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Release of preprotachykinin-A mRNA from rabbit iris upon C-fibre stimulation

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Abstract

It is usually said that axons and nerve terminals do not contain messenger RNA (mRNA) and that peptide transmitters are packaged in granules and transported towards the periphery of the neuron. However, several recent reports challenge this view by showing evidence of the existence of mRNA in axons. In the present study, we demonstrate the existence of mRNA coding for γ-preprotachykinin-A in rabbit iris by Northern blot analysis and Southern blot analysis of the polymerase chain reaction (PCR)-amplified products. Interestingly, mRNA coding for γ-preprotachykinin-A was detected also in aqueous humor from eyes exposed to injury (infrared irradiation of the iris or retrobulbar injection of the C-fibre excitant capsaicin), but not from contralateral eyes and normal eyes of untreated rabbits. Our results suggest that the mRNA coding for γ-preprotachykinin-A occurs in C-fibres in the iris and that it is released into the aqueous humor together with tachykinins in response to C-fibre stimulation.

Keywords: Preprotachykinin; mRNA; Polymerase chain reaction; C-fiber activation; Release

1. Introduction

Neuropeptides are produced as inactive precursors in the cell body, packaged in granules and transported towards the periphery of the neuron (Schwartz, 1991). During transport the precursor is processed, giving rise to bioactive neuropeptides (Schwartz, 1991). Although the synthetic machinery is believed to be operating in the cell body exclusively, numerous observations describe the presence of neuropeptide mRNA in nervous processes. In mammalian neurons, specific mRNAs can be demonstrated in dentritic projections (Garner et al., 1988; Steward and Banker, 1992; Mohr and Richter, 1993) and recently the presence of mRNA coding for oxytocin was detected in the posterior pituitary (Jirikowski et al., 1990; Levy et al., 1990; Mohr et al., 1990); such mRNA is being transported from the hypothalamus to terminals in the posterior pituitary (Mohr et al., 1991). The physiological significance of mRNA in nerve processes is not understood.

Sensory C-fibres are rich in substance P, neurokinin A and calcitonin gene-related peptide. Substance P and neurokinin A are encoded by the preprotachykinin-A gene. The preprotachykinin-A gene is transcribed to hnRNA which undergoes alternative splicing to give rise to at least four forms of mRNA, of which α- and δ-preprotachykinin-A mRNAs encode for substance P only, while the β- and γ-preprotachykinin-A mRNAs encode for both substance P and neurokinin A (Hiroyuki et al., 1983; Krause et al., 1987; Harmar et al., 1990; Mägert et al., 1993). Sensory neuropeptides, such as substance P, neurokinin A and calcitonin gene-related peptide, are known to be released from C-fibres to play an important role in the ocular response to noxious stimuli (see Wahlestedt et al., 1986; Håkanson and Wang, 1995; Wang et al., 1995). In the present study, we have examined whether the mRNA coding for preprotachykinin-A exists in the rabbit iris and whether such mRNAs, like their corresponding peptides, can be released in response to noxious stimuli. As there is no barrier separating the

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iris and the ciliary body from the anterior chamber, any agent that is being released locally will diffuse into the anterior chamber, making the eye an excellent model for studies of transmitter release.

2. Materials and methods

2.1. Infrared irradiation of the iris or retrobulbar injection of capsaicin

Adult pigmented rabbits (1.5–3 kg) of mixed strains and sex were used. Infrared irradiation of the iris for 2 min is thought to excite C-fibres with the consequent release of C-fibre transmitters, such as substance P and neurokinin A, resulting in a minor and reversible damage of the blood-aqueous barrier (Dyster-Aas and Krakau, 1964; Holmdahl et al., 1981). Only one eye of each rabbit was irradiated, leaving the contralateral eye unchallenged. Capsaicin selectively acts on C-fibres, causing release of C-fibre transmitters (Holzer, 1991). Retrobulbar injection of 0.5 ml of 1% capsaicin solution to one eye was given under pentobarbital anaesthesia (Bynke, 1983). The contralateral eye was given the same volume of the vehicle. Samples of aqueous humor (≈ 200 μl from each eye) were collected from the anterior chamber of the eye when inflammatory responses had reached maximum (1 h after the irradiation and 2 h after capsaicin treatment) (Wang et al., 1995). The samples were frozen on dry ice and stored at -80°C until isolation of RNA or assay for substance P and neurokinin A.

2.2. Northern blot analysis

Total RNA was isolated from the trigeminal ganglion and iris of rabbits, using a modified Acid-Guanidinum-Thiocyanate-Phenol-Chloroform procedure (Monstein et al., 1995). The RNA was quantified spectrophotometrically at 260/280 nm and the integrity of the isolated RNA was examined by electrophoretic separation on a denaturating 1% agarose-formaldehyde gel, followed by staining in ethidium bromide. RNA was blotted onto Hybond-N membranes (Amersham, Amersham, Bucks, UK) and subsequently hybridized with a ^{32}P-labelled bovine preprotachykinin-A cRNA probe (Riboprobe) (Monstein et al., 1992). The preprotachykinin-A cRNA probe was prepared by using 1 μg plasmid DNA (pbSP-p3/307) as template. It was transcribed in vitro by RNA polymerase SP-6 using a Riboprobe kit (Promega, Madison, WI, USA) and 50 μCi [α-^{32}P]UTP (400 Ci/mmol). The bovine preprotachykinin-A template (pbSP-p3/307) was obtained by subcloning a 661 bp Pst DNA fragment from clone pbSP-307 (kindly provided by Dr. S. Nakanishi, Kyoto, Japan) into the transcription vector pGem-3 (Promega).

2.3. Isolation of RNA from aqueous humor

Samples of aqueous humor were collected from eyes exposed to infrared irradiation of the iris or subjected to retrobulbar injection of capsacin as well as from the contralateral eyes and normal eyes of untreated rabbits. RNA was isolated using a commercially available kit for the isolation of RNA from liquid samples (Biotecx, Houston, TX, USA). Briefly, each sample (about 1.2 ml from each of six eyes) was mixed with 10 volumes of Ultraspec-3 reagent (Biotecx) and extracted with chloroform. After centrifugation at $12000 \times g$ for 15 min, the upper aqueous phase was transferred to a fresh tube. Isopropanol and RNA Tack resin (Biotecx) were added to the aqueous phase and mixed. After spinning for 1 min in a bench top minicentrifuge (maximum speed), the supernatant was discarded and the resin was washed twice with 75% ethanol. RNA was eluted from the resin by 50 μl diethylpyrocarbonate-treated water.

2.4. The polymerase chain reaction (PCR) amplification of cDNA using rabbit γ-preprotachykinin-A gene specific primers and rat β-actin gene specific primers

1 μg of RNA isolated from tissues or 10 μl of the RNA extract of aqueous humor were reverse-transcribed into single stranded cDNA by using a cDNA synthesis kit (Clontech, Palo Alto, CA, USA). Rabbit and rat brain cDNAs (Clontech) were used as controls. cDNAs were amplified using 200 pmol of a 27-mer sense up-stream primer II, located at position 1 and of a 27-mer antisense down-stream primer III, located at position 348 of the published rabbit preprotachykinin-A cDNA sequence (Mägert et al., 1993) (Table 1 and Fig. 1). Amplification of cDNAs was carried out using a Perkin-Elmer Gene Amp PCR kit at a $MgCl_2$ concentration of 2.5 mM in a total reaction volume of 100 μl. The reactions were carried out in thin-walled 0.5 ml reaction tubes (Perkin-Elmer, Norwalk, CN, USA) using a thermocycler. The PCR step program was 94°C, 45 s; 60°C, 30 s; 72°C, 45 s (5 cycles); 94°C, 30 s; 60°C, 30 s; 72°C, 1 min (30 cycles) with a final extension step at 72°C for 10 min. The PCR-amplified products were separated by electrophoresis on a 1.5% agrose gel, blotted onto Hybond-N membranes (Amersham) and finally hybridized with ^{32}P-5′-end-labelled specific substance P probe IV, located at position 212, and specific neurokinin A probe V-2, located at position 285 of the published rabbit preprotachykinin-A sequence (Mägert et al., 1993) (Table 1 and Fig. 1).

cDNAs from rabbit brain, trigeminal ganglion, iris and aqueous humor were also amplified by reverse-transcribed PCR, using a commercially available rat β-actin primer kit (Clontech, cat. nr. 5506-1), following the instructions given by Clontech. The rat β-actin

amplimer generates a PCR product of about 750 bp. The PCR product was hybridized with a ^{32}P-labelled rat β-actin riboprobe (Clontech).

2.5. Sequencing of PCR products

The PCR products, separated by gel electrophoresis, were stained with ethidium bromide. Each fragment was eluted individually from the gel by incubation in TEN buffer (10 mM TrisHCl, 1 mM EDTA, 250 mM NaCl, pH 7.5) at 37°C overnight. The PCR products were concentrated by adding 2.5 volume 99% ethanol, kept at −70°C for 2 h. After centrifugation at 15 000 × *g* for 15 min, the pellet was washed with ethanol, air-dried and finally dissolved in water. The PCR products were further treated with Exo nuclease-I and Shrimp alkaline phosphatase (Amersham). Prior to sequencing, the products were purified by gel filtration using a Chromo Spin-TE 100 column (Clontech). Sequencing was carried out using a fmol-sequencing kit (Promega). Four 5'-end-labelled primers as indicated in Table 1 (II, III, V-1 and VI) were used for the sequencing, located at positions 1, 348, 99 and 181 of the published rabbit preprotachykinin-A cDNA sequence, respectively (Mägert et al., 1993) (Fig. 1).

2.6. Radioimmunoassay of substance P and neurokinin A in aqueous humor

Substance P- and neurokinin A-like immunoreactivity was measured by the use of RIA kits from Peninsula (Merseyside, St. Helens, UK). Briefly, aqueous humor samples or standard solutions of substance P or neurokinin A in RIA buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 0.1% Triton X-100, 0.01% sodium azide, pH 7.4) were incubated with 100 μl substance P or neurokinin A antiserum at a final dilution of 1:120 000 at 4°C for 24 h. 100 μl iodinated Tyr^8 substance P or iodinated neurokinin A (≈ 10 000 cpm) in RIA buffer was added. Each mixture was incubated for another 24 h at 4°C. Antibody-bound tracer was separated from free tracer by addition of 100 μl goat anti-rabbit IgG serum and 100 μl normal rabbit serum. After incubation at room temperature for 90 min, 500 μl of cold RIA buffer was added, and the samples were centrifuged at 1700 × *g* for 30 min. The tubes were gently aspirated and the radioactivity of the precipitates was measured in a gamma counter (Packard Autogamma Cobra 5005, 80% counting efficiency for ^{125}I). The IC_{50} values (i.e. the concentration of peptide that displaces 50% of the tracer from the antibody) are about 27 and 150 pmol/l for substance P and neurokinin A, respectively. The detection limits are 1 and 8 pmol/l for substance P and neurokinin A, respectively. The substance P antiserum does not cross-react with either neurokinin A or neurokinin B. The neurokinin A antiserum cross-reacts with neurokinin B (80%) but not with substance P.

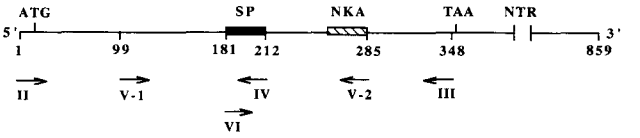

Fig. 1. Schematic illustration of rabbit γ-preprotachykinin-A cDNA. Numbered arrows indicate the primers/probes used in PCR amplifications, DNA sequencing and Southern blot analysis of PCR products (also see Table 1). SP and NKA: sequences coding for substance P and neurokinin A, respectively. → and ←: sense and antisense directions of primers/probes. ATG: initiation codon. TAA: stop codon. NTR: non-translated region.

3. Results

3.1. mRNA coding for γ-preprotachykinin-A in the iris and trigeminal ganglion

Using Northern blot analysis, preprotachykinin-A mRNA was detected in extracts of the iris as well as of the trigeminal ganglion (Fig. 2). The finding was confirmed by Southern blot analysis of PCR-amplified products, using primers II and III (Table 1; Figs. 1 and 3). One large fragment with a size of 348 bp, corresponding to γ-preprotachykinin mRNA, and one small fragment (≈ 270 bp) were found. Partial DNA sequence analysis of the PCR products confirmed the

Table 1
Oligonucleotide primers/probes used in PCR amplification, Southern blot and DNA sequence analysis

No.	Start position in rabbit cDNA	Sequence (5' to 3' orientation)	Estimated T_m (°C)
II	1 →	AAATCCAACATGAAAATCCTCGTGGCC	53
III	348 ←	TCTTCTCTCGTAATTCTGCATGGCGCT	54
IV	212 ←	CATTAATCCAAAGAACTGCTGAGGCTTGGGT	57
V-1	99 →	TGGTCCGACTGGTCCGAC	50
V-2	285 ←	CATTAGTCCAACAAAGGAATCTGTTTTATG	51
VI	181 →	AGACCCAAGCCTCAGCAG	47

→ and ← represent sense and antisense direction of primers/probes, respectively. T_m is the temperature at which the correctly base-paired hybrid dissociates.

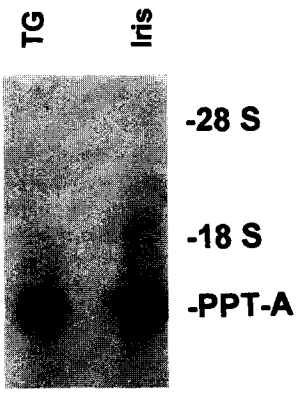

Fig. 2. Northern blot analysis showing the expression of preprotachykinin-A in the rabbit trigeminal ganglion (TG) and iris. Each lane contains 10–30 μg of total RNA. Markers to the right indicate the positions of 28 S and 18 S ribosomal RNA.

expression of γ-preprotachykinin mRNA (large fragment) and α-preprotachykinin mRNA (small fragment) in rabbit brain, trigeminal ganglion and iris (Fig. 3). These findings were confirmed by hybridization experiments, using a specific antisense substance P oligonucleotide probe IV and a specific antisense neurokinin A oligonucleotide probe V-2 (Table 1 and Fig. 1). Only the 348 bp fragment hybridized with both probes (Fig. 3). Since the product was available in very small amounts, only a partial sequence of the small fragment was obtained, which corresponded to the substance P but not to the neurokinin A sequence.

3.2. Release of mRNA coding γ-preprotachykinin-A into the aqueous humor in response to C-fibre activation

Southern blot analysis of PCR-amplified products revealed γ-preprotachykinin-A mRNA in the aqueous humor of eyes exposed to irradiation of the iris or

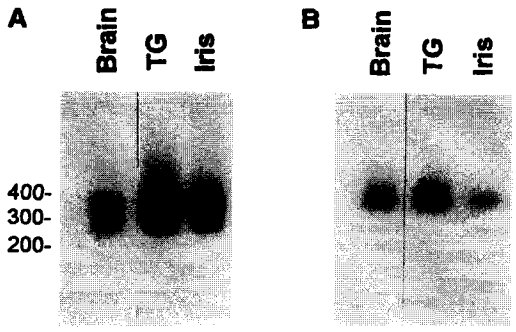

Fig. 3. Southern blot analysis of reverse-transcribed PCR-amplified products showing expression of preprotachykinin-A in the rabbit brain, trigeminal ganglion (TG) and iris. Two PCR-amplified products were evident. Both of them hybridized with an antisense substance P oligonucleotide probe (A). Only the larger of the two fragments hybridized with an antisense neurokinin A oligonucleotide probe (B). Size markers (base pair) to the left.

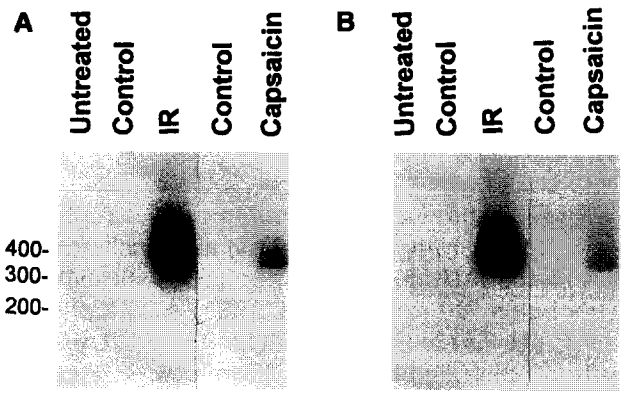

Fig. 4. Southern blot analysis of reverse-transcribed PCR-amplified products revealed the presence of γ-preprotachykinin-A mRNA in aqueous humor of eyes exposed to infrared irradiation (IR) or treated with capsaicin, but not in aqueous humor of contralateral (control) or untreated eyes. In each case the PCR-amplified product was identical in size to the large fragment detected in brain, trigeminal ganglion and iris. It hybridized with the substance P (A) and the neurokinin A (B) antisense oligonucleotide probes. Size markers (base pair) to the left.

treated with capsaicin, but not in the aqueous humor of contralateral or normal eyes (Fig. 4). The PCR product was identical in size to the large fragment seen in the trigeminal ganglion and iris and hybridized with both the substance P and the neurokinin A oligonucleotide probes.

cDNAs from brain, trigeminal ganglion, iris and aqueous humor were also amplified by PCR, using a commercially available rat β-actin primer kit (Clotech). Electrophoresis revealed a β-actin product with a size of about 700 bp in the brain, trigeminal ganglion and iris. This product was absent from the aqueous humor. The PCR product was blotted and hybridized with a rat β-actin riboprobe. Positive signals were seen in brain, trigeminal ganglion and iris, but not in aqueous humor (Fig. 5).

3.3. Release of substance P and neurokinin A into the aqueous humor in response to C-fibre activation

Irradiation of the iris released substance P- and neurokinin A-like immunoreactivity into the aqueous humor; the concentrations were 102.6 ± 23.8 and 230.4 ± 45.6 pmol/l ($n = 10$), respectively. In the aqueous humor of the contralateral non-irradiated eye, the concentration of substance P-like immunoreactivity was 11.2 ± 0.7 pmol/l while neurokinin A-like immunoreactivity could not be detected ($n = 10$). Similarly, retrobulbar injection of capsaicin was found to release substance P- and neurokinin A-like immunoreactivity into the aqueous humor; the concentrations were 24.5 ± 3.1 and 45.2 ± 8.1 pmol/l, respectively ($n = 10$). In the aqueous humor of vehicle-treated eye, the concentration of substance P-like immunoreactivity was 11.2

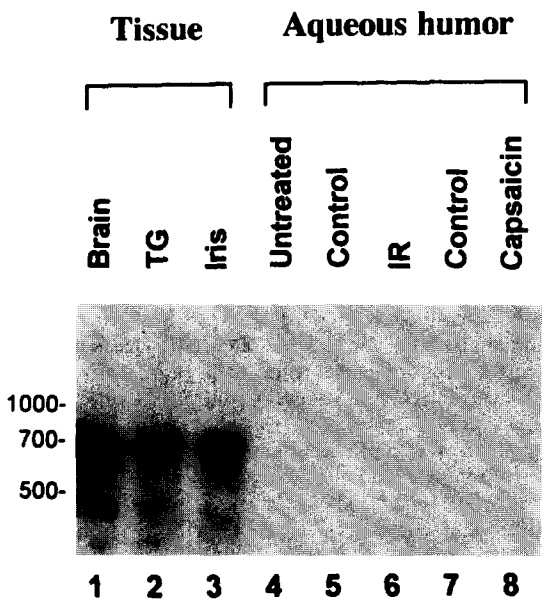

Fig. 5. cDNAs from rabbit brain, trigeminal ganglion (TG), iris and aqueous humor were amplified by PCR, using a commercially available rat β-actin primer kit. The PCR product was hybridized with a rat β-actin riboprobe. Positive signals were seen in brain, TG and iris, but not in the aqueous humor of eyes exposed to infrared irradiation (IR) (lane 6) or treated with capsaicin (lane 8), and not in the aqueous humor of contralateral (control) eyes (lanes 5 and 7) or in normal untreated eyes (lane 4), indicating that only specific mRNAs (such as γ-preprotachykinin-A mRNA) are released in response to C-fibre stimulation. Size markers (base pair) to the left.

± 1.4 pmol/l while neurokinin A-like immunoreactivity could not be detected ($n = 10$).

4. Discussion

In the uvea of the rabbit eye, substance P immunoreactivity is restricted to nerve fibres. Since either destruction of the trigeminal ganglion or capsaicin pretreatment eliminates such nerve fibres, they are thought to originate from neurons in the trigeminal ganglion (Butler et al., 1980; Miller et al., 1981; Tervo et al., 1981; Stjernschantz and Sears, 1982; Stone et al., 1982; Tornqvist et al., 1982; Terenghi et al., 1983). In the present study, the γ-preprotachykinin mRNA was detected in the iris as well as in the trigeminal ganglion. This finding was further confirmed by PCR amplification and sequencing of the PCR products. Conceivably, the mRNAs co-exist with their translated products substance P and neurokinin A, i.e. they may exist in C-fibres in the iris.

C-fibres are committed to react to noxious stimuli as part of their physiological/pathophysiological response to harmful, local influences. Hence, substance P and neurokinin A are released from ocular C-fibres into the aqueous humor as a normal response to such stimulation. Also other C-fibre neuropeptides, such as calcitonin gene-related peptide and pituitary adenylate cyclase-activating peptide, have been shown previously to be released into the aqueous humor in response to, for instance, infrared irradiation of the iris and to retrobulbar injection of capsaicin (Wang et al., 1995). Recently, in situ hybridization demonstrated axonal mRNA coding for oxytocin in neurosecretory vesicles in nerve terminals (Jirikowski et al., 1990), raising the possibility that neuropeptide-coding mRNAs may be released from nerve terminals together with peptide transmitters in response to stimuli. In the present study, mRNAs coding for γ-preprotachykinin-A were detected in the aqueous humor. The hybridization signal for preprotachykinin-A mRNAs (revealed by electrophoresis of PCR products with subsequent hybridization with oligonucleotide probes) was detectable in the aqueous humor from injured eyes but not from control eyes. Moreover, mRNAs coding for β-actin (house-keeping gene) could be detected in the brain, trigeminal ganglion and iris, but not in the aqueous humor. Possibly, only mRNAs coding for secretory proteins and peptides are released in response to specific stimuli. Our results support the view that mRNAs coding for preprotachykinin-A may be present in the C-fibre nerve terminals in the iris and that they are released into the aqueous humor in response to C-fibre stimulation.

Questions emerging from these observations concern the possible physiological role of γ-preprotachykinin-A mRNA in peripheral C-fibres. Indeed, although numerous reports have provided evidence for the presence of mRNA in neuronal projections (Garner et al., 1988; Jirikowski et al., 1990; Levy et al., 1990; Mohr et al., 1990, 1991; Steward and Banker, 1992; Mohr and Richter, 1993), the physiological significance of this phenomenon is poorly understood. Conceivably, local protein synthesis may take place. This has been suggested for the ribosome-rich dendritic compartment (Garner et al., 1988; Jirikowski et al., 1990; Levy et al., 1990; Mohr et al., 1990, 1991; Steward and Banker, 1992; Mohr and Richter, 1993) but also for the axonal compartment, where ribosomes are rare (Giudetta et al., 1990; Mohr et al., 1991). In fact, however, the complete protein synthesizing machinery has not yet been demonstrated in axons. Alternatively, mRNA is being transported peripherally without being translated. Another observation with bearing on the present finding is the demonstration of mRNA in secretory vesicles in neuronal projections (Jirikowski et al., 1990), suggesting that mRNA may be included among the secretory products. Recently, it was reported that injection of mRNA coding for vasopressin into the rat hypothalamus leads to the selective uptake, retrograde transport, and expression of the corresponding peptide in the magnocellular neurons (Jirikowski et al., 1992). The present finding of mRNA in the aqueous humor

after C-fibre stimulation may reflect a phenomenon characteristic of stimulated neurons in general. If so, the implications are that specific mRNAs could be secreted from one set of neuronal projections to be taken up by another neuronal population, leading to transient expression of novel transmitters in the receiving neurons. Thus, the uptake of foreign mRNA and the consequent expression of novel transmitters could represent an additional mode of inter-neuronal communication.

In conclusion, mRNA coding for γ-preprotachykinin-A was detected in the rabbit brain, trigeminal ganglion and iris. Interestingly, mRNA coding for γ-preprotachykinin-A was detected also in aqueous humor from injured eyes but not from contralateral eyes and normal eyes of untreated rabbits. Our results suggest that mRNA coding for γ-preprotachykinin-A may be present in C-fibre terminals in the iris and that it is released from these fibres into the aqueous humor in response to C-fibre stimulation.

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