Calcitonin Gene-Related Peptide Immunoreactive Nerve Fibers in the Rat Conjunctiva

Jussi Luhtala, Arto Palkama, and Hannu Uusitalo

Calcitonin gene-related peptide (CGRP) immunoreactive nerve fibers were studied in the rat conjunctiva by using indirect immunohistochemistry. Their origin was evaluated in a series of experiments where the animals were denervated by electrocoagulating the two first branches of the trigeminal nerve or by surgically extirpating the superior cervical ganglion. The CGRP-immunoreactive nerve fibers were seen mainly as thin varicose fibers in the epithelium and in the stroma. Many of the stromal fibers showed no apparent destination. However, CGRP-immunoreactive fibers were commonly found in association with stromal blood vessels, the smooth muscle of Müller, and the meibomian glands. Approximately 40% of the ganglion cells in the trigeminal ganglion were immunoreactive to CGRP. In the superior cervical ganglion, a few CGRP-immunoreactive fibers were seen although the ganglion cells were negative. After trigeminal denervation, all the epithelial and most of the stromal CGRP-immunoreactive nerve fibers disappeared. Sympathectomy had no effect on the presence of the CGRP-immunoreactive fibers. These observations indicate that most of the CGRP-immunoreactive nerve fibers in the rat conjunctiva are sensory nerves originating in the trigeminal ganglion. A few of the demonstrated fibers are, however, resistant to the sensory denervation and may be parasympathetic in their origin. Invest Ophthal Vis Sci 32:640–645, 1991

Since the introduction of immunohistochemical methods, innervation of various ocular tissues has been widely studied.1-5 Very little is known, however, on the innervation of the conjunctiva, and virtually nothing has been published about neuropeptide immunohistochemistry in this tissue. Based on earlier studies, it is evident that the conjunctiva receives parasympathetic, sympathetic, and sensory innervation originating from pterygopalatine, superior cervical, and trigeminal ganglion, respectively.6 Catecholamine-containing and acetylcholinesterase-positive nerve fibers have previously been demonstrated in the conjunctiva.7

The current immunochemical data states that primary sensory neurons contain various neuropeptides, eg, substance P, cholecystokinin, neurokinin A, and calcitonin gene-related peptide (CGRP).5-11 The CGRP is a 37-amino acid peptide encoded in the same gene as calcitonin.11 It is a putative neurotransmitter and predominantly expressed in the neural tissue.12 In the peripheral nervous system CGRP localizes mainly in sensory and motor nerves.13 Neurotransmitters in the sensory nervous system may be involved in acute inflammatory conditions in various tissues, like bronchial asthma,14,15 cutaneous disease,16 and ocular inflammations.17 Therefore the sensory nerves also might play a role in inflammatory states in the conjunctiva.

The present study was aimed to show the presence and location of CGRP-immunoreactive nerve fibers in the rat conjunctiva. The origin of these fibers was studied with sympathetic and sensory denervations.

Materials and Methods

Wistar Hannover albino rats of both sexes weighing 180–300 g were kept in a constant dark-light cycle and allowed water and food pellets ad libitum. All the experimental procedures in this study were conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

Denervation Experiments

Of the 16 rats used, four were intact; five, unilaterally sensory denervated; and five, unilaterally and two, bilaterally sympathectomized. The animals were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg of body weight; Mebunat, Orion, Finland). Sympathectomies were done by surgically extirpating the right (and left) superior cervical ganglion 12–25 days before fixation. Sensory denervations were done by electrocoagulating the ophthalmic and maxillary branches of the trigeminal ganglion.
through craniotomy under visual control 6–12 days before further processing. The site of the coagulation was verified at autopsy, and the loss of the corneal blink reflex confirmed the success of the operation.

Immunohistochemistry

The rats were anesthetized with sodium pentobarbital and perfusion fixed through the left heart ventricle first with saline and then with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The eyes were dissected out en bloc with both lids and immersion fixed in the same fixative for an additional 2 hr at 4°C. After fixation the tissues were soaked overnight at 4°C in 0.1 M phosphate buffer containing 30% sucrose.

Cryostat sections (8–20 μm) were mounted on chrome-alum-gelatin-coated glass slides, air dried for 60 min at 20°C, and incubated with 0.1% Triton-X in 0.05 M phosphate-buffered saline, pH 7.2 (Triton-PBS) containing 5% normal swine serum for 2 hr at 4°C. This and all other incubations were done in humid chambers. Primary polyclonal antisera to CGRP (RPN 1842; Amersham, UK) was diluted 1:1000 in Triton-PBS and incubated at 4°C for 24–48 hr. The sections were rinsed three times (each for 20 min) with Triton-PBS and incubated at 20°C for 60 min with biotinylated antisera to rabbit immunoglobulin G (RPN 1004; Amersham, UK) diluted 1:100 in PBS. After three 20-min washings with PBS, 1:100 fluorescein-conjugated streptavidin (RPN 1232; Amersham, UK) in PBS was incubated at 20°C for 15 min. The sections were rinsed with PBS three times (each for 20 min). Then glycerin-PBS (3:1) mixture was applied on the glasses, and they were coated with cover slides. The specimens were viewed using a Leitz Orthoplan or Aristoplan fluorescence microscope (Ernst Leitz Wetzlar, Wetzlar, Germany) equipped with an epi-fluor microscope (Ernst Leitz Wetzlar, Wetzlar, Germany) with a specific filter block 12. Photographs were taken with an automatic Leitz Ortho-Variomat microscope camera on Kodak T-max film (Rochester, NY).

Controls

Histochemical control experiments included omission of the primary antiserum, replacement of the primary antiserum with normal rabbit serum, and absorption of the primary antiserum with 1 µM CGRP (Peninsula, Belmont, CA). All these controls were negative, confirming the immunohistochemical specificity of our results.

Results

The CGRP-immunoreactive nerve fibers were seen mainly as thin varicose fluorescent fibers in the epithelium and substantia propria. Some thicker CGRP-immunoreactive nerve bundles were also detected in the stromal part. There was no apparent difference in either the number or distribution of the CGRP-immunoreactive nerve fibers between the upper and lower lids.

Palpebral Conjunctiva

The palpebral epithelium showed a moderate number of CGRP-immunoreactive fibers and free nerve endings. About 90% of all detected epithelial nerve fibers were located in the palpebral conjunctiva, their number seeming to increase toward the mucocutaneous junction (Fig. 1). The stromal part contained an abundance of CGRP-immunoreactive nerve fibers, and most of them had no apparent destination. Many thin fibers were seen following the course of the smooth muscle of Müller (Fig. 2). The stromal blood vessels regularly received CGRP-immunoreactive nerve fibers (Fig. 3). Occasional fibers were detected running between the acini of meibomian glands (Fig. 4).

Fornical Conjunctiva

The Müller’s muscle was loosely surrounded by single, thin CGRP-immunoreactive nerve fibers and thicker bundles (Fig. 5). Many CGRP-immunoreactive nerve fibers were observed around the stromal blood vessels and also running as free fibers with no clear destination. When distinguishable, the lymphoid layer had fewer nerve fibers than the deeper fibrous layer of the conjunctiva (Fig. 6). Epithelial CGRP-immunoreactive nerve fibers were nearly absent from the goblet cell-rich fornices.

Bulbar Conjunctiva

Both the total number and density of the CGRP-immunoreactive nerve fibers were lower in bulbar than palpebral conjunctiva. Nevertheless, nerve fibers immunoreactive to CGRP were found in the substantia propria, mainly associated with blood vessels (Fig. 7). The episcleral veins were regularly surrounded by these fibers (Fig. 8). A few epithelial free nerve endings were detected in the bulbar conjunctiva (Fig. 9).

Denervation Experiments

Most of the CGRP-immunoreactive nerve fibers disappeared after the unilateral coagulation of the two first branches of the trigeminal nerve (Fig. 10). No epithelial nerve fibers were detected in the sensorily denervated specimens. The meibomian glands also lost CGRP-immunoreactive nerve fibers after
Figs. 1-6. Fig. 1. CGRP-immunoreactive free nerve endings in the epithelium (EP) of palpebral conjunctiva. STR, palpebral stroma; MG, meibomian gland; CE, corneal epithelium. Fig. 2. Immunofluorescent fibers running along the smooth muscle of Müller (MM) in the palpebral conjunctiva. The cells (arrowheads) were nonspecifically stained. Fig. 3. Blood vessels (v) in the palpebral stroma surrounded by CGRP-immunoreactive fibers. Fig. 4. Two immunofluorescent nerve fibers (arrows) located close to meibomian gland (MG). Fig. 5. The Müller's muscle (MM) and a blood vessel (v) associated with immunoreactive fibers in the fornical stroma. Note the absence of CGRP-immunoreactive fibers in the epithelium (EP). Fig. 6. An immunofluorescent nerve fiber (arrow) in the fibrous layer (FL) of the fornix. The cells (arrowheads) in the lymphoid layer (LL) were nonspecifically stained. Bars represent 50 μm.
Figs. 7–12. Fig. 7. A stromal blood vessel (v) surrounded by immunofluorescent fibers in the bulbar conjunctiva. EP, epithelium; STR, stroma. Fig. 8. CGRP-immunoreactive fibers around episcleral veins (EV). SCL, sclera. Fig. 9. Immunofluorescent fibers in the bulbar epithelium (EP). The stromal fibers (arrow) are associated with a blood vessel. Fig. 10. No CGRP-immunoreactive fibers visible in the palpebral conjunctiva after sensory denervation. EP, epithelium; STR, stroma; MG, meibomian gland. Fig. 11. Two CGRP-immunoreactive fibers in the bulbar stroma (STR) of a sensorily denervated rat. EP, epithelium. Fig. 12. Epithelial and stromal CGRP-immunoreactive fibers in the palpebral conjunctiva of a bilaterally sympathectomized rat. EP, epithelium; MG, meibomian gland. Bars represent 50 μm.
FIGS. 13, 14. Fig. 13. Cell bodies and nerve fibers immunoreactive to CGRP in the rat trigeminal ganglion. Fig. 14. Immunofluorescent nerve fibers in the superior cervical ganglion. No CGRP-immunoreactive ganglion cells are detectable. Bars represent 50 μm.

sensory denervation. However, a modest number of CGRP-immunoreactive fibers could be seen throughout the substantia propria, their number being highest in the bulbar and fornal conjunctiva and decreasing toward the lid margins (Fig. 11).

Sympathectomies had no significant effect on the number or distribution of CGRP-immunoreactive nerve fibers (Fig. 12).

**Trigeminal Ganglion**

The trigeminal ganglion was strongly immunoreactive to CGRP, this immunoreactivity being principally located in the small-sized ganglion cells and thin, varicose fluorescent fibers located in the ophthalmic and maxillary branches of the trigeminal nerve and between ganglion cells (Fig. 13). It was estimated that up to 40% of all ganglion cells in the trigeminal ganglion displayed CGRP immunoreactivity.

**Superior Cervical Ganglion**

Neither the principal nor the small intensely fluorescent nerve cells in the superior cervical ganglion were immunoreactive to CGRP. A few thin CGRP-immunoreactive nerve fibers were detected between the ganglion cells (Fig. 14).

**Discussion**

In this study CGRP-immunoreactive nerve fibers were found in the rat conjunctiva in the following locations: in the epithelium, associated with the smooth muscle (of Müller), around blood vessels, and near the meibomian glands. Moreover many nerve fibers did not have any particular destination. A similar distribution pattern was found in the rat urinary tract and genitalia. About 40% of all the ganglion cells in the trigeminal ganglion were immunoreactive to CGRP, which agrees with previous results.

The disappearance of all the epithelial fibers and the remarkable decrease in the number of the stromal CGRP-immunoreactive nerve fibers after the coagulation of the ophthalmic and maxillary nerves suggests that most of the CGRP-immunoreactive fibers in the conjunctiva are sensory in nature and originate in the trigeminal ganglion. The presence of CGRP in sensory nerves and ganglia has been widely established in earlier studies.

Interestingly a small portion of the CGRP-immunoreactive nerve fibers persisted in the substantia propria after sensory denervation. Because sympathectomy did not affect the number or distribution of the CGRP-immunoreactive nerve fibers, it is possible that the nontrigeminal CGRP-immunoreactive fibers could be parasympathetic. The pterygoplatine ganglion is known to contain a small amount of CGRP-immunoreactive neurons. Recently ten Tusscher with his co-workers showed that injection of wheat germ agglutinin coupled with horseradish peroxidase into the rat conjunctiva results in a labeling of neuronal cell bodies in the pterygoplatine ganglion (Vrensen GFJM, personal communication). Additionally the extirpation of the pterygoplatine ganglion causes changes in the nerves of the monkey conjunctiva. The exact origin of the nontrigeminal CGRP-immunoreactive nerve fibers in the conjunctiva remains to be determined.

The physiologic role of CGRP in the function of the conjunctiva is unknown. It has been suggested that CGRP together with substance P acts as a mediator of neurogenic inflammatory response. In several tissues, including the eye, CGRP has been shown...
to be a potent vasodilator, and furthermore it increases the permeability of blood vessels. Due to their anatomic location the sensory epithelial CGRP-immunoreactive free nerve endings might be involved in neurogenic inflammation in the conjunctiva. Our observations of CGRP-immunoreactive nerve fibers near conjunctival blood vessels further support the idea of CGRP-containing nerve fibers as a part of this local inflammatory mechanism. Further experiments are needed to clarify the pathophysiologic effects of CGRP in the conjunctiva.

Key words: CGRP, innervation, immunohistochemistry, conjunctiva, trigeminal ganglion, superior cervical ganglion, rat

Acknowledgments

The authors thank Mrs. Paula Hasenson for her skillful technical assistance and Mr. Reijo Karpipinen for producing the photographs.

References