_i$ in cultured MECs, as does BzATP, which normally binds to P2X₇ receptors. The positive response with UTP is indicative of the presence of P2Y₂ and P2Y₄ receptors, whereas the responses with MeSATP and ATP γ S indicate the P2Y₁ and P2Y₁₁ receptors are present. This study demonstrated with RT-PCR, immunofluorescence, and Western blot analysis that MECs from the lacrimal gland express P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ receptors, as does the intact lacrimal gland.

The P2Y₁₁ and P2Y₁₃ purinergic receptors in the cultured MECs were localized in the cytoplasm instead of the plasma membranes, as would be expected for receptors, and were seen in the acini of the lacrimal gland. We believe the intracellular localization of these P2 receptors occurs because these cells normally interact with acinar and ductal cells in the lacrimal gland, allowing the MECs to assume their characteristic stellate shape and maintain polarity. This interaction allows the expression of receptors in the appropriate location of the plasma membrane. In culture, such interactions do not occur. However, MECs are able to respond quickly to agonists, which implies that there is either a small number of purinergic receptors at the plasma membrane that are below the limit of detection by immunofluorescence or that these receptors are able to be recruited to the plasma membrane very quickly. Cytosolic localization of purinergic receptors has been observed in cultured astrocytes.³⁶ This has also been observed with other types of receptors in cultured conjunctival goblet cells.^{37,38}

Of note, BzATP, which in lacrimal gland acini activates P2X₇ receptors,¹⁷ has a limited effect on these receptors in cultured MECs. Indeed, three P2X₇ receptor inhibitors had minor effects on the BzATP-stimulated increase in $[Ca^{2+}]_i$. In contrast to acini, the BzATP-stimulated increase in $[Ca^{2+}]_i$ in MECs is not potentiated in the absence of Mg²⁺, and the response becomes desensitized by repeated applications of BzATP. It is well known that P2X₇ receptors are expressed in different splice

variants, depending on the tissue and cell type.³⁹ These variants have different properties. It is likely then that the P2X₇ receptor in MECs is different from the P2X₇ receptors present in acinar cells, as they have different properties.

There is strong evidence that BzATP activates P2Y receptors on cultured MECs from the lacrimal gland. The PLC inhibitor blocked the BzATP-stimulated increase in $[Ca^{2+}]_i$, and this response was desensitized with repeated applications of BzATP. Finally, specific inhibitors of P2X₁, P2Y₁₁, and P2Y₁₃ blocked the BzATP-induced increase in $[Ca^{2+}]_i$. As the P2Y₁ receptor inhibitor was more potent than the inhibitors of either P2Y₁₁ or P2Y₁₃, we conclude that BzATP activates primarily P2Y₁ receptors rather than the P2X₇ receptors.

In the lacrimal gland, the source of the ATP to activate the purinergic receptors on the MECs could be the nerves that innervate the glands, the acinar cells, or the MECs themselves. ATP is released from nerves along with acetylcholine and norepinephrine.⁴⁰ We have previously shown that cholinergic and α_{1D} -adrenergic agonists can also cause the release of ATP from lacrimal gland.^{18,41} ATP released via these mechanisms can interact with the purinergic receptors on the MECs to stimulate them in a paracrine manner. In addition, mechanical stimulation of cells, such as contraction of MECs, can cause the release of ATP which can act in an autocrine manner, stimulating purinergic receptors on the same cell.

Little information is known regarding MECs and purinergic receptors. It has been reported that in cultured MECs from mammary glands, the purinergic receptor agonists ATP, ADP, UTP, and UDP increase $[Ca^{2+}]_i$ and cause contraction.^{6,42} As the ATP response was inhibited by a P2 receptor antagonist and a PLC inhibitor, the conclusion was that ATP binds to a P2Y receptor. However, the subtype was not further identified. In addition, the increases in $[Ca^{2+}]_i$ caused by submaximum concentrations of oxytocin were further elevated in the presence of ATP, implying that purinergic receptors facilitate milk ejection.^{42,43}

In conclusion, lacrimal gland MECs express P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₃ purinergic receptors. The predominant target of BzATP in cultured MECs is the P2Y₁ receptor. However, BzATP interacts to a limited extent with its expected target, P2X₇ receptors, as well as with P2Y₁₁ and P2Y₁₃ receptors. Activation of these receptors in cultured rat MECs increases $[Ca^{2+}]_i$.

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