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α1-Adrenoceptors relate Ca^{2+} modulation and protein secretions in rat lacrimal gland

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ABSTRACT

Noradrenaline (NA) is a catecholamine with multiple roles including as a hormone and a neurotransmitter. Cellular secretory activities are enhanced by adrenergic stimuli as well as by cholinergic stimuli. The present study aimed to determine which adrenoceptors play a role in controlling intracellular calcium ion ($[Ca^{2+}]_i$) level in acinar cells of rat lacrimal glands. Expression of mRNA for adrenoceptor subtypes in the acinar cells was assessed using RT-PCR. All types except α2c, β1, and β3 were detected. NA induced a $[Ca^{2+}]_i$ increase with a biphasic pattern in the acinar cells. Removal of extracellular Ca^{2+} and use of Ca^{2+}-channel blockers did not inhibit the NA-induced $[Ca^{2+}]_i$ increases. In contrast, U73122 and suramin almost blocked these increases. The α1-adrenoceptor agonist phenylephrine induced a strong increase in $[Ca^{2+}]_i$. However, clonidine and isoproterenol failed to induce a $[Ca^{2+}]_i$ increase. The peroxidase activity was quantified as a measure of mucin secretion. Ca^{2+}-dependent exocytotic secretion of peroxidase was detected in rat lacrimal glands. The RT-PCR results showed that MUC1, MUC4, MUC5AC, MUC5B, and MUC16 were expressed in acinar cells. These findings indicated that NA activates α1-adrenoceptors, which were found to be the main receptors in Ca^{2+}-related cell homeostasis and protein (including mucin) secretion in lacrimal glands.

The lacrimal gland is the main contributor to the aqueous component of the preocular tear film, which contains water, electrolytes, proteins, peroxidase, mucins, and lactoferrin. The lacrimal gland is composed of several cell types, including myoepithelial cells, ductal cells, goblet cells, and acinar cells, which are the major cell type constituting 80% of the gland. Acinar cells are highly polarized and joined by tight junctions at the luminal membrane, creating distinct basolateral and apical membranes (12). Goblet cells are a source of mucus in tears and secrete different types of mucins onto the ocular surface, especially in the conjunctiva. Although the number of goblet cells increases in the nasolacrimal duct, as the diameter of the nasolacrimal duct lumen is narrower than that of the lacrimal sac. Regulation of lacrimal gland fluid secretions is under neural control; activation of the sensory nerves in the cornea and conjunctiva initiates an afferent pathway leading to the central nervous system, which subsequently activates an efferent pathway that stimulates parasympathetic and sympathetic nerves, and ultimately, the lacrimal gland (32). The appropriate amount and composition of lacrimal gland fluid are crucial for a healthy, intact ocular surface (14).

Sympathetic nerves release the catecholamine noradrenaline (NA). Catecholamines bind to adreno-

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ceptors and are the principal mediators of the sympathetic nervous system effects. Adrenoceptors are part of the G-protein-coupled receptors that activate heterotrimeric G-proteins after binding of their ligands. G-proteins typically stimulate (Gs) or inhibit (Gi) the enzyme adenylyl cyclase, or activate (Gq) phospholipase C (PLC) (55). NA signals via a family of adrenergic receptors comprising three major classes, α1, α2, and β, which are coupled to distinct intracellular signaling pathways (11, 29).

NA is an effective stimulus of lacrimal gland secretion, particularly protein secretion (15, 16). However, the adrenergic system in the lacrimal gland has been assumed to play an indirect, minor role in lacrimal function, mostly by regulating the blood flow to the gland (7, 27). In rodents, the lacrimal gland is innervated by the autonomic nervous system, with parasympathetic input predominating (6, 57, 58). Although it is generally accepted that parasympathetic innervation of the lacrimal gland is responsible for inducing tear secretion, there is still some controversy about its functional relevance. Moreover, increasing evidence indicates that the adrenergic system may also play a direct and significant role in regulating tear secretion in some species (8, 50). Recent studies have demonstrated that the α1-adrenergic pathway stimulates lacrimal gland protein secretion from acini (17) and that α1D-adrenergic receptors compared with P2X7 receptors (ionotropic ATP-gated receptors) use different cellular mechanisms to increase $[Ca^{2+}]_i$ and cause protein secretion in rat lacrimal gland acini (18). The authors reported that activation of α1D-adrenoceptors releases ATP, which induces P2X7 receptors to increase $[Ca^{2+}]_i$, but not to stimulate protein secretion. In another study, direct stimulation of either the α1- or the β1-receptor induced significant protein secretion from mouse lacrimal glands (20). Although it is very important to understand these physiological phenomena, their mechanisms remain unclear at present.

Mucus, a major component of the tear film, comprises giant glycoconjugates called mucins, whose protein core is extensively decorated with O-linked oligosaccharide chains (24). Mucins are a class of very high-molecular-weight glycoproteins that are synthesized and secreted by epithelial tissues such as the gastrointestinal, respiratory, and reproductive tracts, and by the conjunctiva (25, 34, 38, 48, 61). At these tissue surfaces, mucins prevent evaporation, provide lubrication, and protect tissues from harmful agents and organisms. At least 22 human mucin genes have been cloned (31, 35, 56, 68). For instance, in case of lungs, 16 (MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC11, MUC13, MUC15, MUC16, MUC18, MUC19, MUC20, MUC21, and MUC22) mucin genes are expressed (19, 31, 35, 56, 63, 68). The predicted amino acid sequences of the cloned mucin genes revealed that there are 2 types of mucin groups in humans: secreted and membrane-tethered mucins. Seven mucin gene products (i.e., MUC2, 5AC, 5B, 6, 7, 8 and 19) are secreted in type, and the remaining 10 are membrane-tethered (31, 65). The membrane-associated mucins MUC1, MUC4, and MUC16 have been identified in the cornea, conjunctiva, and tears in humans, while the soluble secreted mucin MUC5AC is secreted by conjunctival goblet cells in rats (3, 5, 33, 34, 38, 52, 53). Secretion of MUC5AC is stimulated by the calcium ionophore A23187 and by purinergic agonists acting as P2Y2 receptors (37). Tears of patients with Sjögren's syndrome have decreased levels of the goblet-cell mucin MUC5AC (2). Some scientists consider that mucins found on the ocular surface are primarily produced by goblet cells, apical part of cells of the conjunctiva and cornea, and the lacrimal gland. For that reason, there is no report to observe an immediate mucin secretion from acinar cells in lacrimal glands to date. Analysis of the protein secretion mechanism of lacrimal glands is required to unravel the cause of Sjögren's syndrome.

The aim of the present study was to determine the contribution of adrenoceptors to NA-induced changes in $[Ca^{2+}]_i$ in lacrimal gland acinar cells. For this purpose, we examined the $[Ca^{2+}]_i$ dynamics in semi-intact rat acinar cells that retained their essential cellular structure. We recently showed that ATP and protease-activeted receptor 2 participate in a variety of signaling activities in lacrimal glands (40, 49). The present study focused on identification of the types of adrenoceptors that are present in acinar cells and the receptors that mediate the effect of NA on $[Ca^{2+}]_i$ levels in these cells. We examined the effect of NA on the secretion of mucus peroxidase to clarify its mechanism by using ELISA. The mucus that constitutes the mobile mucus layer covering the cornea is a complex mixture of mucins, globular proteins, antimicrobial proteins (including peroxidase) and peptides, salts, and water. It is difficult to directly obtain native mucins from cells and tissues because of their high molecular weight, many disulfide linkages and large variety of sugar structure. And mucins are difficult to quantitate. In this study, therefore, mucin secretion was quantified indirectly by the peroxidase activity in the rat lacrimal gland acinar cells. mRNA levels of α- and β-adrenoceptors

and MUCs were assayed by reverse-transcriptase polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Preparation of rat glandular acini. Protocols and all animal experiments were approved by and conducted under the authority of the Iwate Medical University Institutional Animal Care and Use Committee. Adult Wistar male rats (7–12 weeks old, 150–290 g body weight) were used. The rats were sacrificed using carbon dioxide gas and perfused via the left cardiac ventricle with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.25 mM $CaCl_2$) at room temperature. The lacrimal glands were removed and placed in HEPES-buffered Ringer's solution (HR) containing 118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1 mM NaH_2PO_4, 5.5 mM D-glucose, MEM amino acids solution (Gibco, Grand Island, NY, USA), 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA), and 10 mM HEPES; the pH was adjusted to 7.4 with NaOH. The lacrimal glands were trimmed of excessive connective tissues and digested with purified collagenase (100 U/mL; Elastin Products, Owensville, MO, USA) in HR buffer for 1 h at 37°C. Enzyme digestion was conducted with constant agitation (~200 rpm) in a rotary shaker under an atmosphere of 100% O_2 that was achieved by gassing at 15-min intervals. After digestion, the glands were washed twice and centrifuged at $800 \times g$ for 2 min at room temperature between washes. The pellet was resuspended in 15 mL of HR buffer. The suspension was filtered through a Nitex screen (mesh size 150/inch). The gland cells were washed twice and centrifuged at $800 \times g$ for 2 min at room temperature between washes. The final pellet was resuspended in 3 mL of HR buffer.

Intracellular Ca^{2+} imaging. The lacrimal gland acinar cells were transferred into HR buffer containing 3 μM Indo-1/AM and 0.02% cremophore®-EL (Nacalai Tesque, Kyoto, Japan) and incubated for 1 h at 37°C. Indo-1 is a ratiometric dye that is excited by ultraviolet light and is used for quantitative determination of $[Ca^{2+}]_i$. The emission maximum of Indo-1 shifts from 475 nm in Ca^{2+}-free medium to 400 nm when the dye solution is saturated with Ca^{2+}. The ratio of the emission intensity at a wavelength shorter than 440 nm to that at a wavelength longer than 440 nm can be used to estimate $[Ca^{2+}]_i$; a higher ratio indicates higher $[Ca^{2+}]_i$.

A real-time confocal microscope (RCM/Ab; a modified version of the Nikon model RCM-8000; Nikon, Tokyo, Japan) was used to measure cellular $[Ca^{2+}]_i$ changes. The Indo-1-loaded cells were exposed to an ultraviolet-beam (351 nm). An inverted microscope was equipped with an argon-ion laser (TE-300, Nikon) and the fluorescence emission was passed through a water-immersion objective lens (Nikon C Apo 40×, N.A. 1.15) to a pinhole diaphragm. The acquisition time per image frame was 1/30 s. Images were immediately stored on high-speed hard disks. The digital images in the laser scanning microscopic imaging were composed of 512 × 480 pixels with a density resolution of 8 bits/pixel. The fluorescence intensity was displayed in pseudocolor with 256 colors; red represents high $[Ca^{2+}]_i$, and purple and blue low $[Ca^{2+}]_i$. Additionally, we measured $[Ca^{2+}]_i$ changes in specific, restricted areas of the cell (~0.5-μm^2 spot size).

Stimulation with NA and NA-analogs. The $[Ca^{2+}]_i$ dynamics in acini were examined in a perfusion chamber immediately after the dye-loading procedure. After perfusion with the standard HR solution for a few minutes at room temperature, intact lacrimal glands were selected and examined under the microscope. The specimens were continuously perfused with the HR solution containing the following agonists and/or antagonists: NA (30 μM) from Alfa Aesar (Lancashire, UK), U73122 (3 μM; an inhibitor of PLC), suramin (50 μM; an antagonist of G proteins), and diltiazem (50 μM; an L-type Ca^{2+}-channel blocker) from Sigma, phenylephrine (10 μM; an agonist of α1-adrenoceptors), clonidine (10 μM; an agonist of α2-adrenoceptors), and isoproterenol (10 μM; an agonist of β-adrenoceptors) from Tocris (Bristol, UK), GF109203X (2 μM; a protein kinase C (PKC) antagonist) from Enzo Life Sciences (New York, NY, USA), and $GdCl_3$ (100 μM; a nonspecific cation-channel blocker) from Wako (Osaka, Japan).

RT-PCR. Total RNA was extracted from lacrimal gland acinar cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were determined by spectroscopy at 260 nm. First-strand cDNA synthesis and RT-PCR were carried out using the ReverTra Ace-α-® (TOYOBO, Osaka, Japan) in a thermal cycler (PC-701; ASTEC, Fukuoka, Japan). The primer sequences used to amplify α- and β-adrenoceptor mRNAs expression are shown in Table 1 and have previously been described by Yoshizumi *et al*., Yoon *et al*., and Matsubara *et al.* (45, 69, 70). The primer sequences used to amplify mucins are shown in Table 2 and have previously been described by Zoghbi

Table 1 *Primers used for PCR of the α- and β-adrenoceptors*

Receptor		Sequence 5'-3'	Position	Accession Code	Amplicon
rα1A	F	GCATCATCTCCATCGACCGA	368	NM017191	485 bp
	R	AGCAGCCTCACTGAGAAGTG	800		
rα1B	F	GCTACCCTAGAAGTGCTTGG	696	NM016991	391 bp
	R	TCCTCTTGGCCACGATGTAG	1086		
rα1D	F	CCTACAGACGGTCACCAACT	839	NM024483	321 bp
	R	AGTAGCGGTCCCACAGATAC	1159		
rα2A	F	GGTAAGGTGTGGTGCGAGAT	349	NM012739	229 bp
	R	CAGCGCCCTTCTTCTCTATG	577		
rα2B	F	ACACCGTCTTCAACCAGGAC	1654	NM138505	169 bp
	R	CCCAGAGAAATGGCCATAGA	1822		
rα2C	F	CTGGCAGCCGTGGTGGGTTTCCTC	250	NM138506	426 bp
	R	GTCGGGCCGGCGGTAGAAAGAGAC	675		
rβ1	F	GCTCTGGACTTCGGTAGACG	1652	D00634	248 bp
	R	ACTTGGGGTCGTTGTAGCAG	1899		
rβ2	F	GAGCACAAAGCCCTCAAGAC	1001	NM012492	209 bp
	R	TGGAAGGCAATCCTGAAATC	1209		
rβ3	F	TGCGCCCATCATGAGCCAGTGGTG	721	NM013108	550 bp
	R	GCGAAAGTCCGGGCTGCGGCAGTA	1270		
GAPDH	F	TTCAACGGCACAGTCAAGGC	1009	AF106860	812 bp
	R	TCCACCACCCTGTTGCTGTAGC	1820		

F, forward primer; R, reverse primer. The primers are from Yoshizumi *et al.*, Yoon *et al.*, and Matsubara *et al.* (45, 69, 70). All primers were assessed for specificity using BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

et al., Tian *et al.*, Yu *et al.* (66, 71, 72), except MUC1, 4, and 16. Primers were designed using the web-based tool "Primer3." The thermal cycling protocol was as follows: 42°C for 10 min and 94°C for 1 min, followed by 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gels and were stained with ethidium bromide. Images of the gels were captured using a Polaroid MP4 Land Camera (Polaroid, Minnetonka, MN, USA). RT-PCR was repeated for three independent experiments.

Peroxidase activity. Lacrimal gland acinar cells were preincubated in 2 mL of HR buffer without BSA at 37°C for 5 min and stimulated for 60–80 min with the following compounds: 100–500 μM NA, 200 μM phenylephrine, 200 μM isoproterenol, and 2 μM A23187. Preincubated cells without stimulation (incubated in HR buffer for 60–80 min) were used as controls. The cell suspensions were chilled on ice for 5 min to terminate secretion and centrifuged at 137 × *g* for 5 min at 4°C. The peroxidase activity in aliquots of the supernatants was assessed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen/Thermo Fisher scientific, Waltham, MA, USA) according to the manufacturer's instructions. The absorbance at 560 nm was measured using a Multiskan GO microplate spectrophotometer (Thermo Fischer Scientific).

RESULTS

Expression of adrenoceptors in rat lacrimal gland acinar cells

Lacrimal glands from rats were digested with collagenase in HR buffer as described in Materials and Method. Only acinar cells of lacrimal glands, excluding goblet cells, neurons and mast cells, were collected. Total RNA was extracted from the sample. We initially assessed the expression of adrenoceptor mRNAs in lacrimal gland acinar cells using RT-PCR. RT-PCR of adrenoceptor mRNAs harvested from the rat lacrimal glands yielded products of the predicted size (Table 1). Receptor expression levels were graded from (−), where the PCR product was not detectable by ethidium bromide staining of an agarose gel, to (++), where a very strong band was detected in the gel (Fig. 1). All of the NA-receptor mRNAs that were investigated, except α2C, β1, and β3 mRNAs, were expressed in the lacrimal gland acinar cells (Fig. 1).

Effect of NA and NA analogs on $[Ca^{2+}]_i$ dynamics

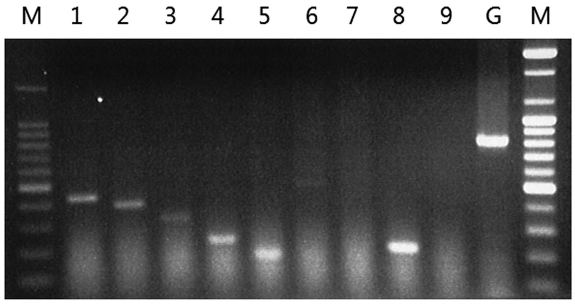
Table 2 *Primers used for PCR of the mucins*

Receptor		Sequence 5'-3'	Position	Accession Code	Amplicon
rMUC1	F	TCGACAGGCAATGGCAGTAG	1952	NM012602	286 bp
	R	TCTGAGAACCACCACTACCC	2237		
rMUC2	F	GCCTCAAACCCGTGCGTGTC	696	XM006230606	613 bp
	R	TCATTCACCAACCACTCATC	1086		
rMUC3	F	TCATCCTGAAGGCCCAGTAC	398	U76551	335 bp
	R	CTGACATTTGCCATAGCTGC	732		
rMUC4	F	CGTACTAGAGAACTTGGACATGC	7655	XM006221167	638 bp
	R	GGTAGGAGAACTTGTTCATGG	8292		
rMUC5AC	F	AGCTCGATCGACTCCTACTCC	4647	XM001063331	321 bp
	R	GGTGTGTTGGGAAAGAACTGA	4967		
rMUC5B	F	TGACCACCTCTATTGGCAAAC	6879	XM006223574	209 bp
	R	GGCTGAAGATGTTGTGGTGAT	7087		
rMUC6	F	TCCTACTTGCCAGGTCTT	5884	XM006230605	294 bp
	R	GGGTGTTGACTTCGGTAT	6177		
rMUC16	F	ACCCTACCACTGTGGCTTTG	16592	XM235886	180 bp
	R	GTGTGGCCAGTGAAATTGTG	16771		
GAPDH	F	TTCAACGGCACAGTCAAGGC	1009	AF106860	812 bp
	R	TCCACCACCCTGTTGCTGTAGC	1820		

F, forward primer; R, reverse primer. The primers are from Zoghbi *et al.*, Tian *et al.*, and Yu *et al.* (66, 71, 72), except those for MUC1, MUC4, and MUC16; these primers were designed using the web-based tool "Primer3." All primers were assessed for specificity using BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

We analyzed the effect of NA and NA analogs on the $[Ca^{2+}]_i$ dynamics in lacrimal acinar cells, which could be clearly observed by confocal microscopy as shown in Fig. 2. No spontaneous $[Ca^{2+}]_i$ changes were observed in the cells. A small number of injured cells, which showed high $[Ca^{2+}]_i$ under basal conditions, were excluded from subsequent analyses.

Exposure of the lacrimal glands to extracellular NA led to an increase in $[Ca^{2+}]_i$ in some acinar cells (n = 10) (Fig. 2a–e). A biphasic increase in $[Ca^{2+}]_i$ was observed; an initial transient increase was followed by a second, sustained increase. Contracted myoepithelial cells, which have been previously observed in guinea pig lacrimal glands (59, 60), were not evident in the rat lacrimal gland because of the relatively coarse net of the myoepithelial envelope. To analyze the mechanism by which NA induces changes in $[Ca^{2+}]_i$, we investigated if ion channels were entirely responsible for the NA-induced changes in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ changes were a little inhibited in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$) (n = 10) (Fig. 3a). Lasting time of the reaction was short: a second plateau phase disappeared (Fig. 3a, dotted arrows). Treatment of the acinar cells with Gd^{3+} (100 μM), a nonspecific cation-channel blocker, was same as in the absence of Ca^{2+} (n = 8) (Fig. 3b). Furthermore, no complete inhibition of NA-induced $[Ca^{2+}]_i$ increases was observed following treatment



Lane	1	2	3	4	5	6	7	8	9
Intensity of bands	α1A	α1B	α1D	α2A	α2B	α2C	β1	β2	β3
	+	+	+-	++	++	-	-	++	-

Fig. 1 RT-PCR analysis of adrenoceptors in rat lacrimal gland acinar cells. All adrenoceptor subtypes except *α*2C, *β* 1, and *β*3 were expressed. G: GAPDH (positive control), M: molecular standards.

with diltiazem (50 μM), an L-type Ca^{2+}-channel blocker (data not shown). These data suggested that receptors other than ion channels might mediate the activity of NA.

Generally, metabotropic receptors are G-protein-linked, and stimulation of G protein activates PLC, which cleaves membrane-bound phosphatidyl-inositol-biphosphate to generate inositol-triphosphate (IP_3) and diacylglycerol. IP_3 subsequently causes Ca^{2+}

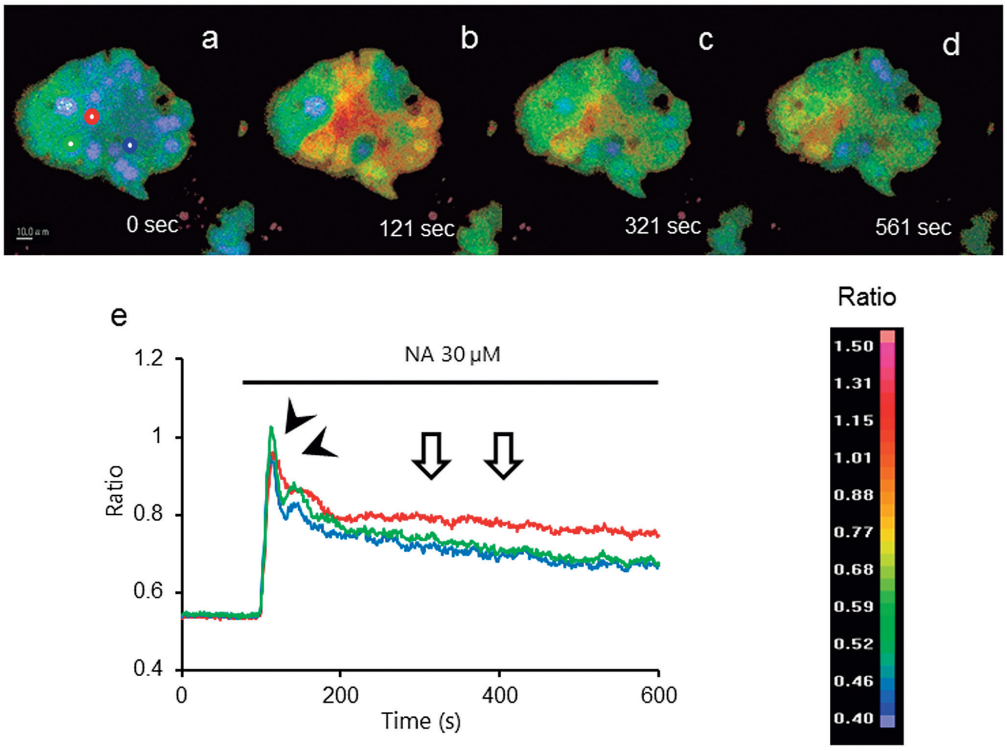


Fig. 2 Spatiotemporal changes in $[Ca^{2+}]_i$ in acinar cells during noradrenaline (NA)-stimulation (n = 10). Changes in $[Ca^{2+}]_i$ are indicated as pseudocolors. (**a**–**d**) Color scale bar: $[Ca^{2+}]_i$ was calculated based on the ratio of dye fluorescence at different wavelengths. (**e**) Time course of NA-induced changes in $[Ca^{2+}]_i$ in specific regions of interest (ROIs) of lacrimal gland acinar cells (~1 μm^2 in size). Note that the $[Ca^{2+}]_i$ changes are biphasic; an initial acute increase (arrow heads) is followed by a plateau phase (arrows).

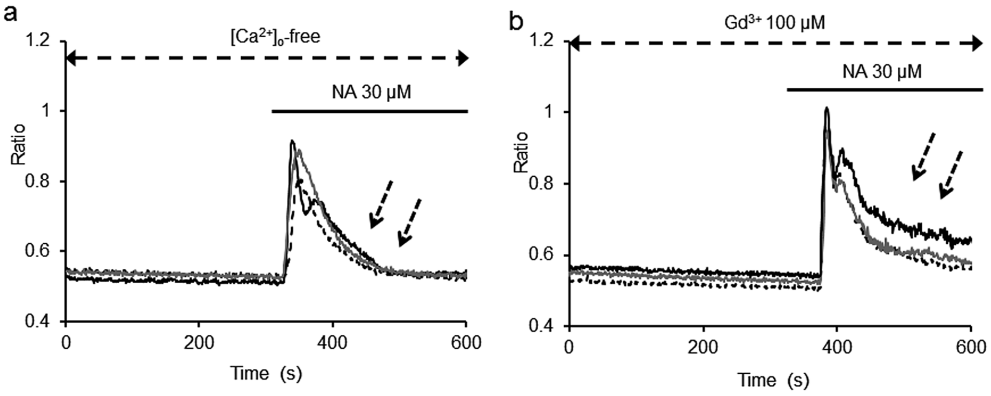


Fig. 3 Ion channels are not entirely responsible for NA-induced $[Ca^{2+}]_i$ changes. The time course of $[Ca^{2+}]_i$ changes induced by NA in specific areas of lacrimal gland acinar cells (~1 μm^2 in size) was analyzed as described for Fig. 2. Under extracellular Ca^{2+}-free conditions ($[Ca^{2+}]_o$-free) (n = 10), the second plateau phase disappeared (**a**, dotted arrows). Gd^{3+} (100 μM) (n = 8) inhibits NA-induced $[Ca^{2+}]_i$ dynamics in the same manner as above (**b**, dotted arrows). Three ROIs were set.

mobilization from internal stores (4). To determine if this mechanism of Ca^{2+} mobilization was involved in the NA-dependent $[Ca^{2+}]_i$ increase, the effects of the PLC inhibitor U73122 and the G-protein antagonist suramin were assayed. U73122 (5 μM; n = 9) and suramin (50 μM; n = 8) almost completely blocked NA-induced increases in $[Ca^{2+}]_i$ (Fig. 4a and b). This finding confirms that NA-induced $[Ca^{2+}]_i$ increases in lacrimal gland acinar cells were caused by the Ca^{2+} release from internal Ca^{2+} stores and the influx

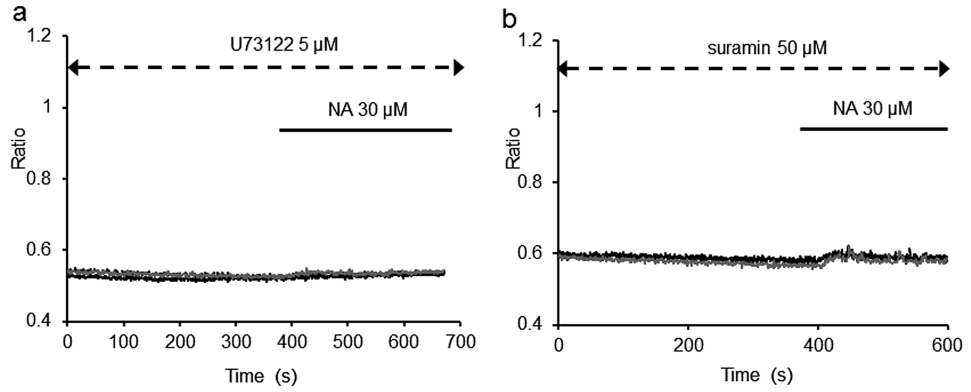


Fig. 4 A role for mobilization of Ca^{2+} from intracellular Ca^{2+} stores in NA-induced $[Ca^{2+}]_i$ changes. (**a**) NA (30 μM) induced only a slight $[Ca^{2+}]_i$ increase in the cells after blocking of PLC by treatment with U73122 (5 μM; n = 9). (**b**) Treatment with a G protein antagonist, suramin (50 μM; n = 8), inhibited NA-induced $[Ca^{2+}]_i$ increases. Three ROIs were set.

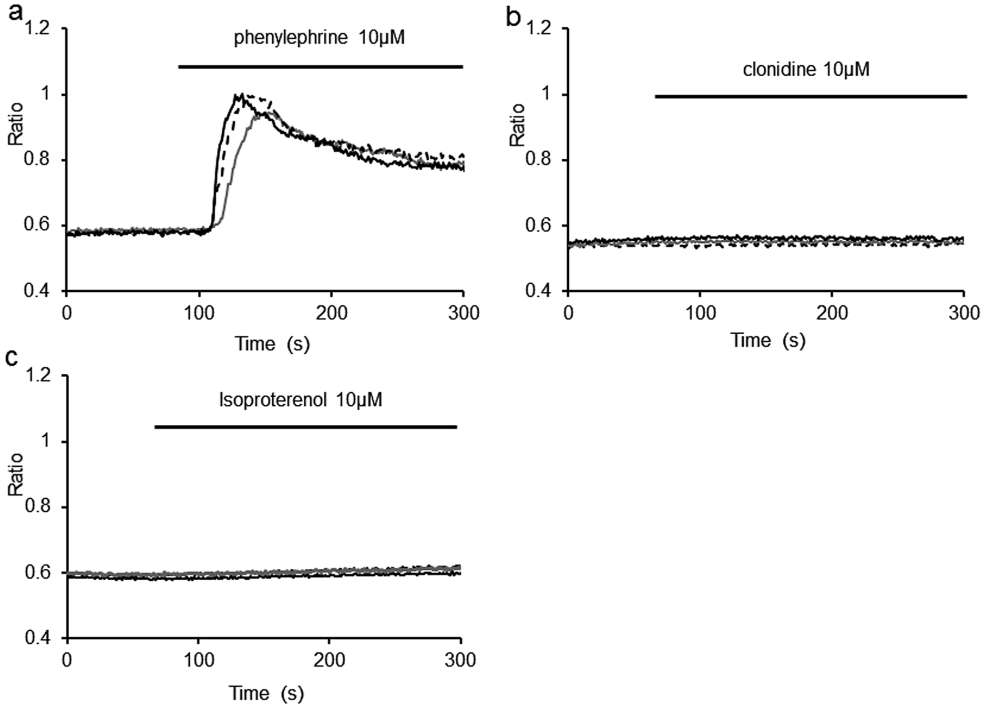


Fig. 5 Effect of adrenoceptor agonists on $[Ca^{2+}]_i$ dynamics. (**a**) The $\alpha 1$ agonist phenylephrine (10 μM; n = 10) caused a strong $[Ca^{2+}]_i$ increase in the cells. (**b**) The $\alpha 2$ agonist clonidine (10 μM; n = 8) caused no $[Ca^{2+}]_i$ increase in the cells. (**c**) The β agonist isoproterenol (10 μM; n = 10) failed to induce an increase in $[Ca^{2+}]_i$. Three ROIs were set.

of extracellular Ca^{2+}. Thus, Ca^{2+} influx from extracellular spaces and IP_3-dependent Ca^{2+} mobilization from intracellular Ca^{2+} stores were induced by NA stimulation, and mobilization of Ca^{2+} from intracellular Ca^{2+} stores may be more significant than Ca^{2+} influx in the NA-induced response.

Next, we studied the involvement of α- and β-adrenoceptors in the effect of NA and its analogs on lacrimal gland acinar cells using specific agonists of these receptors. Phenylephrine, an α1-adrenoceptor agonist, induced a $[Ca^{2+}]_i$ increase in the cells (n = 10) (Fig. 5a). However, both the β-adrenoceptor agonist isoproterenol (n = 8) and the α2-adrenoceptor agonist clonidine (n = 10) failed to induce an increase in $[Ca^{2+}]_i$ in the acinar cells (Fig. 5b and c). These data indicated that the NA-induced response is mainly mediated by the α1-adrenoceptor and involves intracellular Ca^{2+} stores.

PKC plays a major role in both cholinergic- and α1-adrenergic-stimulated lacrimal gland protein secretions (73). Therefore, we investigated whether PKC participates in the NA-induced response in the acinar cells. The PKC antagonist GF109203X (2 μM) failed to inhibit an increase in Ca^{2+} by NA stimulation in these cells (n = 10) (Fig. 6). These data suggested that PKC might not be involved in the α1-adrenoceptor-stimulated increase in $[Ca^{2+}]_i$ in lacrimal gland acinar cells.

Peroxidase secretion

To explore the involvement of NA in mucous secretion, mucus peroxidase secretion was investigated. Acini were preincubated with various concentrations of NA for 60 min. Dose-dependent release of peroxidase was observed (Fig. 7A). Next, we checked the time-dependent increase in peroxidase activity using 400 μM NA. NA-induced peroxidase secretion increased during 80 min of incubation (Fig. 7B). To determine whether $[Ca^{2+}]_i$ is involved in the protein secretion, the effect of the Ca^{2+} ionophore A23187 was investigated. The cells were stimulated with A23817 (2 μM) for 60 min, and peroxidase secretion was measured. Peroxidase secretion was significantly stimulated by A23187 (Fig. 7C). Next, we assessed peroxidase secretion in the presence of α1- and β-receptor agonists. Stimulation of the acini with the α1-adrenergic agonist phenylephrine (200 μM) increased peroxidase secretion. In contrast, the β-adrenergic agonist isoproterenol (200 μM) did not increase peroxidase secretion (Fig. 7C). These results suggested that NA mainly stimulates α1-adrenoceptors that induce the increase in $[Ca^{2+}]_i$ and stimulate protein secretion. Additionally, the protein secretion mechanism is activated by intracellular Ca^{2+}.

MUC mRNA expression in lacrimal gland acinar cells

Finally, we assessed the mRNA expression of MUCs in lacrimal gland acinar cells, excluding goblet cells, using RT-PCR. Receptor expression levels were graded from "−," where no PCR product was detectable by ethidium bromide staining of an agarose gel, to "++," where a very strong band was detected in the gel (Fig. 8). MUC1, MUC4 and MUC16 (membrane-associated mucins), and MUC5AC and 5B (soluble, secreted mucins) were expressed in the lacrimal gland acinar cells (Fig. 8). The band obtained for MUC6 was due to non-specific amplification (a predicted PCR product size: 294 bp).

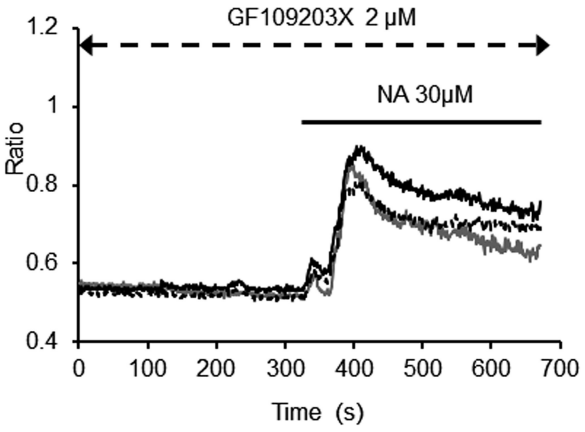


Fig. 6 A role for PKC in mobilization of Ca^{2+} from intracellular Ca^{2+} stores in NA-induced $[Ca^{2+}]_i$ dynamics. GF109203X (2 μM; n = 10), a PKC antagonist, did not inhibit NA-induced $[Ca^{2+}]_i$ increases in these cells. Three ROIs were set.

DISCUSSION

NA, as a ligand of adrenoceptors, plays a role in the secretion of tears from lacrimal gland acinar cells. Most previous studies have reported that the adrenergic system in the lacrimal gland plays an indirect and minor role in lacrimal function, mostly by regulating the blood flow to the gland (7, 27). Based on the data of this study, we would like to propose that NA, particularly α1, plays a significant role in rat lacrimal gland acinar cells.

The present study demonstrated that Ca^{2+} mobilization from intracellular Ca^{2+} stores was induced by extracellular NA, suggesting the presence of metabotropic receptors in rat lacrimal gland acinar cells that are activated by NA. The results of our experiments using U73122 or suramin indicated that the NA-induced Ca^{2+} mobilization was mediated mainly by G protein and the IP_3 receptor. Lacrimal gland secretion is controlled mainly by autonomic nerves. Parasympathetic cholinergic stimuli elicit an IP_3-dependent $[Ca^{2+}]_i$ increase, while sympathetic adrenergic stimulation-induced $[Ca^{2+}]_i$ dynamics are IP_3-independent (13, 28). We previously reported that NA and adrenaline induce an increase in $[Ca^{2+}]_i$ and exocytosis in acinar cells of lacrimal glands, while myoepithelial cells respond only to cholinergic stimuli (60). The results from our current study are consistent with these previous results. Therefore, even under increasing sympathetic nervous system activity, the lacrimal gland can secrete fluid via both an IP_3-dependent calcium signal and myoepithelial cell contraction using the parasympathetic nervous system. Different signaling pathways are involved in ensuring that the

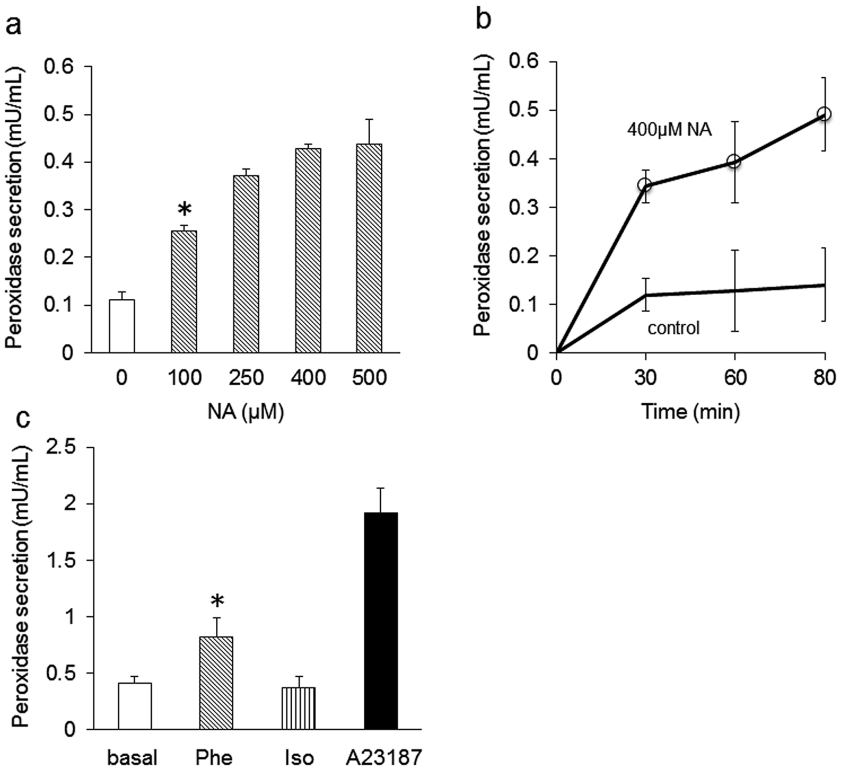
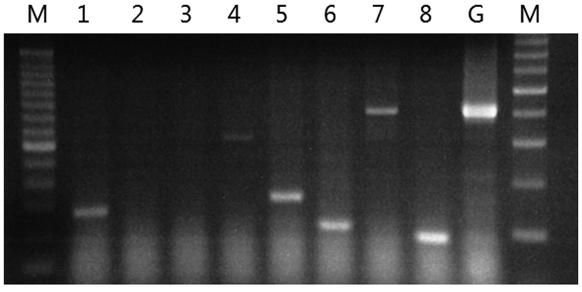


Fig. 7 Stimulus-induced peroxidase release from lacrimal gland acinar cells. (**a**) Dose-dependent effects of NA on peroxidase release from lacrimal gland acinar cells. Cells were stimulated with the indicated concentrations of NA for 60 min. (**b**) Time-course of peroxidase release from NA-stimulated lacrimal gland acinar cells. The cells were stimulated with or without 400 μM NA for the indicated periods. (**c**) Effects of α1- and β-adrenergic agonists on peroxidase release from lacrimal gland acinar cells. The cells were stimulated with 200 μM phenylephrine (Phe), 200 μM isoproterenol (Iso), A23187 (A23187), or buffer (basal) for 60 min. In (a)–(c), peroxidase activity in the supernatants was measured as described in "Materials and Methods." The data shown in (a)–(c) are the mean ± SD of three independent determinations. $^*P < 0.05$, as determined by Student's *t* test compared to cells without stimulation.

corneal surface is moist at all times. If lacrimation is competitively controlled by cholinergic and adrenergic nerves, tear secretion might be stopped because of a hormonal imbalance. As a result, dry eye syndrome will occur.

We recently reported that Ca^{2+} mobilization from intracellular Ca^{2+} stores is induced by extracellular ATP, suggesting the presence of metabotropic receptors that are activated by ATP in rat lacrimal gland acinar cells (40). Purinergic receptors are important signaling molecules that are activated by ATP. These receptors can be divided into 2 main categories: P1 purinoceptors (adenosine receptors) and P2 purinoceptors (ATP receptors) (9). P2 purinoceptors are further grouped into 2 subfamilies: P2X and P2Y. Since P2X purinoceptors are ligand-gated ion channels, they mediate the influx of Ca^{2+}, causing an increase in $[Ca^{2+}]_i$. P2Y purinoceptors are 7-membrane-spanning receptors that are coupled to G-proteins, and their activation results in the mobilization of $[Ca^{2+}]_i$



Lane	1	2	3	4	5	6	7	8
Intensity of bands	rMUC 1	rMUC 2	rMUC 3	rMUC 4	rMUC 5AC	rMUC 5B	rMUC 6	rMUC 16
	+	-	-	+-	++	++	-	++

Fig. 8 RT-PCR analysis of MUC mRNA in rat lacrimal gland acinar cells. MUC1, 4, 5AC, 5B, and 16 are expressed in the cells. There are two types of mucins: secreted and membrane-tethered mucins. MUC5AC and 5B are secreted mucins and MUC1, 4 and 16 are membrane-tethered mucins. G: GAPDH (positive control), M: molecular standards.

from internal Ca^{2+} stores (1, 9, 10, 21, 23, 43). We suggest that under pathological conditions, prolonged stimulation of intact normal cells by ATP that has leaked from injured cells might activate P2X7, resulting in an $[Ca^{2+}]_i$ increase and leakage of various proteins. However, the exocytotic action of nerve endings can result in accumulation of micromolar concentrations of extracellular ATP (26). It is therefore likely that, under physiological conditions, the effect of extracellular ATP on lacrimal glands is mediated by P2Y receptors. Therefore, we hypothesize that under sympathetic nerve stimulation, the lacrimal gland can secrete lacrimal gland fluid via a mechanism involving an IP_3-dependent $[Ca^{2+}]_i$ increase. Direct stimulation of the α1- or β1-receptor by using specific agonists has been shown to induce significant protein secretion from mouse lacrimal glands (20). In contrast, our results showed that the β-adrenoceptor agonist isoproterenol failed to induce an increase in $[Ca^{2+}]_i$ in rat lacrimal glands. However, a Ca^{2+}-independent, cAMP-dependent pathway has been demonstrated to control lacrimal gland protein secretion in rats (12, 46). In our study, stimulation of the acini with α1-adrenergic agonist phenylephrine increased peroxidase secretion, while the β-adrenergic agonist isoproterenol did not. These results suggested that NA mainly stimulates α1-adrenoceptors that induce an increase in $[Ca^{2+}]_i$, which subsequently stimulates protein secretion.

It has been shown that PKC isoforms differentially control lacrimal gland protein secretion and cholinergic-induced Ca^{2+} elevation (74). In Madin-Darby canine kidney (MDCK-D1) cells, agonists acting on α1-adrenoceptors promote hydrolysis of phosphatidylcholine, resulting in rapid generation of diacylglycerol for activation of PKC (62). Our results showed that a PKC antagonist did not inhibit the NA-induced $[Ca^{2+}]_i$ increase in rat lacrimal gland acinar cells, suggesting that PKC is not involved in α1-adrenoceptor stimulation in these cells. However, future studies will be necessary to evaluate the role of various protein kinases in $[Ca^{2+}]_i$ changes in different tissues/organs. Simultaneous activation of α- and β-adrenoceptors is necessary to produce a maximal secretory response to catecholamines and a synergism may exist between both routes of stimulation, leading to an amount of protein discharge higher than that expected in the case of additive effects (47). We hypothesize that α-adrenergic agonists are more effective stimuli of protein secretion than β-adrenergic agonists; however, further experiments will be needed to corroborate this.

Secretory granules in the lacrimal gland acinar cells contain mucin and other proteins. Lacrimal gland secretory proteins such as lactoferrin, lysozyme, β-hexosaminidase, secretory mucins, and peroxidase are secreted by stimulus-induced exocytosis (14). Early studies have demonstrated that stimulation of muscarinic receptors increased the discharge of granule-stored protein, largely peroxidase, from rat exorbital lacrimal gland (30, 41, 54). Phenylephrine activates an α1-adrenergic signaling pathway to stimulate lacrimal gland protein secretion, especially peroxidase, from acini (17). Mucin secretion from goblet cells occurs through Ca^{2+}-dependent exocytosis (22). Therefore, mucin secretion was quantified indirectly by measuring peroxidase activity in the rat lacrimal gland acinar cells. In our experiments, NA stimulated peroxidase secretion in a Ca^{2+}-dependent manner. This result is consistent with the finding that the α1-adrenergic pathway is active in lacrimal glands.

Recently, it has been shown that mucin mRNA expression and peroxidase activity are correlated in human adenocarcinomic human alveolar basal epithelial cells (A549 cells) (44). MUC5AC is synthesized by goblet cells of the normal human conjunctiva and is a component of conjunctival secretions and normal human tears. To date, at least 5 mucins have been found in the ocular surface epithelium (67). MUC1 and MUC4 are produced by both corneal and conjunctival epithelial cells (34, 64). Alterations of the membrane-spanning mucins are also seen in patients with dry eye. MUC5AC and MUC5B are also localized in the acinar cells. In this case, MUC5AC is not only produced by the goblet cells of excretory ducts but also by some acinar cells of the lacrimal gland (51). PCR analysis indicated that MUC1, MUC4, and MUC5B are expressed in human lacrimal tissue, but no *in situ* hybridization analysis has been performed to confirm their localization (39). In humans, MUC16 is present in the whole lacrimal apparatus (36). In our RT-PCR study, MUC1, MUC4 and MUC16 (membrane-associated mucins), and MUC5AC and 5B (soluble, secreted mucins) were detected in the rat lacrimal gland acinar cells, excluding goblet cells. Our data are almost consistent with these previous studies. It is not surprising that membrane-tethered mucins are expressed in lacrimal glands, since these glycoproteins are present at apical surfaces of epithelia and glands that empty onto surface epithelia. These mucins have also been proposed to be required for maintenance of a patent acinar and ductal lumen, because they prevent the adherence of apical surface (42). In this study, we found some evidences for a relation-

ship between mucin secretion and peroxidase secretion. Methods to assay mucins in normal and diseased eyes are being developed and cell lines of the ocular surface epithelia and lacrimal glands have been developed; the mucin expression in these cells remains to be assessed in future studies.

In conclusion, although previous studies have suggested a minor role for the adrenergic system in lacrimal function, based on our data, we propose that NA, particularly α1 receptor, does play a significant role. These findings will allow further characterization of alterations in the mucins in disease and lead to an understanding of the tissue-specific regulation of mucins by lacrimal gland acinar cells.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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