Neurokinin A Is a Main Constituent of Sensory Neurons Innervating the Anterior Segment of the Eye

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Purpose. To study the innervation pattern of the anterior segment of the eye by neurokinin (NK)-A–immunoreactive nerves and to determine their sensory origin.

Methods. The presence and distribution of NKA was examined in human eyes by radioimmunoassay and immunofluorescence. The source of nerves was determined by measuring the concentration of NKA in the trigeminal ganglion (TG) in comparison with that of the classic sensory peptides substance P (SP) and calcitonin gene-related peptide (CGRP) and in eye tissues in capsaicin-pretreated rats versus control subjects. The NKA-like immunoreactivities were further characterized by reversed phase HPLC in the rat TG and the human iris–ciliary body complex. The presence of γ-PPT-A mRNA was studied in the rat TG by in situ hybridization.

Results. The levels of NKA in human eye tissues were approximately 10 times higher than those of SP but lower than those of CGRP. Nerve fibers were visualized in the cornea, the trabecular meshwork, the iridial stroma, and, prominently, in the sphincter muscle, the ciliary body stroma and muscle and processes, and the choroidal stroma and surrounding blood vessels. In the rat TG, the concentration of NKA was approximately five times higher than that of SP. Capsaicin led to a >60% decrease of the concentration of the peptide in the rat TG and rat eye tissues except for the retina. NKA-like immunoreactivities were present in a single peak corresponding to synthetic NKA, both in the rat TG and in the human iris–ciliary body complex, and numerous ganglion cells of small size were labeled by a γ-PPT-A probe in the rat TG.

Conclusions. The present results clearly demonstrate that NKA is a main constituent of sensory neurons innervating the anterior segment of the eye. The presence of the peptide in C fibers in ocular tissues indicates a participation in sensory transmission and an involvement in the irritative response in the eye, a model for neurogenic inflammation in lower mammals. (*Invest Ophthalmol Vis Sci.* 2005;46:268–274) DOI:10.1167/iovs.04-0608

Neurokinin (NK)-A, a 10-amino-acid neuropeptide, belongs to the group of tachykinins that share the common C-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2[1] and that have a variety of similar biological effects. The best known tachykinin is the undecapeptide substance P (SP), which is a well-known constituent of sensory nerves (for review, see Ref. 2). For a long time, SP was thought to be the only tachykinin but so far, more than 40 tachykinins have been isolated from invertebrate (insects, worms, and molluscs), protochordate, and vertebrate (skin, gastrointestinal tract, peripheral, and central nervous system) tissues.[3] Three main tachykinins have been identified in mammals, apart from SP: NKA; neuropeptide K (NPK), an N-terminally extended form of NKA; and NKB.[4–7]

SP and NKA are expressed by the same gene, which gives rise to α-, β- or γ-preprotachykinin (PPT)-A as a result of alternative splicing of a common transcription product. SP alone is present in the α-PPT-A sequence, whereas β- and γ-PPT-A contain both SP and NKA. In contrast, NKB is expressed by a second gene that gives rise to PPT-B. PPT-A and -B are structurally very similar, suggesting evolution from a common ancestor gene.[8–11] In accordance with the existence of a common precursor for SP and NKA, NKA is known to coexist with SP in sensory neurons in a large number of tissues.[12,13] The tachykinins exert their effects by interacting with specific receptors termed the NK receptors. There have been three NK-receptors identified: the NK-1 receptor having the highest affinity for SP, the NK-2 receptor having the highest affinity for NKA, and the NK-3 receptor with the highest affinity for NKB (for review, see Ref. 14).

With respect to the eye, SP has been extensively studied. In the anterior segment, it represents a classic sensory neuropeptide that features a distinct innervation pattern (for review, see Ref. 15). There is little knowledge about the two other mammalian tachykinins NKA and -B. High levels of NKA were reported in the porcine retina indicating participation in visual processing.[16] Furthermore, NKA innervates the rabbit iris,[17,18] and it has been detected by immunohistochemistry in the rat trigeminal ganglion (TG) supporting the idea that it also may be a sensory peptide for cranial tissues.[19] Detailed studies on the innervation pattern of the anterior segment of the eye have not been performed, and experimental evidence is lacking about whether this peptide indeed contributes to the sensory innervation of the eye.

In the present study, we investigated the innervation pattern of the anterior segment of the eye by NKA. We evaluated the presence and distribution of the peptide in human eyes in detail and investigated the source of nerves, by using animal experiments. The common precursor for SP and NKA and the detection of NKA in the rat TG[19] indicate a sensory origin of NKA-positive ocular nerves, which should be confirmed by this study. Capsaicin is an ideal tool to characterize sensory neu-

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Supported by Grant P14022-Med from the Austrian Science Foundation (JT).

Submitted for publication May 27, 2004; revised July 14, 2004; accepted July 26, 2004.

Disclosure: **E. Schmid**, None; **J. Leierer**, None; **A. Doblinger**, None; **A. Laslop**, None; **R. Fischer-Colbrie**, None; **C. Humpel**, None; **E. Theodorsson**, None; **B. Teuchner**, None; **D. Lalehabbasi**, None; **E. Dragositz**, None; **C. Kunze**, None; **W. Philipp**, None; **W. Göttinger**, None; **J. Troger**, None

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rons, because it is known to provoke selectively the loss of 50% to 90% of these neurons when injected into newborn rats, in particular small neurons giving rise to unmyelinated C-fibers.[20–22] The levels of NKA, SP, and calcitonin gene-related peptide (CGRP) were measured in the rat TG and in human eye tissues in a comparative manner. The expression of γ-PPT-A mRNA was studied in the rat TG by in situ hybridization.

Materials and Methods

Capsaicin Treatment

Newborn Sprague-Dawley rats were injected at the first day after birth with a single dosage of 50 mg/kg capsaicin (Sigma-Aldrich, Vienna, Austria), which was dissolved in saline containing 10% ethanol and 10% Tween-80. Untreated rats served as control subjects. The injection was performed subcutaneously, under the neck fold. The animals were housed in cages with a dark–light cycle of 12 hours each (lights on at 7 AM and off at 7 PM in 23 ± 1°C) and fed a commercial chow and water ad libitum. All experimental and animal care procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the animal experiments were approved by the Ministry of Science in Austria. The animals were allowed to grow, and they were killed by an overdose of CO_2 after 3 months. The eyes then were removed, followed by dissection of the cornea, the iris-ciliary body complex, retina, and choroid-sclera. Furthermore, the TGs were removed, and each tissue was weighed.

Preparation of Human Eyes

Data of four eye donors were evaluated (two men, two women, 52–81 years of age). None of the patients had neurologic disorders before death, and the eyes were enucleated at routine autopsy at a postmortem interval of 5 to 18 hours. None of the eyes had any signs of diseases in the anterior and posterior segment of the eye and were not infectious or pseudophakic. Donor eyes were obtained in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue.

At the beginning, an incision was made at the limbus, and the cornea including the limbus was circumferentially excised. Next, the sclera was gently detached and a piece cut off for determination of the concentration of the peptides. After preparation of the cornea and the sclera, the eyecup was turned around and the choroid was removed, the retina was dissected, and finally the iris-ciliary body complex was excised. Each tissue was weighed before the analytical procedures.

Radioimmunoassay

The RIA was performed as described by us in detail.[23] Briefly, the various specimens and TGs were sonicated in 1 mL of 2 M acetic acid and centrifuged at 3500 rpm for 10 minutes, and the supernatant analyzed for the presence of NKA-like immunoreactivities (LIs), SP-LIs, and CGRP-LIs. The remaining pellet served to measure the protein content by the method of Lowry et al.[24] One hundred microliters of the clear supernatant was used for the SP, CGRP- and NKA RIAs. The RIA was performed with specific antisera: RD2 for SP (a gift from Susan E. Leeman, Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA), RAS6009 for CGRP (Peninsula Laboratories, San Carlos, CA), and K12 for NKA (a gift from Elvar Theodorsson, Department of Clinical Chemistry, University Hospital, Linkoping, Sweden). Nonradioactive peptides were purchased from Peninsula Laboratories and radioactively labeled peptides from Amersham Biosciences (Freiburg, Germany): [^{125}I]-Bolton Hunter-SP; [^{125}I]-iodohistidyl-CGRP; 2-[^{125}I] iodohistidyl^{1}-neurokinin A.

Incubation was performed for 48 hours without and a further 48 hours with the tracers added. Separation of bound and free radioactivity was performed with dextran-coated charcoal. Bound activity was counted by a γ-counter. Under these conditions, the detection limit was 0.1 to 0.2 femtomoles. Differences between control and capsaicin data were calculated with the Mann-Whitney U test.

Reversed Phase HPLC

The antibody K12 used for the measurements by RIA recognizes both NKA and certain other tachykinins, including NKB, neuropeptide K, NKA (3-10), and NKA (4-10). To differentiate between NKA and other immunoreactive peptides, we separated the tissue extracts of the rat TG and the human iris-ciliary body complex by reversed phase HPLC, as described recently.[23] For this purpose, the peptides were extracted by sonication in 2 M acetic acid, the supernatant was lyophilized and reconstituted in 500 μL distilled water. A total of 100 μL was loaded into a reversed phase HPLC column (LiChrospher WP 300 RP-18, 5 μm; Merck, Darmstadt, Germany) and eluted with a gradient ranging from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid-water over 50 minutes at a flow rate of 1 mL/min. Fractions (1.0 mL) were collected, lyophilized, reconstituted in assay buffer, and analyzed for NKA by RIA, as just described. The elution position of NKA was determined in a separate run with synthetic NKA as standard (Peninsula Laboratories).

In Situ Hybridization

For in situ hybridization, the ganglia of three untreated rats were dissected, mounted on optimal cutting temperature (OCT) medium (Tissue-Tec; Sakura, Loeterwoude, The Netherlands), and frozen in 2-methylbutane (−30°C). Serial sections (20 μm) were cut on a cryostat (Microm, Heidelberg, Germany), mounted onto polylysine-coated slides (Menzel-Glazer, Braunschweig, Germany) and stored at −20°C. The sections then were fixed for 10 minutes in 2% paraformaldehyde and rinsed twice in phosphate-buffered saline (PBS) followed by 0.25% acetic anhydride in 0.1 M triethanolamine/0.95% sodium chloride (pH 8) for 10 minutes. Tissues were dehydrated through a series of ethanol dilutions, delipidated in chloroform for 5 minutes, rehydrated, and air dried. The PPT-A oligonucleotide (5′-GCTCCGGCATTGCCTCCTT-GATTTGGTCACTGTCGGACCAGTCGGACC) was 3′ labeled with terminal deoxynucleotidyl transferase (Roche, Mannheim, Germany) and ^{35}S-α-thio-dATP (NEG 034H; NEN, Boston, MA) at 37°C for 30 minutes. Labeled probe (1.5×10^6 cpm) was applied to individual sections in 50 μL of hybridization buffer containing 50% formamide.[25] The sections were placed in humid chambers and incubated for 18 hours at 42°C. Posthybridization washes included four changes at 15-minute intervals of 300 mM sodium chloride/30 mM sodium citrate with 50% formamide at 42°C, preceded and followed by 150 mM sodium chloride/15 mM sodium citrate at room temperature. Finally, slides were rinsed in water and 70% ethanol and left to dry. Dried sections were exposed to ^{3}H-sensitive film (Kodak Biomax MR; Eastman Kodak, Rochester, NY), then dipped in photographic emulsion (NTB2, diluted 1:1 with water; Eastman Kodak) and exposed at 4°C for 14 to 28 days, counterstained with cresyl violet, and coverslipped.

Immunofluorescence

Both eyes of a 75-year-old patient were removed immediately after death caused by bronchial carcinoma and were placed in an ice-cold solution of 4% paraformaldehyde in PBS buffer for 4 hours. The eyes were separated into four parts, and after removal of the lens and vitreous, the tissues were immersed with 4% paraformaldehyde in PBS buffer for another 4 hours. The blocks were placed in a solution containing 20% sucrose in PBS for at least 24 hours and then frozen in cold (−60°C) isopentane and stored at −70°C. Six- to 15-μm-thick sections were cut from the specimens on a cryostat (Reichert Jung; Leica-Reichert, Vienna, Austria) at −20°C and mounted on poly-L-lysine–coated slides. Fixed human eye sections were washed for 1 hour at room temperature in Tris-buffered saline (TBS; 50 mM Tris [pH 7.5] and 0.9% NaCl) with 0.3% Triton X-100. Sections were then preincubated for 1 hour with 20% normal goat serum (NGS) in TBS with 0.3% Triton X-100 in disposable immunostaining chambers (cat. no. 7211013, Shandon Coverplate; Thermo Electron Corp., Woburn, MA) and subsequently incubated for 72 hours at 4°C with the antibody

TABLE 1. Concentrations of NKA, SP and CGRP in Various Human Eye Tissues

	NKA	SP	CGRP
Femtomoles per milligram protein			
Cornea	46.66 ± 10.28	3.35 ± 1.06	93.7 ± 16.42
Iris-ciliary body complex	304.75 ± 41.39	43.19 ± 11.28	423.02 ± 76.09
Retina	1009.98 ± 205.25	185.91 ± 44.39	0.53 ± 0.27
Choroid	151.32 ± 30.54	8.3 ± 4.13	170.45 ± 34.03
Sclera	60.19 ± 9.68	2.24 ± 0.63	262.88 ± 59.49
Femtomoles per milligram wet weight			
Cornea	0.47 ± 0.09	0.032 ± 0.01	0.98 ± 0.17
Iris-ciliary body complex	3.33 ± 0.52	0.47 ± 0.13	4.62 ± 0.88
Retina	9.59 ± 1.92	1.79 ± 0.44	0.003 ± 0.002
Choroid	1.81 ± 0.38	0.10 ± 0.05	2.07 ± 0.47
Sclera	0.50 ± 0.19	0.01 ± 0.003	0.94 ± 0.24

Values are given both as femtomoles per milligram protein and as femtomoles per milligram wet weight and represent the mean ± SEM of results in seven eyes.

SK2 at a dilution of 1:1000 in TBS containing 2% NGS and 0.3% Triton X-100. After three washes with TBS, the sections were incubated with the secondary antibody (Cy3-conjugated goat anti-rabbit IgG; AffiniPure, cat no. 111-165-006; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:2000 for 24 hours at 4°C. Stained sections were washed three times with TBS, mounted with 0.5% gelatin containing 0.05% chrom(III)sulfate, and coverslipped. Sections were visualized with an optical microscope (Axioplan; Carl Zeiss Meditec, Jena, Germany) and micrographs obtained (AxioCam HR camera; Carl Zeiss Meditec). In control experiments, no immunoreactivity was detected with antibodies adsorbed with an excess of NKA (1 μM) or when the primary antibody was omitted.

RESULTS

Concentration of NKA-LIs in Comparison with SP-LIs and CGRP-LIs in Human Eye Tissues

NKA-LI was present in each of the tissues studied (see Table 1). The highest amounts were found in the retina, with 1009.98 ± 205.25 fmol/mg protein and 9.59 ± 1.92 fmol/mg wet weight, but the choroid and the iris-ciliary body complex also contained the peptide in significant amounts, and it was detectable even in the cornea and sclera.

The two other sensory peptides were also found to be present in human eye tissues (see Table 1). For SP, lower levels were measured, averaging approximately one tenth those of NKA, but the ratio between the various eye tissues was similar to that of NKA. On the contrary, CGRP was present at higher levels than NKA in all the tissues studied except the retina, where it was absent.

Immunofluorescence of NKA in the Anterior Segment of the Human Eye

NKA was found to be distinctly distributed throughout the anterior segment of the human eye (Fig. 1). In the cornea, NKA-positive nerve fibers were visualized in the upper and deeper stroma (Fig. 1A) and at the corneoscleral limbus, sometimes in association with blood vessels (not shown). Immunoreactivities were also observed within the trabecular meshwork (Fig. 1B) and around Schlemm's canal (Fig. 1C). In the iris, abundant nerve fibers were present in the stroma (Figs. 1D, 1F) and also surrounding blood vessels (Fig. 1E). An association of immunoreactivities with the dilator muscle (Fig. 1F) and a prominent innervation of the sphincter muscle were seen (Fig. 1G). In the ciliary body, NKA-positive fibers were visualized in the ciliary muscle, most prominently adjacent to the sclera (Fig. 1H), but also at the insertion of the muscle on the scleral spur (Fig. 1B). A dense network was observed in the stroma at the base of the ciliary processes (Fig. 1I) and nerves were also found in the stroma of the ciliary processes (Fig. 1I). Blood vessels again were surrounded by immunoreactive nerves (Fig. 1J). Finally, in the choroid NKA-positive nerves were present in the stroma and in association with blood vessels (Fig. 1K).

Concentration of NKA-LIs in Comparison with SP-LIs and CGRP-LIs in the Rat TG

The concentrations of the peptides in the rat TG are shown in Table 2. NKA-LI was present in significant amounts within the ganglion. The peptide averaged 938.5 ± 85.1 fmol/mg protein and 19.58 ± 2.24 fmol/mg wet weight in untreated animals. The comparison of the levels with those of classic sensory peptides revealed that the concentration of SP-LI was much lower, both when measured as femtomoles per milligram wet weight and as femtomoles per milligram protein, and levels of CGRP-LI were found to be higher. The ratio of the concentration of SP, NKA, and CGRP was approximately 1:5:10.

Capsaicin treatment significantly reduced the levels of each peptide. For NKA, the reduction averaged 75.58% ± 1.42% when given as femtomoles per milligram protein and 60.62% ± 5.12% when given as femtomoles per milligram wet weight. For SP, the decrease was 81.50% ± 2.89% and 71.58% ± 3.20%, respectively, and for CGRP 62.19% ± 4.97% and 47.42% ± 5.53%, respectively.

Concentration of NKA-LIs in Various Rat Eye Tissues

NKA-LIs were found to be present in each of the tissues studied (Table 3). The highest amounts were detected in the retina, but the iris–ciliary body complex and cornea also contained significant amounts. The peptide was detectable even in the choroid-sclera.

Capsaicin pretreatment significantly lowered the levels of NKA in each tissue except the retina. In the iris-ciliary body, a 83.9% ± 6.86% decrease was found, in the cornea the decrease averaged 76.0% ± 6.0%, and in the choroid-sclera it averaged 87.02% ± 5.09%.

Expression of γ-PPT-A mRNA within the TG

γ-PPT-A mRNA was found to be abundant in the rat TG, and the ganglion cells were specifically labeled as evidenced by the presence of silver grains both in the dark- and bright-field images (Fig. 2). In particular, numerous neurons were distinctly labeled by the PPT-A probe and the hybridization signal appeared over the cytoplasmic portion of cells with various diameters, most prominently in small-sized neurons with a

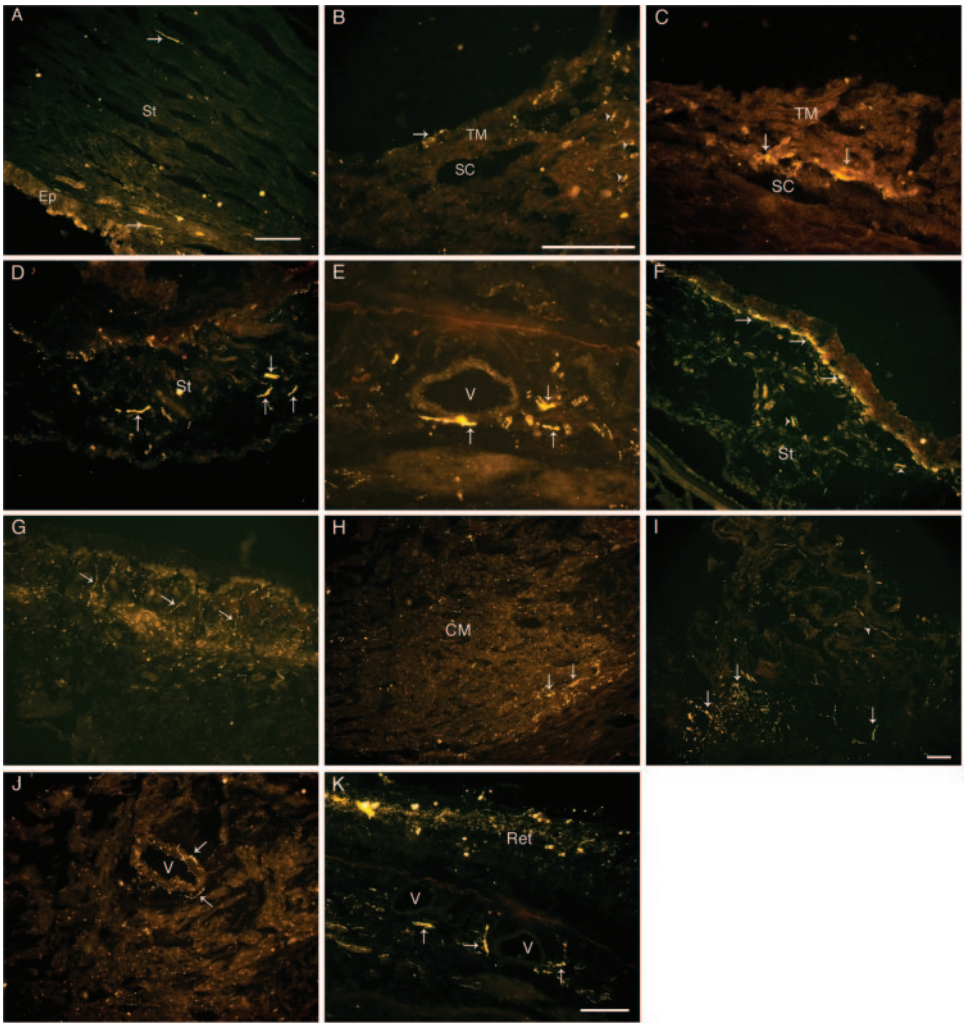

FIGURE 1. Innervation of the anterior segment of the eye by NKA. In the cornea, NKA-immunoreactive nerves were visualized in the upper and deeper stroma (**A**), and immunoreactivities were also observed in the trabecular meshwork (**B**, *arrow*), at the insertion of the ciliary muscle on the scleral spur (**B**, *arrowheads*), and around Schlemm's canal (**C**, *arrows*). Nerve fibers were also present in the iridial stroma (**D**, *arrows*; **F**, *arrowheads*) and surrounding blood vessels (**E**, *arrows*), and a prominent innervation of the sphincter muscle was seen (**G**, *arrows*; *arrowheads*: autofluorescence), whereas a minor association with the dilator muscle was evident (**F**, *arrows*). In the ciliary muscle, NKA-positive nerve fibers were visualized predominantly adjacent to the sclera (**H**, *arrows*) and a dense network was observed in the ciliary body stroma at the base of the ciliary processes (**I**, *arrows*). A few nerves were present in the stroma of the ciliary processes (**I**, *arrowhead*). The innervation of blood vessels was also visible (**J**, *arrows*). In the choroid, nerves were present in the stroma and in association with blood vessels (**K**, *arrows*). St, stroma; Ep, epithelium; TM, trabecular meshwork; SC, Schlemm's canal; V, vessel; CM, ciliary muscle; Ret, retina. Scale bar, 100 μm; in (**A**) it is also valid for (**D**, **F**, **G**, **H**, **J**); in (**K**) for (**I**); and in (**B**) for (**C**, **E**).

diameter of up to 30 μm (Fig. 2). The cells were found to be scattered throughout the ganglion (Fig. 2A). The probe did not label any nerve fibers or non-neuronal cells.

Reversed Phase HPLC

The molecular form of NKA-LI was further analyzed in the rat TG and the human iris-ciliary body complex by reversed phase HPLC (Fig. 3). In the rat TG, one major peak was found in the position of NKA and a negligible one after NKA. In the human iris-ciliary body complex (ICB), a major peak was apparent in the position of NKA and a second minor one thereafter which has not been further characterized. Thus, the results indicate that the quantitative values of NKA obtained by RIA represent authentic NKA.

DISCUSSION

There is unequivocal evidence that NKA is a constituent of capsaicin-sensitive sensory neurons innervating the anterior segment of the eye, since the peptide was present in significant amounts in the rat TG and since capsaicin significantly reduced the levels in the TG and rat eye tissues. It must be emphasized that the levels of NKA are approximately five times higher than those of SP in the rat TG, and SP is known to be a main sensory peptide. There is no doubt that the RIA results are highly predicative, as NKA-LIs (Fig. 2), SP-LIs[26] and CGRP-LIs[26] were further characterized by reversed phase HPLC, which revealed that the concentrations measured by RIA are equivalent to those of the free peptides. The levels of CGRP were twice as

TABLE 2. Concentration of NKA in Comparison with SP and CGRP in the Rat TG

	NKA	SP	CGRP
Femtomoles per milligram protein			
Control subjects	938.5 ± 85.1	228.1 ± 17.2	2473.1 ± 292.9
Capsaicin-treated	229.2 ± 13.3*	42.2 ± 6.6*	935.2 ± 123.0†
Femtomoles per milligram wet weight			
Control subjects	19.58 ± 2.24	4.68 ± 0.29	53.92 ± 6.11
Capsaicin-treated	7.71 ± 1.0*	1.33 ± 0.15*	28.35 ± 2.98†

Data represent the mean ± SEM in controls versus capsaicin-treated specimens ($n = 6$). Differences between control and capsaicin data were calculated with the Mann-Whitney U test (* $P < 0.001$; † $P < 0.01$).

TABLE 3. Concentration of NKA in Various Rat Eye Tissues in Control and Capsaicin-Pretreated Specimens

	NKA (fmol/mg wet weight)	
	Control	**Capsaicin-Treated**
Cornea	7.5 ± 0.74	1.8 ± 0.45*
Iris/ciliary body complex	21.86 ± 1.71	3.52 ± 1.50*
Retina	155.26 ± 57.08	103.54 ± 22.86
Choroid/sclera	3.93 ± 0.58	0.51 ± 0.20*

Data represent the mean ± SEM ($n = 6$). Differences between both groups were calculated by the Mann-Whitney U test (* $P < 0.001$).

high as those of NKA, which is in agreement with the lower number of ganglion cells expressing PPT-A mRNA (10%–20%)[27] versus those expressing CGRP mRNA (40%–50%).[28] In prior studies, in situ hybridization[27] and the results obtained with immunohistochemistry demonstrating that 10% to 20% of the TG cells in the rat contain SP[29] and show equally strong immunoreactivity to both SP and NKA[19] are not in agreement with the levels in our study, in which immunoreactivity of SP was much lower than that of NKA. One has to bear in mind that immunohistochemistry provides a qualitative approach only, which allows no conclusions about the absolute levels. In contrast, SP may be less stable than NKA in microenvironments and thus may be more rapidly degraded. In situ hybridization experiments mostly used γ-PPT-A probes, which contain the message both for SP and NKA, suggesting an overlap in the signal between SP and NKA. Another argument demonstrating the significance of NKA refers to the concentrations of SP and NKA in human eye tissues. As in the rat TG, the concentrations of NKA were much higher there than those of SP. Finally, the CGRP-to-SP ratio has been found to be 8.11 in the rat TG,[30] which is similar to our results. Thus, one can conclude that NKA, at least quantitatively, represents a main constituent of the ganglion, and this peptide may therefore participate in cranial sensory transmission as do SP and CGRP. There are several further neuropeptides present in the TG including cholecystokinin, somatostatin, opioid peptides, galanin, pituitary adenylate cyclase activating polypeptides (PACAPs), and neuropeptide Y (for review, see Ref. 28). Besides CGRP, NKA can now be regarded as the predominant neuropeptide of the ganglion. Chromatographic separation failed to produce a peak in the position of NKB, both in the rat TG and the human iris-ciliary body complex. This indicates that NKB is not a constituent of cranial sensory neurons which is similar to observations made in sensory dorsal root ganglia.[31] It also seems not to be present in the anterior segment of the eye, which is in agreement with results obtained in the rabbit iris-ciliary body.[18]

Capsaicin led to a significant decrease of NKA in the rat TG, ranging higher than 60%, indicating the presence of NKA-LIs in small-diameter neurons which give rise to unmyelinated C fibers.[20–22] The in situ hybridization experiments confirmed these RIA data, as predominantly small-sized neurons contained the signal in the rat TG. Pretreatment with capsaicin also decreased the levels of the peptide in each rat eye tissue by >70%, except for the retina, which confirms the presence of NKA predominantly in unmyelinated C fibers and the contribution of NKA to the sensory innervation of the anterior segment of the eye. We also measured the concentration of the peptide in the superior cervical ganglion and found very low levels there (unpublished observations, February 2004) indicating that NKA does not contribute to the sympathetic innervation of the eye. Participation in the parasympathetic transmission cannot be excluded. However, the data revealed from the RIA experiments and the common precursor for NKA and SP clearly indicate that NKA, like SP and CGRP, represents a constituent exclusively of sensory neurons. The levels of the other peptides also decreased in the TG because of capsaicin treatment, an observation that has already been described for SP[32] and CGRP.[30]

In human eyes, the levels of NKA in each tissue were 10 times higher than those of SP, but lower than those of CGRP. The distribution pattern of NKA in the anterior segment of the eye is similar to that of SP[15] and CGRP,[15] including the intense innervation of the sphincter muscle, and particularly blood vessels in each of the tissues, respectively. This may indicate colocalization with SP or CGRP. The decrease of the peptide in capsaicin-pretreated rat corneal specimens argues for the presence of NKA in the unmyelinated C fiber afferents that transmit information of primarily nociceptors after noxious mechanical, thermal, and/or chemical stimulation.[22] This peptide may therefore participate in pain transmission due to corneal irritation, a function that is proposed for SP.[15] In contrast to SP, NKA was not found to exert trophic effects, particularly regarding the influence on the migration of corneal epithelial cells.[33] In the retina, there are excessively high levels of NKA, and it is important to analyze the immunoreactivities by reversed phase HPLC to find out whether they indeed are attributable to NKA. The high concentration of NKA indicates a main function of this peptide in visual processing. This is consistent with the high levels of NKA in the porcine retina[16] and with the demonstration of a prominent expression of SP and NKA mRNAs in the rat retina.[34] However, the exact distribution of NKA in the retina must be investigated in a further study.

In the human iris-ciliary body complex the abundance of nerve fibers reflects the significant amounts of the peptide there. The prominent innervation of the iris sphincter muscle is of relevance, as NKA has been shown to contract this muscle in the rat,[35] rabbit,[36–41] and pig[42] via activation of NK-1 receptors, at least in the rabbit.[41] Miosis induced by peptides deriving from the TG is well known to occur in response to topical noxious stimulation in lower mammals and has been shown to be mainly mediated by SP (see review, see Ref. 43). C fiber peptides, in particular SP and CGRP[43] but also PACAPs,[44] are released from uveal nerve endings after topical noxious stimulation, and although the release of NKA has not been shown in this response so far, the present results clearly demonstrate that this peptide is also a constituent of the C fiber nerves in the iris-ciliary body that mediate the response, and NKA may therefore be another candidate for the provocation of miosis besides or in cooperation with SP. The irritative response in the eye represents a model for neurogenic inflammation and is mediated by sensory peptides, whereas prosta-

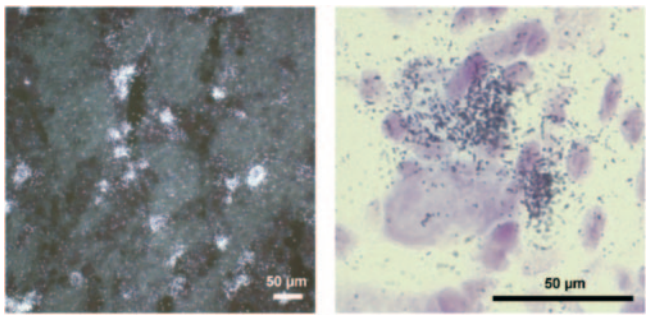

FIGURE 2. Demonstration of the expression of γ-PPTA by in situ hybridization in the rat TG. The cells labeled by the probe appeared typically scattered throughout the ganglion (**A**, dark-field image) and the ganglion cells were mainly small (**B**, bright-field image).

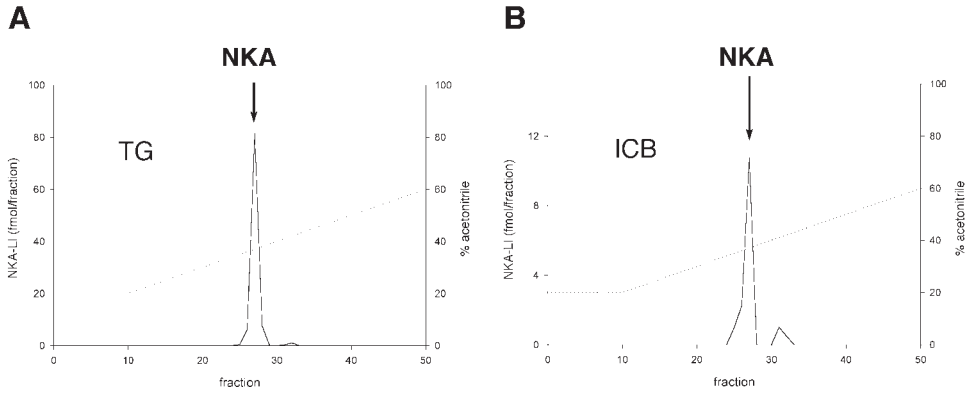

FIGURE 3. Analysis of NKA-LI in the rat TG (TG; *left*) and the human iris-ciliary body (ICB; *right*) by reversed phase HPLC. One hundred microliters of tissue extracts were loaded into a reversed phase HPLC column and eluted with 0.1% trifluoroacetic acid-water. *Dotted line*: gradient profile (percent acetonitrile, right ordinate). One-milliliter-per-minute fractions were collected, lyophilized, reconstituted in assay buffer, and analyzed for NKA by RIA. The elution position of NKA is indicated above the peaks. NKA-LI was found in both tissues in one major peak coeluting with the synthetic peptide, indicating that the quantitative data measured by RIA represent authentic NKA.

glandins act in a modulatory way. It consists not only of miosis but also of uveal vasodilation, breakdown of the blood–aqueous barrier, and an increase in intraocular pressure, and although there are species differences, the vascular effects are thought to be accomplished by CGRP, at least in the rabbit (for review, see Ref. 43). PACAPs mimic the symptoms of inflammation, suggesting that they also take part in the inflammatory response.[45] Furthermore, the involvement of nitric oxide has been evidenced at least in the inflammation induced by electroconvulsive treatment[46] and by intravitreally injected endotoxin,[47] suggesting that nitric oxide activates C fibers and mediates the vascular effects of sensory peptides in the eye.[46,47] NKA is well known to act in a vasoregulatory way and contributes to neurogenic inflammation in the skin, as it constitutes a main messenger for the increase in vascular permeability that takes place in response to sensory nerve stimulation.[48] The notion of participation of NKA in the vascular effects of the irritative response is strengthened by the observation that immunoreactivities are associated with blood vessels in the eye; and, indeed, NKA has been shown to induce a breakdown of the blood–aqueous barrier in the rat,[35] whereas this peptide is inactive in the rabbit.[38,49]

In conclusion, there is unequivocal evidence that NKA represents a constituent of capsaicin-sensitive sensory neurons innervating the anterior segment of the eye. Moreover, this peptide can now be regarded as a main constituent of sensory neurons, at least quantitatively, and it may therefore participate in cranial sensory transmission including the eye, since prominent innervation of the eye is made apparent by this neuropeptide.

Acknowledgments

The authors thank Iris Berger and Hildegunde Knaus for excellent technical assistance.

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