Comparison of the Responses to the Sensory Neuropeptides, Substance P, Neurokinin A, Neurokinin B and Calcitonin Gene-Related Peptide and to Trigeminal Nerve Stimulation in the Iris Sphincter Muscle of the Rabbit

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Abstract—Three mammalian tachykinins (substance P, neurokinin A and B) and two non-mammalian ones (eledoisin and physalaemin) produced potent contractions of the isolated rabbit iris sphincter muscle. The rank order of potencies was eledoisin > neurokinin B = physalaemin > substance P > neurokinin A. The maximum efficacy was much the same. The contractile responses to neurokinin A and eledoisin developed more rapidly than did those to the other tachykinins used and were selectively attenuated by [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP. Electrical transmural stimulation produced a contraction consisting of cholinergic and tachykininergic components. The tachykininergic component was abolished by pretreatment with capsaicin or by trigeminal denervation (Fujiwara et al., 1984). [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP attenuated the tachykininergic component, but not the cholinergic one. KCl and capsaicin also produced a tachykininergic contraction which was inhibited by [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP. Calcitonin gene-related peptide affected neither the iris sphincter muscle nor the response to electrical transmural stimulation. These results suggest that the tachykininergic responses induced by electrical transmural stimulation, KCl and capsaicin are predominantly mediated by neurokinin A, probably released from the peripheral endings of trigeminal nerves.

Substance P (SP) was thought to be the only tachykinin which acts as a mediator of sensory transmission (1–3). However, recent biochemical and immunocytochemical studies have revealed the coexistence of several neuropeptides in sensory nerves (4–7). Among them, neurokinin A (NKA, also called substance K or neuromedin L) and neurokinin B (NKB, also called neurokinin β or neuromedin K) are new mammalian tachykinins which share the C-terminal amino acid sequence common to SP (8, 9). NKA is also present in one of the two types of bovine brain SP precursors, β-preprotachykinin (5). On the other hand, calcitonin gene-related peptide (CGRP) has been found by analysis of the calcitonin gene coding sequence and is present concomitantly with the tachykinins mentioned above, in the same sensory nerves (7, 10). Therefore, the functions of sensory nerves may be multiply regulated by these neuropeptides.

The rabbit iris sphincter muscle is innervated by trigeminal, sensory nerve and produces a tachykininergic contraction in response to stimulation (11–14). In the present study, we compared the effects of four sensory neuropeptides (SP, NKA, NKB...
and CGRP) on the rabbit iris sphincter muscle and examined the possible involvement of such peptides in the response to trigeminal nerve stimulation.

Materials and Methods

Albino rabbits of either sex, weighing 2.0 to 3.0 kg, were exsanguinated while under pentobarbital anesthesia, and the eyes were immediately enucleated. Strips of the sphincter was prepared as described previously (14) and then mounted in a bath of 10-ml capacity which contained modified Krebs-Henseleit solution gassed with 95% O2 and 5% CO2. The temperature of the bath was maintained at 37.5±0.5°C. The composition of the modified Krebs-Henseleit solution was as follows (millimolar): NaCl, 112; KCl, 5.9; MgCl2, 1.2; CaCl2, 2; NaHCO3, 25; Na2HPO4, 1.2 and glucose 11.5.

The free end of the preparation was connected to a force-displacement transducer, and the isometric tension was recorded on a strip chart. A resting tension of 150 mg was applied and maintained throughout the experiments. All preparations were allowed to equilibrate for about 90 min in the Krebs-Henseleit solution before the start of the experiments. Cumulative concentration-response curves were obtained by increasing concentrations of drugs as soon as a steady response to the previous administration had been achieved. Electrical transmural stimulation was applied by means of a pair of platinum electrodes. Stimulus parameters used were squarewave pulses of 0.2 msec duration and 7.5 V intensity for 10 sec.

The following drugs were used: atropine sulfate and capsaicin (Merck Sharp & Dohme, West Point, PA, U.S.A.); substance P, Physalaemin (Protein Research Foundation, Osaka, Japan); carbachol (Sigma Chemical Co., St. Louis, MO, U.S.A.); Neurokinin B, [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP, calcitonin gene-related peptide, eledoisin (Peninsula Laboratories, Inc., San Carlos, CA, U.S.A.). Neurokinin A was donated by Shionogi Research Laboratories, Osaka, Japan. All peptides were dissolved in 0.1% gelatin solution immediately before use.

All values measured were expressed as the mean±S.E. Statistical analysis was performed using Student's t-test for paired or unpaired comparison.

Results

Responses to tachykinins and CGRP: SP, NKA and NKB produced a potent contraction of the rabbit iris sphincter muscle. The responses were concentration-dependent; the EC50 values showed that NKB was the most potent of the three mammalian tachykinins used (Table 1). Figure 1 shows representative responses to three tachykinins, in which the concentrations used corresponded approximately to the EC75 values. NKA elicited a rapid response, as compared with NKB and SP. Time to the peak response at such concentrations was 0.97±0.03 min for NKA (6 experiments), 5.2±0.3 min for NKB (5 experiments) and 8.5±1.2 min for SP (7 experiments). Time course of the recovery to the original resting tension after washing varied among the tachykinins; the NKA response was abolished within several min after washing, while

| Tachykinin       | Maximum response | EC50 (×10⁻⁶ M) | control | +antagonist
|------------------|-----------------|----------------|---------|-----------
| Substance P      | 0.98±0.01       | 2.5±0.3        | 6.0±3.1 |
| Neurokinin A     | 1.00±0.01       | 6.7±1.5        | 31.4±3.1 |
| Neurokinin B     | 1.00±0.01       | 1.1±0.3        | 1.1±0.4 |
| Physalaemin      | 0.89±0.02       | 1.6±0.2        | 2.2±0.2 |
| Eledoisin        | 1.00±0.01       | 0.71±0.09      | 4.0±0.23 |

a Maximum contraction induced by tachykinin was compared with that induced by 10⁻⁶ M carbachol in each preparation.

b EC50 in the presence of 10⁻⁶ M [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP.

c significantly different from the control (P<0.01). Mean±S.E. of 4–8 experiments.
approximately 2 hr were required for complete relaxation following the response to SP. Repeated application of NKA or NKB produced reproducible responses in the same preparation. On the other hand, the second response to SP was much smaller than the first one; thus the first concentration-response curve of SP in each preparation was taken as datum in the following experiments.

Two non-mammalian tachykinins, eledoisin and physalaemin, also produced a potent contraction. The time course of the response to eledoisin was more rapid than that of physalaemin; time to the peak response induced at EC75 was 2.3±0.2 min for eledoisin (6 experiments) and 5.8±0.6 min for physalaemin (4 experiments). Table 1 shows the EC50 values and maximum efficacies of all the tachykinins tested. Eledoisin was the most potent and NKA was the weakest; however, the maximum efficacy of the five tachykinins was much the same. CGRP (10⁻⁸ and 10⁻⁷ M) produced neither contractile nor relaxing responses of the rabbit iris sphincter muscle. The contractile responses induced by electrical transmural stimulation were not affected by 10⁻⁷ M CGRP (Fig. 2).

**Effects of [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP on tachykinin responses:** The contractile responses to NKA were inhibited by [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP.
However, the responses to SP and NKB were not significantly affected by the antagonist. Among the two non-mammalian tachykinins, the response to eledoisin was also antagonized by [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP. Figure 3 shows the concentration-response curves of NKA and eledoisin, in the absence and presence of [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP. At concentrations greater than $10^{-7}$ M, the antagonist attenuated the contractile responses to NKA and eledoisin. The EC50 values of the five tachykinins in the presence of $10^{-6}$ M antagonist are listed in Table 1.

**Effects of [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP on the responses to electrical transmural stimulation:** Electrical transmural stimulation contracted the rabbit iris sphincter muscle; the response developed rapidly and reached a peak at the end of stimulation. This rapid phase was followed by the slowly decaying phase, during which a shoulder or a second peak frequently occurred. [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP attenuated the contractile response (Fig. 4A). Our previous studies have shown that the contractile

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**Fig. 3.** Effects of [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP on the concentration-response curves of neurokinin A (NKA) and eledoisin. Maximum contraction induced by NKA or eledoisin before treatment with the antagonist was taken as 100%. •: before; □, △ and ○: after treatment with $10^{-7}$, $10^{-6}$ and $10^{-5}$ M [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP, respectively. Mean±S.E. of 4–6 experiments.

**Fig. 4.** Effects of [D-Arg¹, D-Pro², D-Trp⁷⁻⁹, Leu¹¹]·SP on the response to electrical transmural stimulation in the rabbit iris sphincter muscle. Stimulus parameters were the same as those of Fig. 2. In B, $10^{-6}$ M atropine was present throughout the experiment.
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Fig. 5. Effects of [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-SP on the cholinergic and tachykinergic responses to electrical transmural stimulation (5 Hz, 10 sec) in the rabbit iris sphincter muscle. The contractile response before treatment with the antagonist was taken as 100%. ○: tachykinergic responses recorded in the presence of 10⁻⁶ M atropine (7 experiments). ●: cholinergic response in the preparations which had been exposed to 10⁻⁵ M capsaicin and then washed for 1 hr (4 experiments). #: significantly different from the control (P<0.05).

The response consists of fast, cholinergic and slow, tachykinergic components (12, 14). Therefore, the effects of [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-SP on both components were examined separately. In the presence of 10⁻⁶ M atropine, electrical transmural stimulation evoked only a slow response, which was inhibited by [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-SP, in a concentration-dependent manner (Fig. 4B and Fig. 5, open circles). On the other hand, the antagonist had no significant effect on the cholinergic response evoked in preparations which had been exposed to 10⁻⁵ M capsaicin for 20 min in order to abolish the tachykinergic response (14) (Fig. 5, closed circles).

Effects of [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-SP on the responses to KCl and capsaicin: Following treatment with 10⁻⁶ M atropine and 3x10⁻⁶ M guanethidine, an increase in the concentration of KCl in the bathing medium produced a concentration-dependent contraction. This response was attenuated by 10⁻⁶ M [D-Arg¹, D-Pro², D-

Fig. 6. Effects of 10⁻⁶ M [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-SP on the contractile responses to KCl and capsaicin in the rabbit iris sphincter muscle. A: KCl response was recorded in the presence of 10⁻⁶ M atropine and 3x10⁻⁶ M guanethidine. The maximum contraction induced by KCl before treatment with the antagonist was taken as 100%. Mean±S.E. of 6 experiments. B: Since repeatedly applied capsaicin produced no reproducible responses, the response of each preparation induced by the first application of capsaicin in the presence or absence of the antagonist was taken as a percentage of the maximum contraction induced by 10⁻⁴ M carbachol. Mean±S.E. of 8 experiments. #: significantly different from the control (P<0.05).
Fig. 7. Effects of capsaicin on the contractile responses to KCl and neurokinin A (NKA) in the rabbit iris sphincter muscle. KCl response was obtained in the presence of $10^{-6}$ M atropine and $3 \times 10^{-6}$ M guanethidine. Closed circles: responses before treatment with capsaicin. Open circles: responses in the preparations which had been exposed to $10^{-5}$ M capsaicin for 20 min and then washed for 1 hr. Open triangles in A: effects of $10^{-6}$ M [D-Arg', D-Pro2, D-Trp7,9, Leu11]-SP on the response to KCl in capsaicin-exposed preparations. Mean±S.E. of 5–8 experiments. *: significantly different from the control (P<0.01).

Capsaicin produced a concentration-dependent contraction, and this response was not reproducible upon repeated application of this drug. Therefore, the effect of [D-Arg1, D-Pro2, D-Trp7,9, Leu11]-SP (Fig. 7A, open triangles) was examined using separate preparations. As shown in Fig. 6B, the antagonist at $10^{-6}$ M significantly depressed the contractile response to capsaicin.

Previous exposure to capsaicin for 20 min caused a complete loss of the responses to a second application of capsaicin. However, such treatment did not affect the contractile responses to NKA and carbachol. Figure 7B shows the responses to NKA, before and after exposure to $10^{-5}$ M capsaicin.

Discussion

In addition to two non-mammalian tachykinins (eledoisin and physalaemin), three mammalian tachykinins (SP, NKA and NKB) produced a potent contraction of the rabbit iris sphincter muscle. The maximum efficacy was much the same, but the potencies varied slightly with the tachykinin. The rank order of potencies was eledoisin>NKB>physalaemin>SP>NKA, which does not fit the order for the SP-P and SP-E receptor subtypes deduced from the biological activities (15) or the three classes of tachykinin receptors proposed from the ligand binding experiments (16, 17). This suggests that 1) tachykinin receptors in the iris sphincter muscle may show a unique selectivity to tachykinins or 2) that more than two types of receptors may occur, resulting in a complex order of potencies.

The results obtained in the present study indicate that at least two subtypes exist in the iris sphincter muscle. First, based on time course of the tachykinin responses, the
tachykinins used can be classified into two groups; one group includes NKA and elenoidin which produced a rapid response, whereas the remaining tachykinins showed a slower time course. Secondly, the responses to NKA and elenoidin were preferentially inhibited by [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP, whereas those to SP, physalaemin and NKB were not significantly inhibited. Hosoki et al. (18) demonstrated that the response to elenoidin, but not to SP and physalaemin, in the rabbit iris sphincter muscle was selectively attenuated by the treatment with phenoxybenzamine. This agent selectively alkylates the SP-E receptor (19). Furthermore, [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP is an antagonist at all SP-E subtypes studied, with the exception of the hamster urinary bladder (20). Therefore, NKA and elenoidin seem to produce contraction through a SP-E receptor subtype in the iris sphincter muscle, while SP, NKB and physalaemin probably act on other subtype(s).

Electrical transmural stimulation, KCl and capsaicin produce trigeminal nerve-mediated tachykininergic contraction of the rabbit iris sphincter muscle (14, 21). In the present study, also, the responses to such stimuli were either abolished or were markedly attenuated in preparations which had been exposed to capsaicin. Inasmuch as [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP selectively inhibited the response to NKA among the three mammalian tachykinins, we then examined the effect of the antagonist on the trigeminal nerve-mediated responses. The results obtained clearly show that the responses induced by all three types of stimuli were significantly attenuated by the antagonist. It is not likely that this inhibition is due to non-specific effects because the cholineric responses to electrical transmural stimulation and carbachol and the residual response to KCl in capsaicin-treated preparations were not significantly depressed by the antagonist. Furthermore, the contractile responses to NKB and physalaemin were not at all affected by the antagonist, and the inhibition of SP response was feeble or not significant. Thus, it is likely that the attenuation of trigeminal, tachykininergic responses by the SP-antagonist is predominantly accounted for by an inhibitory action on the response to NKA or related substance(s) which are probably released from the trigeminal nerve; the possible involvement of the other tachykinins is not, however, ruled out.

CGRP coexists together with tachykinins in the sensory nerve (7). However, in the iris sphincter muscle, CGRP was without effect on the resting tension and on the responses to electrical transmural stimulation. Thus, CGRP appears to have no appreciable effect, either pre- or postsynaptically on the iris sphincter muscle.

It has been demonstrated that several substances coexist in the same neuron and that they may exert multiple neurofunctions (4, 22, 23). In the rabbit iris sphincter muscle, the existence of SP, NKA and NKB has been reported (24); therefore, physiological involvement of such tachykinins in the peripheral actions of the sensory nerve has to be given attention (25). The present observations suggest that the trigeminal nerve-mediated contractile response in the iris sphincter muscle is predominantly mediated by NKA. Further studies are being directed to elucidating why NKA preferentially produces the trigeminal response and to clarify the physiological functions of the coexisting tachykinins.

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