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Neurologic Evaluation of Acute Lacrimomimetic Effect of Cyclosporine in an Experimental Rabbit Dry Eye Model

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Abstract

PURPOSE—To evaluate neurologically acute lacrimation caused by cyclosporine (CsA) eyedrops in rabbit.

METHODS—Normal adult male New Zealand White rabbits and those that underwent parasympathectomy each received a single instillation of 0.1% CsA or vehicle eyedrops. Schirmer tear test (STT) results, flow rate of lacrimal gland (LG) fluid from the excretory lacrimal duct of the main LG, and blink rate (over a 3-minute period) were measured before and after instillation of CsA or vehicle. Light microscopy was performed to examine the main LG in vitro. Protein release from LG fragments was assessed after incubation with CsA for 30 minutes.

RESULTS—In normal rabbits, the STT value and the flow rate of LG fluid were significantly increased after treatment with CsA compared with vehicle ($P < 0.05$). In contrast, no changes were found in denervated eyes. The blink rate of CsA-treated eyes was significantly higher than that of vehicle- treated eyes in normal rabbits ($P < 0.005$), whereas that of denervated eyes decreased significantly after CsA instillation compared with before administration ($P < 0.005$). Light microscopy showed that the cytoplasm of acinar cells was packed with secretory granules in denervated LG tissue 7 days after parasympathectomy. The same finding was observed 3 hours after CsA instillation. CsA had no stimulatory effect on protein release by acinar cells in LG fragments at all concentrations tested.

CONCLUSIONS—These results suggest that CsA has no direct effect on tear fluid secretion from the LG in an acute model. Instead, CsA increases reflex tear flow.

A topical preparation of cyclosporine (CsA) has been developed to treat eyes while reducing the side effects associated with systemic administration.[1–6] Although the main effect of CsA is immunosuppression, other actions have been reported, such as stimulation of lacrimation and increased conjunctival goblet cell density.[1–16] These effects are beneficial for the treatment of dry eye in patients with autoimmune diseases such as Sjögren syndrome and in patients with non-Sjögren dry eye and keratoconjunctivitis sicca.[7–9,17,18]

A lacrimomimetic effect of CsA eyedrops has also been reported in experimental animal models of dry eye.[19–21] However, little information is available to explain the mechanism

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underlying this action.[7,19–21] We previously reported that lacrimation might be promoted by an effect of CsA on sensory (afferent) and cholinergic (efferent) nerves, and we hypothesized that its lacrimatory activity may result from an increase of reflex tear flow.[20] This hypothesis has also been supported by another group.[21] To clarify the lacrimomimetic mechanism of CsA, we used rabbits with parasympathetic denervation in which the efferent greater superficial petrosal nerve (GSPN) was surgically transected.[22] We examined the tear flow and morphologic changes of the main lacrimal gland (LG) in intact and denervated rabbits after CsA administration. Because there have been no reports of CsA treatment altering protein secretion from the main LG fragments, we also examined the effect of CsA on protein secretion by acinar cells in LG fragments.

MATERIALS AND METHODS

Animals

Twenty-six male adult New Zealand White rabbits (2.0 –3.5 kg) with no evidence of ocular abnormalities were used for this study, which was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fifteen unoperated rabbits formed the normal control group, and 11 rabbits underwent unilateral parasympathetic denervation under deep general anesthesia with intramuscular injection of 100 mg/kg ketamine (Schering-Plough Animal Health, Union, NJ) and 10 mg/kg xylazine (Ben Venue Laboratories, Bedford, OH), as described previously.[22] Contralateral unoperated eyes served as intact controls. After postoperative day 7, animals were subjected to the tear stimulation study.

Chemicals

CsA eyedrops (0.1%) and the vehicle were provided by Santen Pharmaceuticals (Osaka, Japan). We tested 0.1% CsA because that concentration had the maximal effect on lacrimation in our previous study.[20] Vehicle consisted of ethanol, EDTA, sodium chloride, sodium dihydrogen phosphate, polyoxyl 40 stearate, dissolution properties of hydroxypropylmethylcellulose 2906 (which were different from those of prescription eyedrops [Restasis 0.05%; Allergan Inc., Irvine, CA]) glycerin, castor oil, polysorbate 80, carbomer 1342, and sodium hydroxide. Carbamyl choline (carbachol) was purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Procedure

Administration of CsA—Seven normal rabbits and seven rabbits that underwent parasympathectomy were used for this study. Each rabbit received a single instillation (approximately 40 μL) of 0.1% CsA or vehicle. In normal rabbits, one eye was treated with CsA, and the contralateral eye received vehicle. In denervated rabbits, both eyes were treated with CsA eyedrops. The CsA concentration of 0.1% was chosen on the basis of previous reports about tear secretion after a single instillation of CsA.[20,21]

Tear Flow—Schirmer tear test (STT) was performed without topical anesthesia before surgery and 1, 3, 5, 8, and 24 hours after the administration of CsA. The strip was placed between the lower eyelid and the eyeball for 5 minutes, and the length of wetting was measured with a ruler that had 0.5-mm gradations.[20,22]

Tear Fluid Analysis—Five microliters of tear fluid samples were collected from tear meniscus by micropipette in all eyes before and 5 hours after CsA or vehicle administration in the normal control group ($n = 7$). Samples were placed in 0.5-mL microcentrifuge tubes (Eppendorf, Fremont, CA), diluted 100-fold with balanced salt solution, and immediately frozen at −80°C until analysis. Enzyme-linked immunosorbent assay (ELISA) for calcitonin

gene-related peptide (CGRP) was performed with commercial kits (SPIBio, Massay Cedex, France). In accordance with the manufacturer's instructions, color was developed and absorbance was read at 405 nm, after which the concentration of CGRP in each sample was determined from standard curves.

Blink Rate—Blink rate was determined by counting the number of blinks in a 3-minute period 3 hours after eyedrop instillation while the rabbit was secured in a holding bag.[22–24]

Flow Rate of Lacrimal Gland Fluid—Seven days after GSPN transection, four normal and four denervated rabbits were placed under general anesthesia. Flow rate of LG fluid from the main lacrimal duct, which opened into the conjunctival sac, was measured before and 3 hours after the instillation of CsA or vehicle. In normal rabbits, one eye received CsA, and the contralateral eye received vehicle eyedrops. In denervated rabbits, both eyes received CsA eyedrops. The lacrimal duct was cannulated according to the technique previously reported for the collection of LG fluid.[25,26] Fluid was collected in 5-μL glass capillary tubes from the end of the lacrimal duct cannula. The time required to collect each microliter of fluid was then averaged, and flow rate per second was calculated.[22]

Microscopy—Four normal and four denervated rabbits were killed with overdose of sodium pentobarbital after the measurement of tear flow. Another four denervated rabbits were used as an untreated control group. The main LG was removed and immersed in aldehyde fixative (2% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for light microscopy.[27,28] Then 1-μm–thick sections were prepared and were stained with toluidine blue and basic fuschin.

Images were captured with a light microscope (E600, Eclipse; Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (3-CCD, DXC-970 MD; Sony, Tokyo, Japan) controlled by ratio imaging (MetaView; Universal Imaging, West Chester, PA). Color images were converted to grayscale images (Photoshop, version 5; Adobe, San Jose, CA) and were printed on a color printer (8650 PS; Eastman Kodak, Rochester, NY).[28]

Protein Secretion

Protein secretion by LG fragments was investigated in vitro, as described by Bromberg et al.[29] LGs of four normal rabbits were used for this study. First, the glands were cut into 1- to 2-mm^3 fragments, and aliquots were added to 10-mL Erlenmeyer flasks containing balanced salt solution bubbled with O_2 at 37°C. After equilibration for 15 minutes in a shaking water bath with continuous exposure to O_2, the medium was removed and replaced with 2.0 mL fresh medium. After 15 minutes, 500 μL medium was removed to determine baseline protein secretion. Then carbachol (10 nM–100 μM), CsA (10 pM–83 μM), or vehicle was added to the flasks. After 30 minutes, 500 μL medium was removed and was used to assess stimulated protein secretion according to the Bradford method.[30] The amount of protein secretion above baseline was normalized to the tissue wet weight.

Statistical Analysis

Data are shown as the mean ± SEM. Statistical comparisons were made by ANOVA with repeated measures and a least significant difference–planned comparisons test or paired *t*-test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Tear Flow

STT values obtained after the instillation of CsA or vehicle in normal rabbits are shown in Figure 1A. CsA eyedrops significantly increased tear fluid secretion between 3 hours and 5 hours after instillation compared with control ($P < 0.05$). Tear fluid secretion was also increased between 1 hour and 5 hours compared with preinstillation levels in CsA-treated rabbits ($P < 0.005$). Maximal tear flow occurred 3 hours after instillation in CsA-treated eyes (24.71 ± 1.44 mm [mean ± SEM]). The vehicle had no lacrimomimetic effect on the contralateral eye of any rabbit. STT values of denervated rabbits obtained after instillation of CsA eyedrops into both eyes are shown in Figure 1B. Although an increase of tear secretion was observed in the contralateral intact eye, there was no increase in the denervated eye.

Quantification of CGRP in Tear Fluid

CGRP in tears increased significantly, from 91.4 ± 18.1 pM/mL to 294.0 ± 60.3 pM/mL ($P < 0.05$; $n = 7$) 5 hours after CsA administration. In contrast, there were no significant changes 5 hours after vehicle administration (121.3 ± 36.6 pM/mL) compared with before vehicle administration (114.0 ± 30.4 pM/mL).

Blink Rate

In normal rabbits, the blink rate of CsA-treated eyes (4.86 ± 0.86 blinks per 3 minutes) was significantly higher than that of contralateral vehicle-treated eyes (1.71 ± 0.42 blinks per 3 minutes) 3 hours after instillation ($P < 0.005$; Fig. 2A). Contralateral intact eyes of denervated rabbits also showed a significant increase in the blink rate after CsA administration compared with the blink rate before administration ($P < 0.05$). Before CsA administration, the blink rate of denervated eyes was significantly higher than that of contralateral intact eyes ($P < 0.005$; Fig. 2B). However, the blink rate of the denervated eyes showed a significant decrease 3 hours after CsA instillation compared with the rate before administration ($P < 0.005$).

Flow Rate of Lacrimal Gland Fluid

In normal rabbits, the flow rate of LG fluid from the excretory lacrimal duct was significantly increased in CsA-treated eyes (78.37 ± 7.98 nL/s) compared with that before CsA administration (45.94 ± 7.49 nL/s) and was also significantly greater than the rate before (55.94 ± 6.54 nL/s) and after (51.44 ± 1.56 nL/s) administration of the vehicle in the contralateral control eyes ($P < 0.05$; Fig. 3A).

In denervated rabbits, the LG fluid flow rate increased significantly after CsA instillation into the intact eye (100.40 ± 10.18 nL/s) compared with the flow rate before CsA instillation (60.36 ± 7.38 nL/s; $P < 0.005$; Fig. 3B). The LG fluid flow rate of denervated eyes (5.02 ± 1.96 nL/s) was significantly lower than that of the contralateral intact eyes (60.36 ± 7.38 nL/s; $P < 0.005$), and there was no significant increase in flow rate in denervated eyes after CsA instillation (6.38 ± 4.13 nL/s).

Histology

Light microscopy of CsA-treated and vehicle-treated LGs from normal rabbits showed a typical acinar pattern and a heterogeneous mixture of light and electron-dense secretory granules. Figure 4 shows LG tissue samples obtained from the intact side before (Fig. 4A) and 3 hours after (Fig. 4C) topical application of CsA. In contrast, marked changes were observed in denervated LG tissues 7 days after parasympathectomy, as previously reported (Fig. 4B).[22] The cytoplasm of acinar cells was filled with secretory granules, whereas the

nuclei of these cells were flattened and shifted toward the basal region. Similar findings were observed 3 hours after CsA instillation in denervated tissue (Fig. 4D).

Protein Secretion

Dose-response curves for protein release from LG fragments were generated by treatment with 0.1% CsA (10 pM–83 μM) or with carbachol (10 nM–100 μM), which was used as a positive control (Fig. 5). The ED_{50} of carbachol was 509.3 nM, and maximal secretion (27.58 ± 2.45 U/mg protein) was observed at 10 μM. On the other hand, CsA had no stimulatory effect on secretion at any concentration tested compared with the effect of the vehicle. Additionally, preliminary experiments of multiple time points were conducted for treatment with 1 μM CsA at 10 minutes, 30 minutes, 1 hour, and 3 hours. No stimulatory effect on secretion was observed at any time point (data not shown).

DISCUSSION

The present study showed that CsA had a negligible effect on protein release from LG fragments compared with the stimulatory effect of carbachol, a cholinergic agonist.[31–33] Hence, CsA did not directly stimulate LG fluid release from acinar cells. In several studies,[3,17,19] the amount of CsA reached the main LG after topical administration was investigated in rabbits. Although they showed beneficial results, the amount was small compared with the volume used in the present study.

In the present study, a single drop of 0.1% CsA increased lacrimal tear flow in vivo. The tear flow response was the same as previously reported 3 hours after CsA administration, and an ipsilateral increase of flow continued for 8 hours in normal rabbits as measured by the STT.[20,21] Similar results were found when LG fluid flow from the excretory lacrimal duct was measured after topical administration of CsA eyedrops in normal rabbits. This lacrimatory response was too rapid to be attributable to the immunosuppressive action of CsA,[4,5,8,34,35] and it seemed to differ from the typical immunologic and anti-inflammatory actions of this drug. In humans, the suppression of inflammation and immune reactions by CsA occurs slowly. Long-term administration of CsA in humans may reduce chronic inflammation, and it has been reported that inflammation impairs the ability of the LG to secrete and inhibits neural transmission.[36] In contrast, acute inflammation developed in the present rabbit model. Regarding the immediate secretory response to topical CsA in this experimental animal model and the much longer time course of the response in humans, one possible explanation may be a species-specific response, as has been reported for high ocular sensitivity in rabbits and rats.[37]

An irritant effect of CsA has been reported.[34,35,38,39] Solch et al.[40] found that CsA eyedrops caused itching in volunteers during a clinical trial. To ascertain whether an uncomfortable sensation occurred in rabbits, the blink rate was monitored.[41] It increased ipsilaterally after instillation of CsA in normal rabbits (Fig. 2), suggesting that sensory nerves were stimulated by topical CsA. The irritative side effect of CsA eyedrops in humans has also been reported and notified in the interview form of CsA, ophthalmic solution (Papilock Mini 0.1%; Santen Pharmaceutical Company, Osaka, Japan), which was approved only for vernal keratoconjunctivitis in Japan. Ocular irritation was noted in 11.1% of subjects in the present study, and lacrimation was observed in 2.2% of subjects in clinical trials. Based on these data and those of the present study, it can be thought that itching, but not lacrimation, is a common side effect of CsA in humans and rabbits. Furthermore, the reactivity of CsA in humans may be different, depending on the person.

In the present study, CGRP in tear fluid increased significantly after instillation of CsA. A similar result was reported by Mertaniemi et al.[42] after excimer laser keratectomy.

Furthermore, we have previously reported that intraocular pressure was increased at 1 hour after the application of CsA eyedrops and that the blood-aqueous barrier was disrupted with an increase of anterior flare and higher expression of CGRP.[39] These findings suggest an irritant effect of CsA or the induction of neurogenic inflammation. We suggest that the reason for the return of tear flow after its suppression by inflammation is that the irritant effect of CsA may diminish as time passes, as we have reported previously.[20,39] Another possibility is that the administration of CsA may prevent the disruption of acinar structure and promote LG fluid production by acinar cells.[36] On the other hand, the blink rate was decreased in denervated eyes at 3 hours after CsA treatment compared with before CsA administration. This suggests that the depletion of neurotransmitters from sensory nerves[21] might have led to desensitization or anesthesia of the ocular surface. It is also possible that tear film composition and stability were abnormal because of GSPN transection so that the reduced blink rate was associated with an adaptive response of the cornea. In other words, GSPN transection did not reduce the blink rate directly but through a secondary effect.

Still another possibility is that the origin or cause of inflammation induced by a single application of CsA was different from the chronic inflammation associated with dry eye. The former may be neurogenic, and the acute lacrimomimetic effect of CsA eyedrops is caused by increased reflex tear flow. In contrast, the latter may be caused by such factors as autoimmune reaction, environment, increased evaporation, and decreased lacrimation. The latter may also be treated by continuous application of CsA and would enable patients to recover from chronic inflammation associated with dry eye by preventing T-cell activation and epithelial apoptosis and by inhibiting the upregulation of NF-κB. This solution could be more effective than the former and would prove invaluable for patients with chronic inflammation of the ocular surface.

In general, an increase of reflex tear flow occurs after stimulation of the main LG by efferent parasympathetic nerves,[43–45] whereas sympathetic nerves are thought to have no effect on the LG, as shown in a sympathetic denervation study conducted by Meneray et al.[46] They also reported that after sensory denervation, there was no difference in response to adrenergic receptor stimulation between control and sensory denervated glands. In the present rabbit model of dry eye with GSPN efferent nerve transection, there was no significant increase of tear flow after topical administration of CsA and no increase of tear fluid secretion from the excretory lacrimal duct (Fig. 3b). These results suggest that a reflex tearing response is involved in the increase in lacrimation after the application of CsA. Furthermore, in our previous studies, atrophic changes were detected on the ocular surfaces and the LGs after the GSPN was cut,[47,48] and we suggest that continuous neural drive of the pterygopalatine ganglion is necessary to maintain adequate tear flow and mucin secretion.[22] It is likely that the trigeminal system is the afferent origin of this continuous neural tone, and this nerve seemed to be having a trophic effect. A single application of CsA might stimulate it on the intact eye. After GSPN denervation, the drug response might be also eliminated.

Histologic examination of the LG in denervated rabbits after CsA administration showed dense secretory granules in the acinar cells that resembled the denervated LG before CsA treatment.[22] These findings suggest that the mechanism for the release of secretory granules might not have been functioning and that the efferent pathway through the GSPN was necessary to promote lacrimation by CsA. The accessory and main LGs are functionally similar, as Hunt et al.[49] report, and these glands are also thought to be structurally and neurologically similar.[50,51] Accordingly, CsA may not act on the accessory LGs either. Chronic inflammation may interfere with neurally mediated secretion by the main and the accessory LGs.[39]

Previous reports have indicated that patients experience itching when they start treatment with CsA eyedrops but that the itching gradually decreases,[38,44] possibly because patients develop tolerance. Although the stimulation of sensory nerves may cause an uncomfortable sensation, CsA eyedrops should still be used if the benefits outweigh the side effects. It has been reported that cyclophilin, a cyclosporine-binding protein, is expressed in the eye, and it is possible that cyclophilin may be involved in the ocular response to CsA.[52,53] There may also be a difference in the reaction to CsA between rabbits and humans because rabbits seem to show higher ocular sensitivity to irritant substances.[37] Furthermore, the vehicle used to deliver CSA in this study was different from that in prescription eyedrops (Restasis 0.05%; Allergan Inc.). Although that vehicle had no effect on lacrimation in our previous study,[20] the present vehicle might have caused indirect stimulation when combined with CsA.

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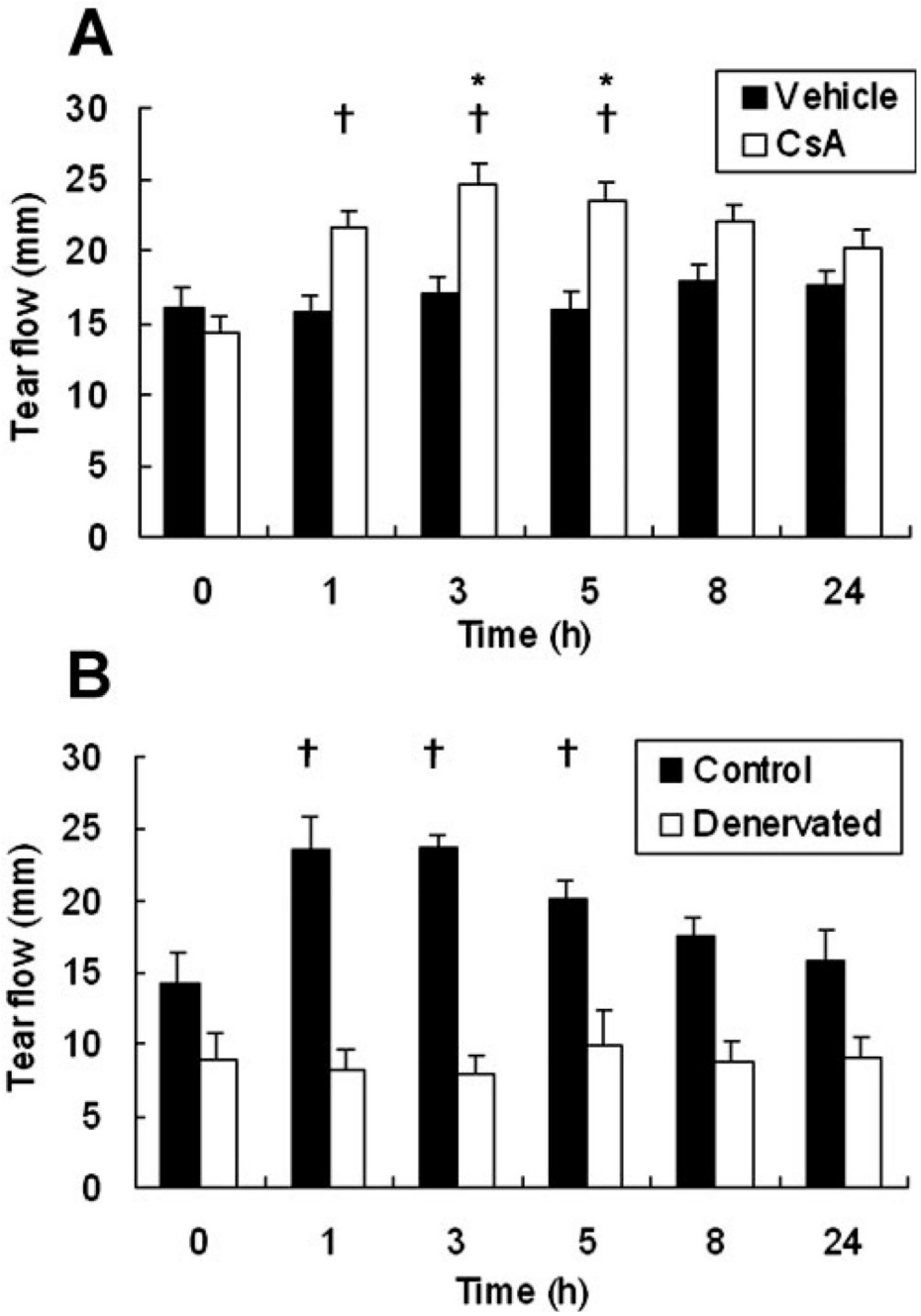

FIGURE 1.

Tear flow measured by the STT in both eyes before and after topical administration of CsA or vehicle in (**A**) normal rabbits ($n = 7$) and (**B**) denervated rabbits ($n = 7$). (**A**) Tear flow reached a maximum 3 hours after CsA treatment and then gradually returned to baseline. There were significant differences at 3 and 5 hours after CsA treatment compared with the control side (*$P < 0.05$). Tear flow also increased between 1 hour and 5 hours compared with flow before instillation in CsA-treated eyes (†$P < 0.005$). No difference was found in the contralateral vehicle-treated eyes. (**B**) Similar changes seen in the CsA-treated eyes of normal rabbits were exhibited by the CsA-treated contralateral intact eyes of denervated

rabbits compared with before instillation (†$P < 0.005$), whereas no significant increase of tear flow occurred in denervated eyes.

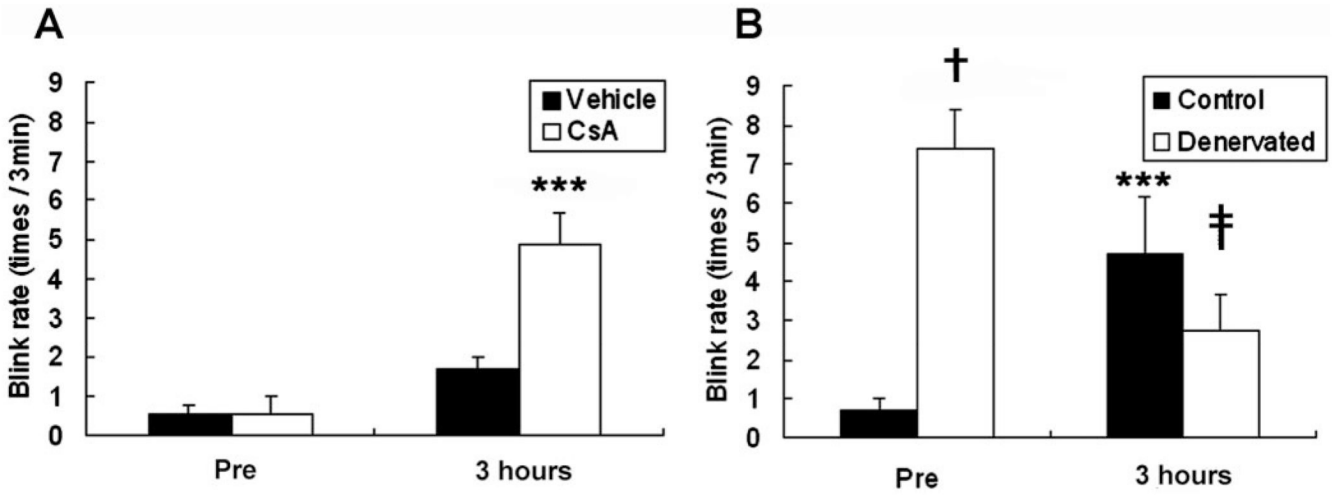

FIGURE 2.

Blink rate at 3 hours after topical administration of CsA or vehicle in (**A**) normal rabbits (n = 7) and (**B**) denervated rabbits (n = 7). (**A**) The blink rate was significantly increased in CsA-treated eyes after vehicle administration and was significantly greater than in the contralateral eyes before and after vehicle administration (***$P < 0.005$). (**B**) Intact contralateral eyes showed an increase at 3 hours after CsA administration compared with before CsA treatment (***$P < 0.005$). In the denervated eyes, the blink rate before CsA administration was significantly higher than in contralateral vehicle-treated eyes (†$P < 0.005$). The blink rate of denervated eyes showed a significant decrease at 3 hours after CsA treatment compared with the rate before CsA administration (‡$P < 0.005$).

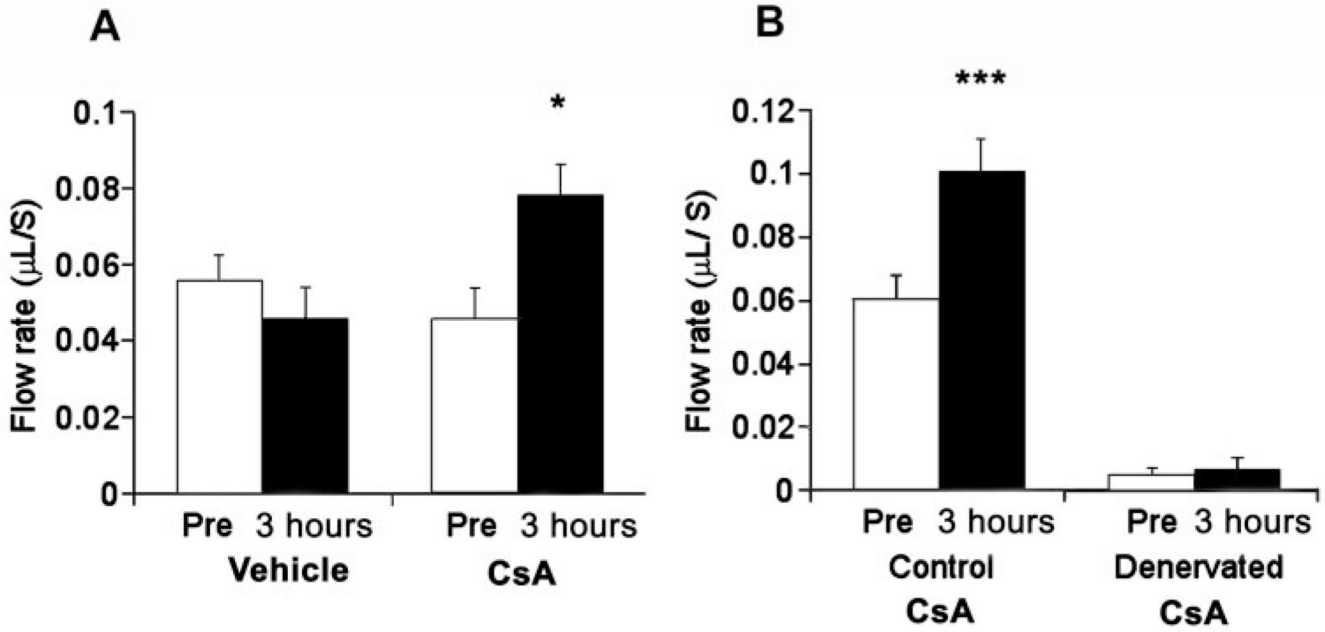

FIGURE 3.

Flow rate of LG fluid from the main lacrimal duct before and 3 hours after topical administration of CsA or vehicle to one eye each in (**A**) normal rabbits ($n = 4$) and (**B**) denervated rabbits that received CsA in both eyes ($n = 4$). (**A**) Flow rate was significantly increased in CsA-treated eyes compared with before administration and before and after vehicle administration in the contralateral eyes of normal rabbits (*$P < 0.05$). (**B**) Similar changes were seen in the contralateral intact eyes 3 hours after CsA administration compared with before CsA treatment (***$P < 0.005$). In the denervated eyes, tear flow was very low before and after administration of CsA.

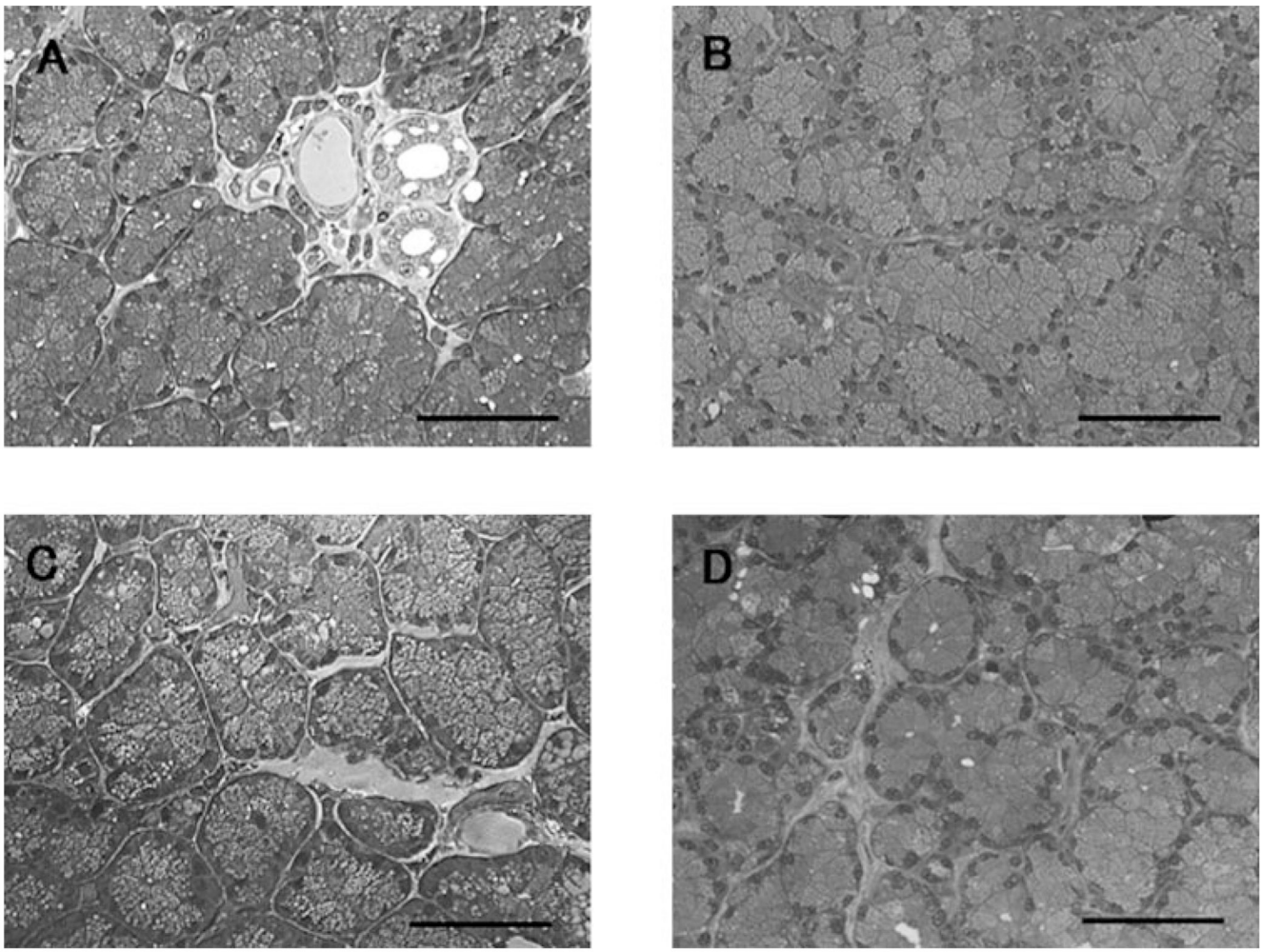

FIGURE 4.
Light micrographs showing acini of the main LG from the contralateral control intact side (**A**) and the denervated side (**B**) before CsA administration and from the contralateral side after CsA treatment (**C**) and the denervated side after CsA treatment.(**D**). Scale bars, 50 μm.

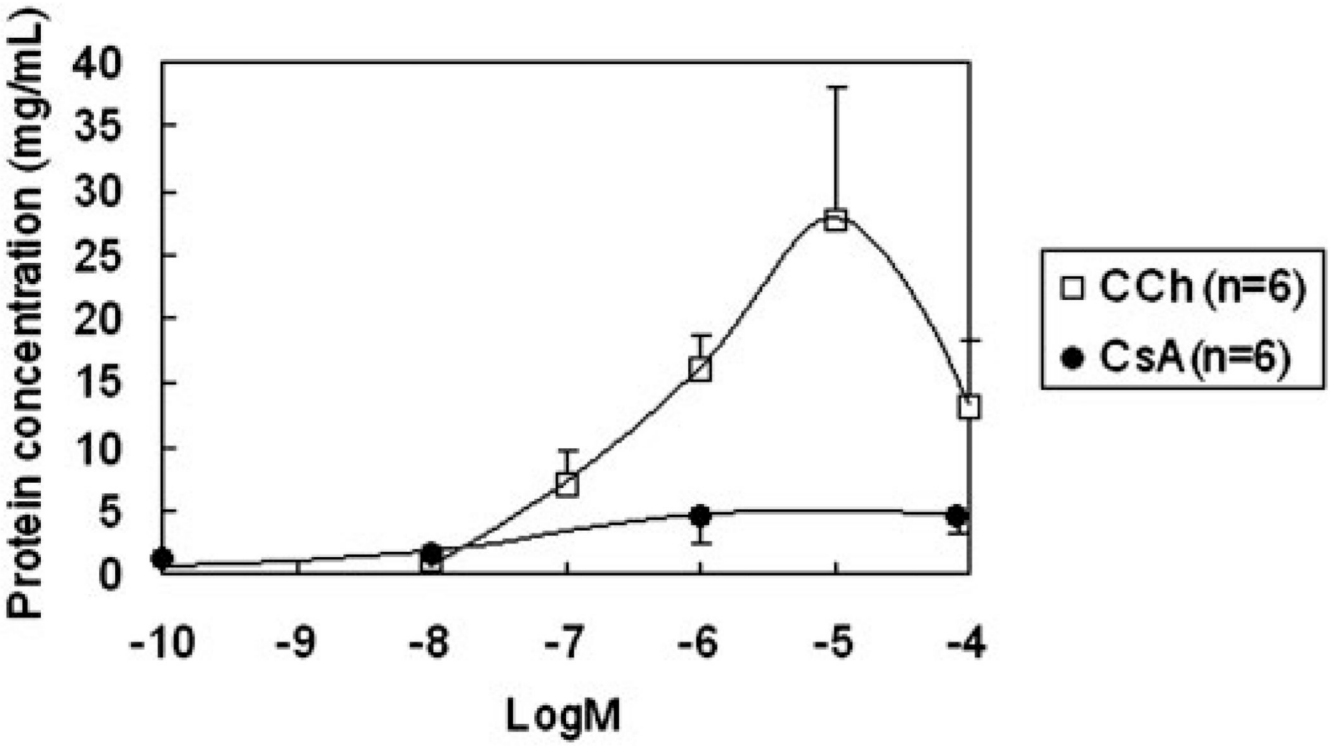

FIGURE 5.
Dose-response curve for protein secretion by main LG fragments in response to carbachol (CCh) (□) and CsA (○).