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Substance P, neurokinin A and neurokinin B in the ocular response to injury in the rabbit

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1 Substance P (SP) and neurokinin A- (NKA)/neurokinin B (NKB)-like immunoreactivity (LI) were measured by radioimmunoassay in extracts of the rabbit uvea. The iris-ciliary body complex contained 3–4 times more NKA/NKB-LI than SP-LI. Tachykinins are thought to mediate many of the responses to ocular injury in the rabbit. Their possible role in the miosis and breakdown of the blood-aqueous barrier (BAB) was studied *in vitro* and *in vivo*.

2 *In vitro*, NKA had a more short-lasting contractile effect on the sphincter pupillae muscle than either SP or NKB, but SP was more potent than the other two. The tachykinin antagonist, spantide, dose-dependently suppressed the response to electrical stimulation (by 90% at 10^{-4} M) and to the three tachykinins. An antiserum against SP (no cross-reaction with NKA or NKB) greatly suppressed the response to SP (by 90%) as well as to electrical field stimulation (by 40%). The responses to NKA and NKB were unaffected.

3 *In vivo* studies revealed that SP was more potent than NKA and NKB as a miotic. SP evoked a moderate breakdown of the BAB at high doses while NKA and NKB were virtually inactive.

4 We conclude that besides SP other tachykinins might play a role in the mediation of miosis in the rabbit eye but, of the three peptides investigated, only SP can be of importance for the breakdown of the BAB.

Introduction

The response to ocular injury in the rabbit consists of hyperaemia, lacrimation, miosis, breakdown of the blood-aqueous barrier (BAB) and ocular hypertension. Miosis and breakdown of the BAB are thought to be mediated by antidromic reflexes in sensory nerve fibres originating in the trigeminal ganglion (Tervo *et al.*, 1981; 1982; Ehinger *et al.*, 1983), and tachykinins, such as substance P (SP), are thought to mediate such reflexes (Lembeck & Holzer, 1979). Stimulation of the trigeminal nerve induces release of SP-like immunoreactivity (SP-LI) into the aqueous humour concomitantly with miosis and break-down of the BAB (Bill *et al.*, 1979), and exogenously applied SP evokes the same response (Stjernschantz *et al.*, 1981; Holmdahl *et al.*, 1981). Pretreatment with tachykinin antagonists has been shown to suppress responses not only to exogenous SP but also to ocular injury (Holmdahl *et al.*, 1981).

SP belongs to the tachykinin family of peptides characterized by a common C-terminal Phe-X-Gly-Leu-Met-NH_2 sequence (Erspamer, 1981). Recently, tachykinins other than SP, namely neurokinin A and B (NKA and NKB), have been demonstrated in mammalian tissues (Kimura *et al.*, 1983; Kangawa *et al.*, 1983; Minamino *et al.*, 1984) and immunocytochemical studies have demonstrated the coexistence of SP- and NKA-like peptides in certain sensory nerve fibres (Dalsgaard *et al.*, 1985; Hua *et al.*, 1985; Sundler *et al.*, 1985).

SP may be derived from either of three tachykinin precursors (α- β- and γ-preprotachykinin A) (Nawa *et al.*, 1983; Nakanishi, 1986; Krause *et al.*, 1987). α-Preprotachykinin A contains one copy of SP while β- and γ-preprotachykinin A each contain one copy of SP and one of NKA. NKB on the other hand has a separate precursor (preprotachykinin B) (Kotani *et al.*, 1986). Erspamer and collaborators (see Erspamer, 1981) were the first to point out that while members of the tachykinin family share a common spectrum of biological actions they differ with respect to potency in different test systems. Lee *et al.* (1982) proposed the existence of two types of receptors, namely one with preference for physalaemin

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and SP and another with preference for eledoisin and kassinin. Later studies have indicated the existence of three types of tachykinin receptor with preference among the mammalian tachykinins for SP (NK-1 receptor), NKA (NK-2 receptor) and NKB (NK-3 receptor), respectively (Buch *et al.*, 1984; Regoli *et al.*, 1985; Henry *et al.*, 1986; Dion *et al.*, 1987).

The aim of the present study was to investigate the possibility that not only SP but also NKA and NKB are involved in the sensory nerve-mediated reflex to ocular injury. The tissue concentrations of SP-LI and NKA/NKB-LI were determined in various regions of the rabbit uvea. In addition, the ocular effects of the three tachykinins were studied *in vivo* and *in vitro*, as well as the suppressant effects of a tachykinin antagonist and of immunoneutralization with an SP-antiserum on the sphincter pupillae contraction evoked *in vitro* by SP, NKA, NKB or electrical stimulation.

Methods

Animals

Adult pigmented rabbits of either sex weighing 1.5–3.0 kg were used.

Tissue collection, extraction and radioimmunoassay of substance P- and neurokinin A/neurokinin B-like immunoreactivity

Six rabbits were killed by a blow on the neck and exsanguinated. The eyes were removed and the posterior uvea, cornea, iris and ciliary body dissected out on ice. The corresponding tissues from both sides were pooled, weighed, frozen on dry ice and stored at −80°C until extracted. At the extraction the frozen tissue samples were immersed in test tubes containing at least 20 volumes of hot distilled water, and the tubes were kept in a boiling water bath for 10 min. Following homogenization (Polytron), the samples were centrifuged (1000 *g* for 10 min) and the supernatants taken off. The pellets were then resuspended in 1.0 M acetic acid, heated for another 10 min period in boiling water, centrifuged, and the supernatants taken off. Both the neutral and acid extracts were lyophilized and reconstituted in 0.9% saline containing 0.5% bovine serum albumin (BSA) just before radioimmuno-assay (RIA).

SP-LI and NKA/NKB-LI were determined by RIA, as described previously using antisera SP2 and NKA5 (Brodin *et al.*, 1986). [^{125}I]-[Tyr^{8}]-SP and [^{125}I]-Bolton-Hunter labelled NKA were used as radioligands, respectively. Antiserum SP2 does not cross-react with NKA or NKB (<0.01%), while antiserum NKA5 cross-reacts with NKB (71%) but not with SP (0.1%) (Brodin *et al.*, 1986).

In vivo studies

The rabbits were anaesthetized with methohexitone sodium (5 mg kg^{-1} body weight) i.v. before the intraocular injections. The animals recovered from the anaesthesia after 10–15 min and no further anaesthesia was used during the rest of the experiment. Synthetic SP and NKA were dissolved in 0.9% saline to a concentration of 4×10^{-5} M. NKB was dissolved in 0.5% formic acid and 0.9% saline. The peptides were frozen and stored in small portions. Serial dilution in 0.9% saline gave the same volume (10 µl) for the different doses used. Peptide solutions were prepared on a daily basis from frozen stock solution, and 10 µl volumes were injected into the vitreous chamber of the left eye by means of a Hamilton precision syringe 3–4 mm behind the limbus. The right eye received the same volume of 0.9% saline or vehicle and served as a control. The breakdown of the BAB was measured as the aqueous flare response (AFR) by a photoelectric method (Bengtsson *et al.*, 1975). The aqueous flare is a Tyndall phenomenon reflecting protein leakage across the BAB. A linear correlation between the AFR and the protein concentration in the anterior chamber has been established (Anjou & Krakau, 1961). The AFR is expressed in arbitrary units with reference to a standard. The pupillary diameter was measured with a clear plastic ruler under standardized light conditions. The AFR and pupil size were measured every 30 min starting 30 min after IR or after injection or topical application of peptides if not otherwise stated. Infrared irradiation (IR) of the iris for 2 min was applied as a standardized minimal trauma to the eye (Dyster-Aas & Krakau, 1964).

In vitro studies

The rabbits were killed by a blow on the neck and exsanguinated. The eyes were taken out and opened. The sphincter pupillae muscle was excised, cut in half and mounted vertically on perspex holders in a 8 ml tissue bath maintained at 35°C. The bathing fluid was a modified Krebs solution (mM): NaCl 133, $NaHCO_3$ 16.3, KCl 4.7, $MgCl_2$ 1.0, NaH_2PO_4 1.4, $CaCl_2$ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 93% O_2 plus 7% CO_2 giving a pH of 7.2–7.3. The mechanical activity was recorded isometrically using a Grass FTO3 force displacement transducer and a Grass model 7 polygraph. Before starting the experiments the sphincter muscle was allowed to equilibrate for 60–90 min under a constant load of 150 mg. The same load was then used throughout the experiments. Electrical field stimulation with square wave pulses (14–17 V over the electrodes, 0.3 ms duration) was applied by means of a pair of platinum electrodes, connected to a Grass

Table 1 Tissue concentrations of neurokinin A (NKA)/NKB-like immunoreactivity (LI) and substance P (SP)-LI in various parts of the rabbit eye

	NKA/NKB-LI (pmol g^{-1})	*SP-LI* (pmol g^{-1})	*Molar ratio NKA-LI/SP-LI*
Cornea	0.28 ± 0.05	0.14 ± 0.3	2.00
Iris	1.52 ± 0.38	0.47 ± 0.04	3.23
Ciliary body	1.82 ± 0.24	0.39 ± 0.04	4.67
Posterior uvea	0.99 ± 0.26	0.23 ± 0.05	4.30

Data shown are means ± s.e.mean ($n = 6$)

S4C stimulator. The muscles were stimulated with trains of pulses lasting 10 s, the pulse frequency being 20 Hz. KCl-enriched buffer solution (137 mM, no NaCl) was used to evoke standard contractions which were set at 100%. Concentration-response curves for SP, NKA and NKB were constructed by adding only one or two concentrations of either of the peptides to each preparation. Before applying a second concentration the preparation was washed extensively. Antiserum, tachykinin antagonists, atropine and guanethidine were applied, in concentrations specified in the Results section, 10–20 min before SP, NKA, NKB or electrical stimulation.

Chemicals

The tachykinin antagonist spantide, [D-Arg[1], D-Trp[7,9], Leu[11]]-SP, was provided by Ferring, Malmö, Sweden. Synthetic SP was purchased from Beckman (Geneva, Switzerland) and NKA and NKB from Peninsula (Belmont, CA). Other drugs included atropine sulphate (Alcon, Forth Worth, Texas, U.S.A.), guanethidine (Ciba-Geigy, Basel, Switzerland) and methohexitone sodium (Brietal, Lilly, Indiana, U.S.A.). For immunoneutralization, SP antiserum (code no. 8127) was provided by MILAB, Malmö, Sweden. The cross-reactivity of this antiserum with NKA as determined by RIA using [^{125}I]-[Tyr[8]]-SP as a tracer was 0.001%, with NKB 0.001%, with eledoisin 0.002%, and with gastrin releasing peptide 0.001%, calculated from the molar concentration. The working dilution of this antiserum in the RIA is 1 : 33,000.

Results

Concentrations of substance P- and neurokinin A/neurokinin B-like immunoreactivity in the rabbit uvea

The tissue concentrations of SP-LI and NKA/NKB-LI were determined by RIA separately in the cornea, iris, ciliary body and posterior uvea, following acid and neutral extraction, respectively. There was two to four times more NKA/NKB-LI than SP-LI on a molar basis. The highest concentrations of the peptides were found in the iris and ciliary body (Table 1). In a separate study the NKA/NKB-LI in the iris and ciliary body of the rabbit was analysed by high performance liquid chromatography and found to represent predominantly material coeluting with synthetic NKA (Beding-Barnekow *et al.*, unpublished observations).

Effects of tachykinins and infrared irradiation of the iris of the rabbit eye

Although SP, NKA and NKB elicited similar maximal miotic responses (amounting to 6.2 ± 0.4 mm (mean ± s.e.mean, $n = 5$), 5.8 ± 0.3 mm, ($n = 3$) and 5.4 ± 0.4 mm ($n = 8$), respectively) at a dose of 300 pmol, SP appeared to be somewhat more potent (Figure 1a). The peak miotic response to either SP, NKA or NKB occurred after 2–3 h; for all three peptides the miosis lasted for more than 24 h at a dose of 300 pmol (Figure 2). These results should be compared with the effects of infrared irradiation of the iris where the miotic effect was prompt (2.9 ± 0.2 mm; mean ± s.e.mean, $n = 4$), and rather transient, the pupil returning to its initial size after about 30 min (Figure 3a).

There was a clear difference in the ability of SP, NKA and NKB to disrupt the BAB. SP produced a weak to moderate, long-lasting aqueous flare response (Figure 1b). NKA and NKB were virtually inactive at the lower doses and produced only a slight aqueous flare response even at 300 pmol (Figure 1c). The aqueous flare response could not, for technical reasons, be measured properly until the pupillary constriction had subsided, which occurred 6–9 h after injection. Visually estimated aqueous flare responses before that time revealed no significant response. The aqueous flare response to irradiation reached its maximum about one h after stimulation (Figure 3b) and lasted for a few hours only.

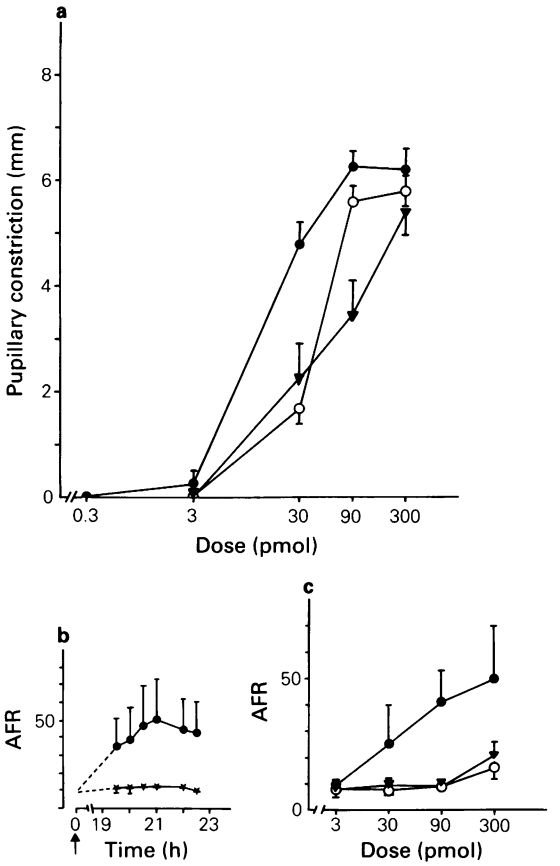

Figure 1 (a) Dose-response curves illustrating the pupillary constriction evoked by the intravitreal injection of substance P (SP), neurokinin A (NKA) or NKB. The pupillary constriction was registered after 2–3 h, when the miosis was maximal. Each value is the mean of 3–9 experiments; vertical lines represent s.e.mean. SP (●), NKA (○), NKB (▼). (b) Time course of the aqueous flare response (AFR) evoked by the intravitreal injection into the left eye of 300 pmol SP (●) ($n = 4$) at zero time (arrow). The maximum occurred after approximately 21 h. The onset and decline of the response were quite slow. For comparison, 0.9% saline was injected into the right eye (*). (c) Dose-response curves illustrating the AFR evoked by injection of SP (●), NKA (○) or NKB (▼). SP produced a response while NKA and NKB were virtually inactive ($n = 3$).

Effects of tachykinins and electrical nerve stimulation on the isolated sphincter muscle

Electrical stimulation (0.3 ms, 20 Hz for 10 s) in the presence of atropine and guanethidine evoked a slow contraction which was virtually blocked by 10^{-5} M spantide, suggesting the involvement of a tachykinin-

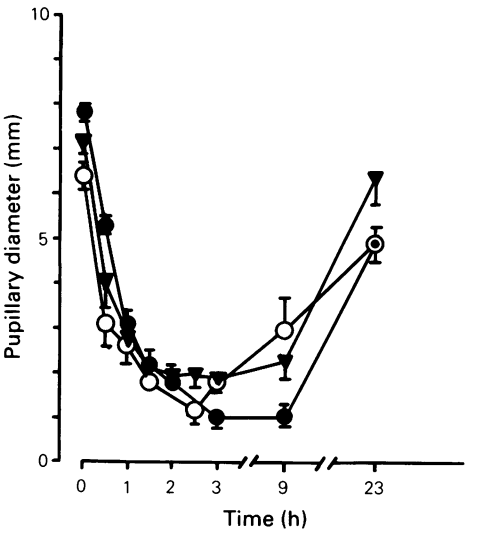

Figure 2 The time course of the pupillary constriction evoked by intravitreal injection of 300 pmol of either substance P (SP; ●), neurokinin A (NKA; ○) or NKB (▼) (injected at arrow). Mean values are shown; $n = 6$–9 for SP, 3 for NKA and NKB. Vertical lines indicate s.e.mean.

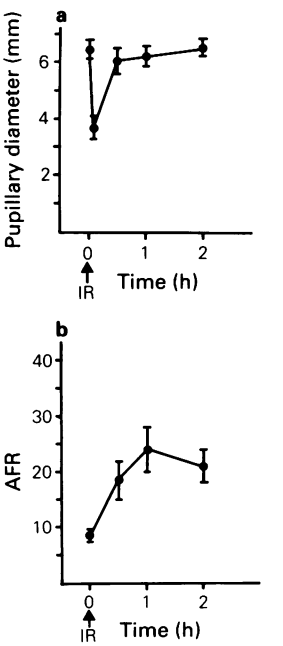

Figure 3 Time course of pupillary constriction (a) and aqueous flare (AFR, b) evoked by a minimal eye trauma (infrared irradiation (IR) of the iris, at arrow). The effects were prompt and comparatively shortlasting. Mean values of four separate experiments are shown; vertical lines indicate s.e.mean.

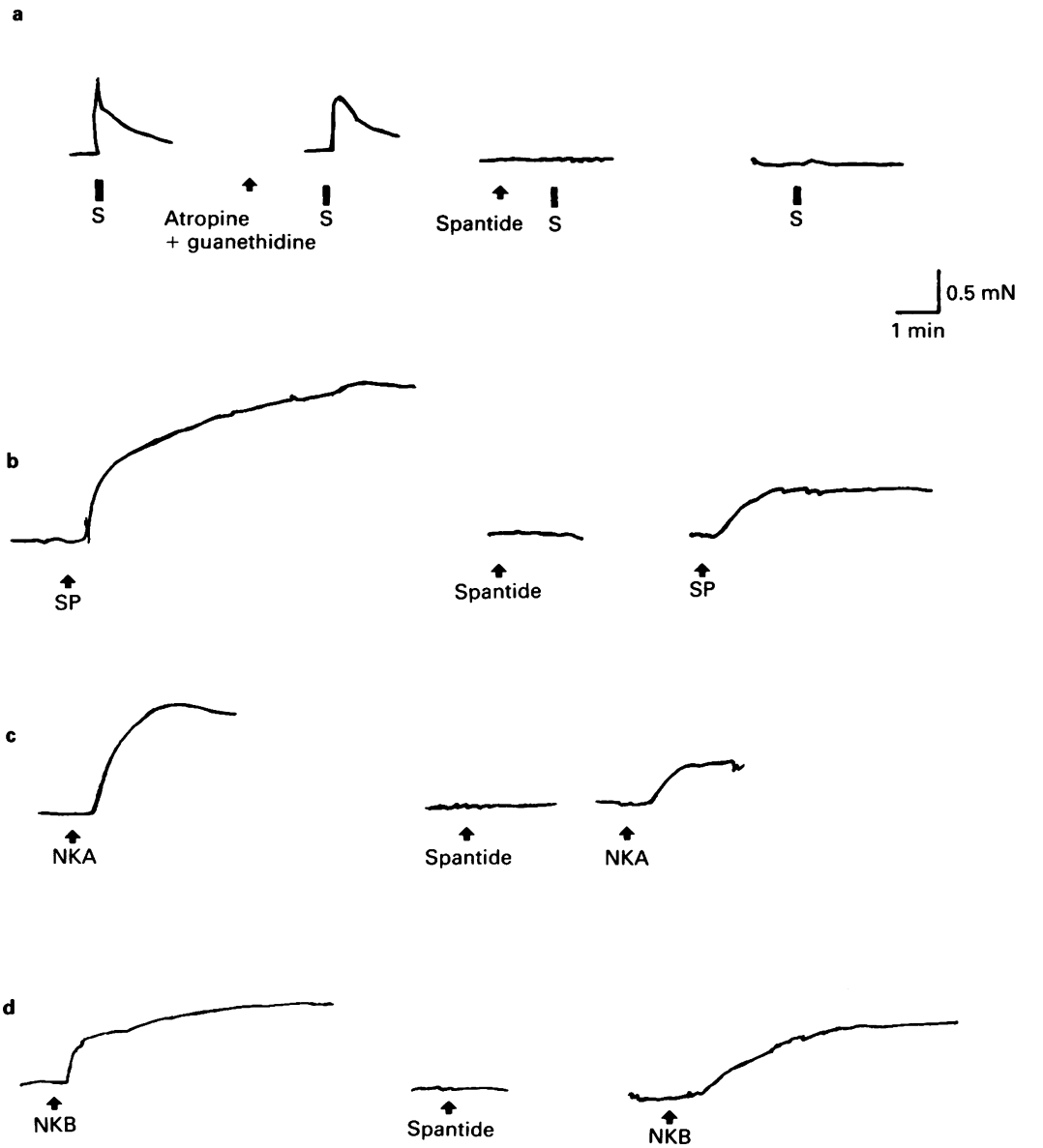

Figure 4 (a) The contractile response of the rabbit isolated iris sphincter muscle to electrical field stimulation (S; 20 Hz, 0.3 ms, 25 V for 10 s) before and after addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M) (arrow). The atropine-resistant contractile response was almost completely blocked by the tachykinin antagonist spantide (10^{-4} M; arrow-head). (b) The contractile response of the rabbit isolated iris sphincter muscle to substance P (SP) 3×10^{-8} M. The response was inhibited by spantide 10^{-5} M. (c) The contractile response of the rabbit isolated iris sphincter muscle to 10^{-7} M neurokinin A (NKA) (arrow). Note that the contractile response, which was inhibited by spantide 10^{-5} M, was more short-lasting than that evoked by SP. (d) The contractile response to 10^{-7} M NKB. The response was poorly inhibited by spantide 10^{-5} M, but was more effectively inhibited by 10^{-4} M spantide (not shown, see Figure 5).

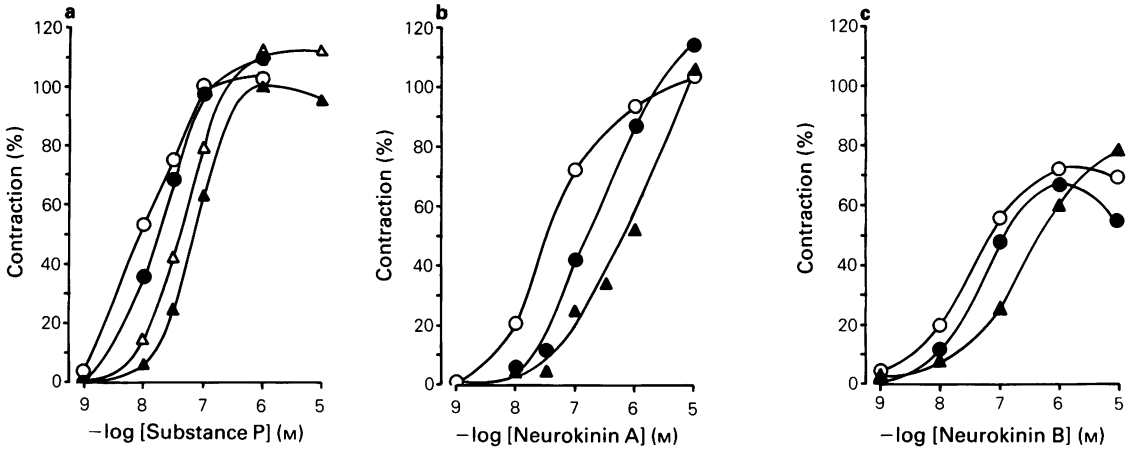

Figure 5 Concentration-response curves illustrating the contractile effects, on the rabbit isolated iris sphincter, of substance P (SP, a), (NKA, b) and NKB (c) in the absence (○) and presence of different concentrations of spantide: 10^{5} (●), 3×10^{5} (△) and 10^{-4} (▲) M. Each value represents the mean of 4–16 experiments. The EC_{50} value for SP was 7.9×10^{-9} M, for NKA 3.6×10^{-8} M and for NKB 2.8×10^{-8} M. The pA_2 value for spantide with SP as agonist was 5.1, the regression coefficient being −0.9954; with NKA 5.8, the regression coefficient being −0.9420; and with NKB 5.0, the regression coefficient being −1.036.

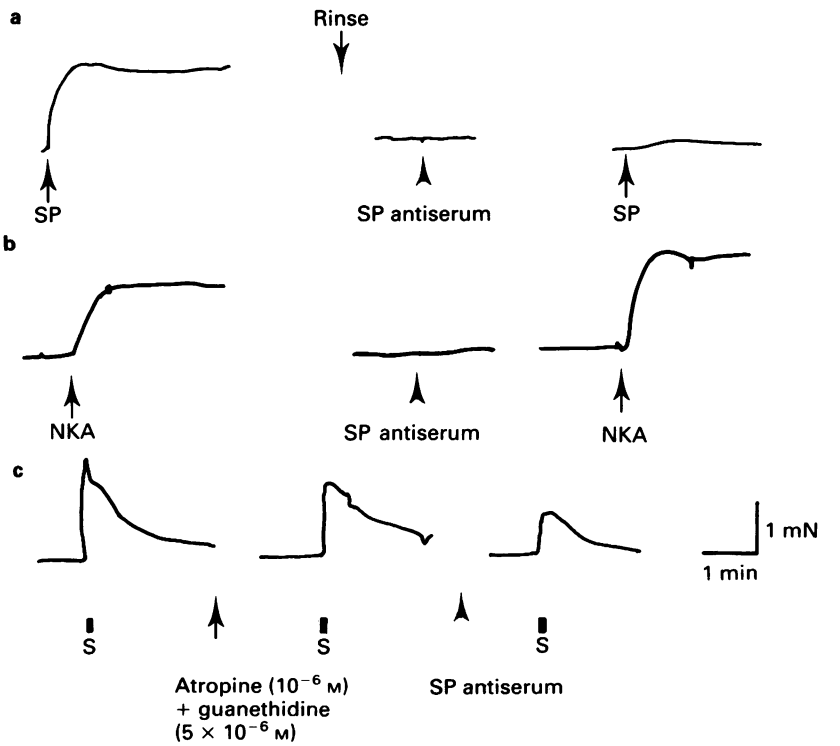

Figure 6 Contractions of the rabbit isolated iris sphincter in response to substance P (SP, 10^{-8} M) (a), neurokinin A (NKA, 10^{-8} M) (b) or electrical field stimulation (S; 20 Hz, 0.3 ms, 25 V for 10 s) (c) before and after the addition of 0.2 ml of an SP antiserum (code no. 8127). The antiserum, which was without effect on the responses to NKA and NKB (not shown), almost completely blocked the contractile response to SP and greatly reduced the response to electrical field stimulation.

like neurotransmitter. Atropine blocked the fast cholinergic component of the contraction and guanthedine was included to prevent adrenergically mediated responses (Figure 4a). Exogenously applied SP (Figure 4b), NKA (Figure 4c) and NKB (Figure 4d) evoked contractile effects; the EC_{50} value for SP was 7.9×10^{-9} M (Figure 4b), for NKA 3.6×10^{-8} M and for NKB 2.8×10^{-8} M. SP and NKB evoked long-lasting contractions, while the response to NKA was comparatively short-lasting. The contractile responses to all three tachykinins were suppressed by spantide with a rightward shift of the concentration-response curves (Figure 5).

Attempts were made to neutralize the effects of exogenous SP and of electrical stimulation by adding 0.2 ml of SP antiserum (code no. 8127) to the bath. The SP antiserum reduced the response to exogenous SP by $90 \pm 2.1\%$ (mean $\pm$ s.e.mean, $n = 5$) and the response to electrical field stimulation by $40 \pm 6.2\%$ ($n = 15$) (Figure 6). The NKA (Figure 6) and NKB (not shown) responses ($n = 10$) were unaffected as was the response to 10^{-4} M carbachol ($n = 4$). The latter experiment was performed without atropine in the bath.

Discussion

Tachykinins are implicated in sensory nerve-mediated reflexes evoked by injury. The aim of our study was to explore if not only SP but also NKA, which has two precursors in common with SP (Nawa *et al.*, 1983, Nakanishi, 1986; Krause *et al.*, 1987) and which coexists with SP in primary afferent neurones (for references see Introduction), might be involved as a mediator in sensory nerve-mediated responses to ocular injury. The cornea, iris, ciliary body and posterior uvea were all found to contain both SP-LI and NKA/NKB-LI. Recently, NKA and NKB were demonstrated to occur in similar concentrations in the rabbit iris (Taniguchi *et al.*, 1986) with a molar ratio between SP and NKA/NKB of 4.3 and 5.4, respectively. With the extraction and assay methods used in this study (see also Brodin *et al.*, 1986) there were approximately two to four times more NKA/NKB-LI than SP-LI on a molar basis, with the highest ratios in the ciliary body. In a separate study it could be shown that NKB did not contribute significantly to the NKA/NKB-LI in extracts of the rabbit uvea (Beding-Barnekow *et al.*, unpublished data). Similar ratios between tissue levels of NKA-LI and SP-LI were recently found in rabbit cerebral arteries, which are also innervated by trigeminal sensory nerve endings (Brodin *et al.*, 1987). NKA and NKB had a miotic effect similar to that of SP upon intravitreal injection. The pupillary constriction was dose-dependent and of about the same magnitude for all three tachykinins, although SP seemed to be slightly more potent than NKA and NKB. The time course for the effect of the three peptides was about the same. Unlike SP, NKA and NKB did not disrupt the BAB at the lower doses and produced a minimal aqueous flare response at the highest dose. Thus none of the tachykinins tested reproduced exactly the rapid ocular response evoked by infrared irradiation of the iris. The difference in time course may well be explained by the different methods of inducing the injury. Infrared irradiation probably causes a release of the transmitter substance in the vicinity of the receptors, while after intravitreal administration peptides reach the receptors only after diffusion.

The results from *in vitro* experiments confirm that NKA and NKB have an effect similar to that of SP on the rabbit iris sphincter muscle. *In vitro*, SP was clearly more potent than NKA and NKB, while NKA had a more short-lasting contractile effect (a few min only) and was more readily washed out than SP, which is in agreement with the recent findings by Ueda *et al.* (1986). NKB was less potent than the two others and was not washed away as readily as NKA. In studies on the rabbit isolated iris muscle Ueda *et al.* (1986) observed that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP antagonized NKA but not SP and NKB. In our hands the contractile effects of SP, NKA and NKB on the iris were blocked by the closely related tachykinin antagonist spantide (Folkers *et al.*, 1984) in a concentration-dependent manner. The rank order potencies of the three tachykinins tested suggest that we are dealing with a NK-1-type receptor on the iris sphincter which responds to SP, NKA and NKB in that order and which is blocked by spantide. There was no apparent difference in the time course of the effect between SP, NKA and NKB *in vivo*, the miosis in all three cases lasting for more than 24 h. The marked discrepancy in the duration of the effect on the iris between *in vitro* and *in vivo* conditions with a rapid and relatively transient effect *in vitro* (particularly with NKA) and a long-lasting effect *in vivo* might be explained by the different modes of application. The *in vitro* method reveals the direct effect of the peptides on the iris sphincter muscle, while the effect of *in vivo* application is complicated by diffusion and degradation processes.

The SP antiserum failed to suppress the *in vitro* response of the iris to exogenous NKA and NKB but greatly suppressed the response to SP (by 90%) and the non-cholinergic response to electrical stimulation (by 40%). The observation that a significant proportion of the non-cholinergic contractile response of the iris sphincter to electrical stimulation was resistant to immunoneutralization but could be inhibited by spantide, suggests that not only SP but also a closely related tachykinin, e.g. NKA or NKB,

might be involved. In this respect, the results agree with the findings of Ueda et al. (1986). They suggested that the non-cholinergic, non-adrenergic contraction of the rabbit sphincter iris muscle is at least partly mediated by NKA.

Our results suggest that both NKA and SP play a role in the mediation of miosis in the rabbit eye but that of the two only SP might be involved in the disruption of the BAB. NKB is not likely to participate in the miotic or aqueous flare response, since only small amounts could be detected in the iris and ciliary body (Beding-Barnekow, unpublished data).

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