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Evidence for Multiple Tachykinin Receptor Subtypes on the Rabbit Iris Sphincter Muscle

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SUMMARY

The actions of mammalian tachykinins (substance P, substance K/neurokinin a, neuromedin K/neurokinin b) and non-mammalian tachykinins (eledoisin, kassinin, physalaemin) were compared on the rabbit pupillary sphincter. All acted as direct spasmogens with potencies in the order: eledoisin > physalaemin = neurokinin b = substance P > kassinin > neurokinin a. However, their actions could be divided into at least two categories on the basis of similar kinetics of contractions, differential sensitivity to the tachykinin antagonist (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) substance P and specific cross-protection against phenoxybenzamine inactivation by structurally related tachykinins. The relationship between these observations and the suggested "P" and "E" subtypes of tachykinin receptors is discussed.

Substance P (SP), a mammalian undecapeptide first isolated from equine brain and intestine (1), belongs to the tachykinin family of biologically active peptides sharing a common conserved C-terminal sequence: -Phe-*X*-Gly-Leu-Met-NH_2 (where *X* is either an aromatic or a branched aliphatic amino acid). Until recently, SP was thought to be the only mammalian tachykinin (2). Two other mammalian tachykinins, NK_a (neuromedin L) and NK_b (neuromedin K), have since been isolated and identified as dodecapeptide amides (3–6).

The new mammalian tachykinins, albeit sharing C-terminal sequence homologies with SP, have been shown to possess pharmacological effects different from those of SP (7–9). Based on pharmacological evidence, the existence of two SP receptor subtypes has been suggested, "SP-P" and "SP-E" (10). The "SP-P" receptor type is exemplified by the guinea pig ileum where the rank order of potency of the tachykinins is: Phy>/SP>/Ele Ele = Kass, and the "SP-E" type which is exemplified by the rat vas deference, with the rank order of potency Kass >/Ele ≫ SP = Phy. It has been suggested that NK_a may be the natural ligand for the purported "SP-E" receptor subtype and SP for the "SP-P" receptor subtype (11). Therefore, the two receptors may be more appropriately considered not as multiple SP receptors, but rather as multiple tachykinin receptors specialized for different tachykinins. The "SP-P"/SP-E" nomenclature which was originally developed for the mammalian smooth muscles has the value of distinguishing two major classes of tachykinins (those possessing aromatic and those with aliphatic amino acid residues at position 4 from the C-terminal) and is probably an oversimplification of the complexity of the tachykinin receptor subtypes. Suggestions have recently been forwarded for the existence of more than two tachykinin receptor types (12, 13).

The present study investigates the possibility of the existence of multiple tachykinin receptors on an isolated smooth muscle, the rabbit iris sphincter muscle. Several procedures have been used: treatment with a b-haloalkylamine to alkylate differentially the different receptors, application of the SP analogue, (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP to antagonize differentially the pharmacological effects of different tachykinins, and cross-protection by specific tachykinins against alkylation. The apparent dissociation constants of the various tachykinins were determined with Furchgott's procedure (14), and the validity of this method was also examined.

Experimental Procedures

The isolated rabbit iris sphincter muscle (15) was suspended in a 10-ml bath at 35° with KRB (in mM: glucose, 11.10; $NaHCO_3$, 25.01; NaCl, 118.05; KCl, 4.69; $NaH_2PO_4 \cdot 2H_2O$, 1.01; $MgCl_2 \cdot 6H_2O$, 0.54; $CaCl_2 \cdot 6H_2O$, 2.51) and was continuously gassed with 95% O_2/5% CO_2. Indomethacin at a concentration of 1 μg/ml was included in the KRB to suppress endogenous production of prostaglandins, which have been shown to contract the iris sphincter muscle (16). Isotonic contractions were recorded with an isotonic muscle transducer (Harvard apparatus, Cambridge, MA) coupled to a pen recorder (Rikadenki, Japan). The

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ABBREVIATIONS: SP, substance P; NK_a, neurokinin a; NK_b, neurokinin b; Phy or Ph, physalaeimin; Ele, eledoisin; Kass, kassinin; KRB, Krebs-Ringer bicarbonate; CCh, carbachol; PBZ, phenoxybenzamine; EDTA, ethylenediaminetetraacetic acid.

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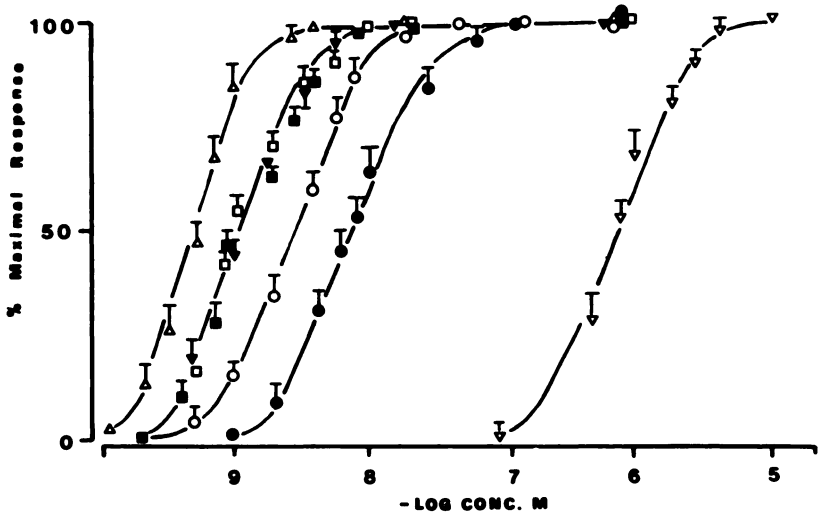

Fig. 1. Concentration response curves of various tachykinins on the contraction of the rabbit iris sphincter muscles. △, Ele; □, SP; ■, Phy; ▼, NK_b; ○, Kass; ●, NK_a; ▽, CCh. CCh was included for comparison. The results were expressed as means ± standard error. The estimated EC_{50} values of the tachykinins are found in Table 1.

TABLE 1

Comparison of the pharmacological potencies of various tachykinins on the rabbit iris sphincter muscle

Results are expressed as means ± standard errors.

Tachykinins	EC_{50}	N
	nM	
SP	1.60 ± 0.52	15
Ph	1.26 ± 0.07	8
Kass	3.11 ± 1.09	11
Ele	0.50 ± 0.10	15
NK_a	7.23 ± 0.51	8
NK_b	1.31 ± 0.71	6

tissue was initially allowed to equilibrate under a load of 150 ml for about an hour, during which the medium was replaced several times. The load was then replaced by a 50-mg counterweight which was used throughout the remainder of the experiment.

The muscle strip was initially exposed three times to supramaximal concentrations of CCh (20 μM), at 10-min intervals each. This procedure was found to stabilize the tissue to further responses to drugs and no desensitization to the effects of any of the tachykinins or CCh were observed. Subsequently, the preparation was exposed to different concentrations of the drugs. Both the order and concentrations of the different drug additions were randomized. At intervals during the sequence of applications of the drugs, the responses to doses of CCh (about 50% of the maximal response) were also obtained in order to monitor the stability of the tissue. Drugs were added in volumes not exceeding 2% of the bath volume and were allowed to be in contact with the tissue for 4 min, after which the tissue was washed twice with fresh KRB solution. A dosing cycle of 12–20 min was used in this study.

Antagonists were added to the bath 6–10 min before the addition of the test agonists. A dose of each tachykinin eliciting 50–80% of the maximal response was tested against increasing concentrations of the SP analogue, (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11}) SP. The effects of this antagonist were then expressed as the reductions in the response of the test agonist relative to the predetermined response in the absence of the antagonist.

To study the alkylating effects of PBZ, complete or partial dose response curves were established prior to alkylation. The strip was then exposed to PBZ for 5 min followed by four washouts and was allowed to reequilibrate for a further 30 min with repeated washings every 5 min. Partial or complete dose response curves were then reestablished. To decrease time-dependent changes in the contractility, paired test peptides with CCh of either SP and Ele, or Kass and Phy were studied in each strip.

In protection experiments, complete or partial dose response curves

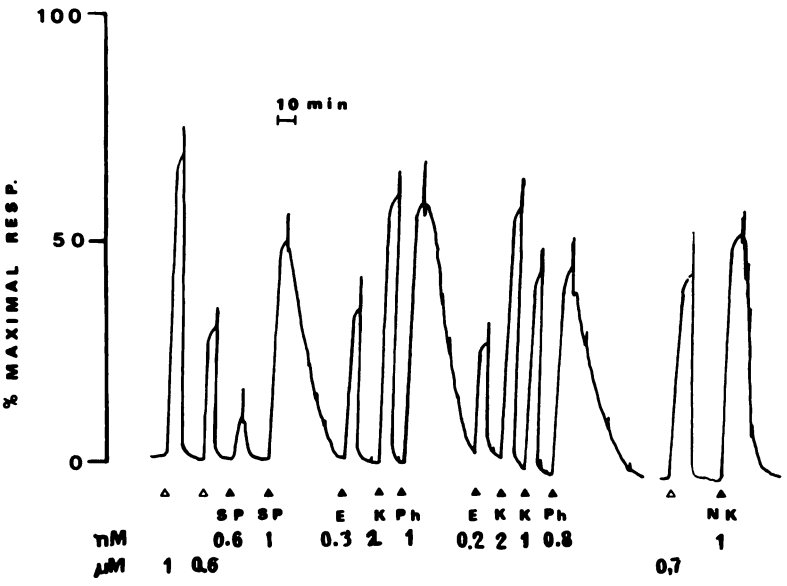

Fig. 2. Trace of tachykinin-induced contractions of the rabbit iris sphincter muscle. Note the slowly relaxation back to baseline after washout with SP and Ph. The Kass (*K*)- and Ele (*E*)-induced contractions were rapid in onset and offset. NK_a-induced contractions are identical to that of either Kass or Ele (not shown). CCh (△) was included for comparison. The contractions induced by NK_b (NK) were rapid in onset but intermediate to that of SP and Ph in offset. The concentrations of the drugs used were as shown in the figure.

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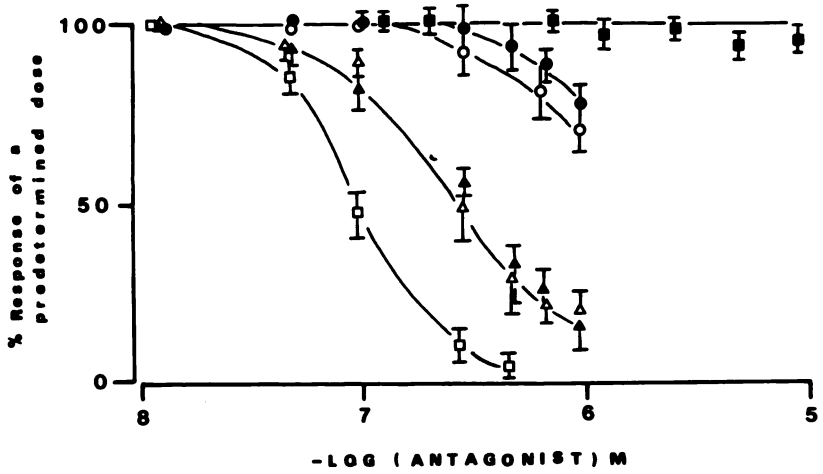

Fig. 3. Inhibition of various tachykinin contractions by the SP analogue, (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11])SP. Ele (□, 0.6 nM), Kass (△, 4 nM), and NK_a (▲, 9 nM) were inhibited to a greater extent than either SP (○, 2 nM) or Ph (●, 2 nM). NK_b-induced contractions (■, 2 nM) were not inhibited by this analogue at concentrations as high as 10 μM. Results were expressed as means ± standard errors of the reduction in the contractions of predetermined doses of each tachykinin ($N = 4$). The doses of the tachykinins used elicited 50–80% of the maximal response.

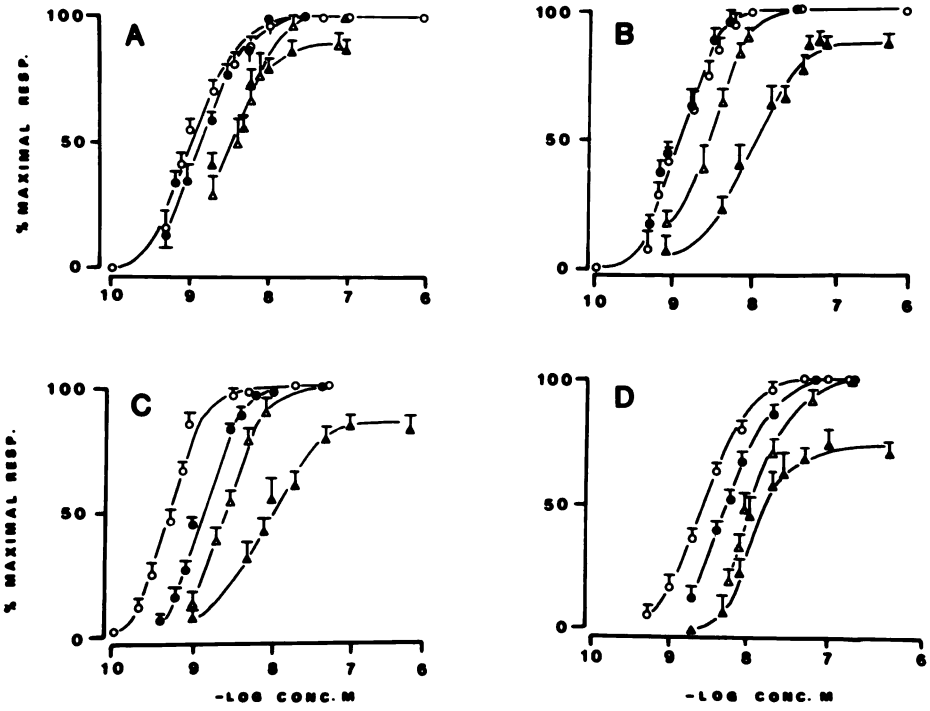

Fig. 4. Effects of various concentrations of PBZ on Ph (A)-, SP (B)-, Ele (C)-, and Kass (D)-induced contractions. ○, control, ($N = 6$); ●, effect of 20 μM PBZ in alcohol ($N = 5$); △, effect of 20 μM PBZ in HCl ($N = 6$); and ▲, effect of 200 μM PBZ ($N = 16$). Results were expressed as means ± standard errors.

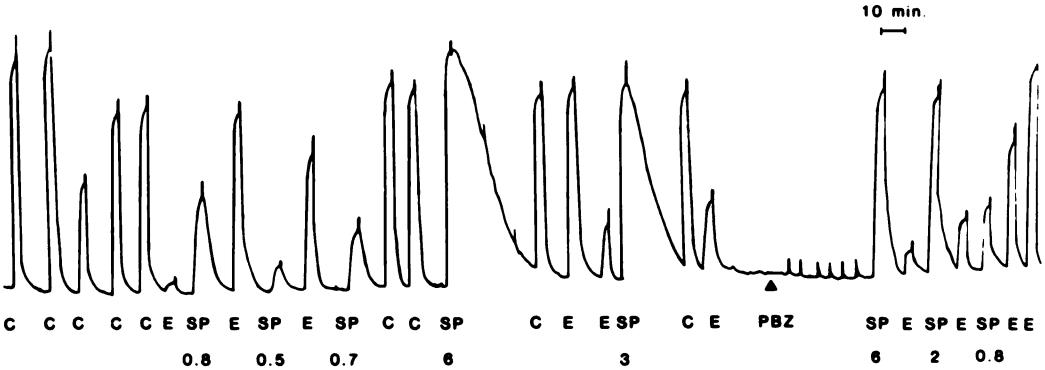

Fig. 5. The effects of 20 μM PBZ (dissolved in alcohol) on the contractions induced by SP and Ele (*E*). The concentrations of SP employed are given in nM. Note that the offset of contractions induced by SP were faster after PBZ treatment. CCh (*C*) was included to monitor the stability of the tissue.

were established initially. Ele (1 μM) or CCh (1 mM) was then applied to the bath 30 sec before the exposure to 200 μM PBZ for a further 5 min. The strip was then washed four times followed by a 30-min reequilibration period during which the strip was washed every 5 min. Dose response curves were subsequently reestablished. With tissues that have not fully relaxed after exposure to high concentrations of either Ele or CCh, further repeated washings at 5-min intervals were carried out until the baseline was reestablished before dose response curves were constructed.

The apparent equilibrium dissociation constants for the agonists were calculated from the dose response curves before and after fractional inactivation of the receptors by PBZ, using the equation derived by Furchgott (14):

$$1/A = 1/q \cdot 1/A' + (1 - q)/q \cdot 1/K_d$$

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TABLE 2

The effect of different concentrations of PBZ on the q values (fraction of pharmacologically active receptors remaining after inactivation by PBZ) and the apparent dissociation constants (K_d) of various tachykinins and CCh as calculated from Furchgott's equation

Substances	PBZ	q	K_d
	μM		nM
SP	20 (alc.)*	ND	ND
	20 (acid)	0.73 ± 0.06	6.82 ± 1.58
	200 (alc.)	0.25 ± 0.07	7.32 ± 0.53
Ph	20 (alc.)	ND	ND
	20 (acid)	0.37 ± 0.01	37.8 ± 5.6
	200 (alc.)	0.37 ± 0.04	20.8 ± 3.6
Ele	20 (alc.)	0.35 ± 0.03	7.38 ± 2.22
	20 (acid)	0.33 ± 0.01	7.11 ± 1.56
	200 (alc.)	0.16 ± 0.01	5.28 ± 1.41
Kass	20 (alc.)	0.46 ± 0.03	163 ± 43
	20 (acid)	0.34 ± 0.04	98 ± 11
	200 (alc.)	0.18 ± 0.06	167 ± 93
CCh	20 (acid)	0.01 ± 0.01	252 ± 63

* (alc.), PBZ dissolved in alcohol; ND, not determined; (acid), PBZ dissolved in acid (0.05 M HCl).

where A and A' are equipotent concentrations of the agonist before and after PBZ alkylation, respectively. The apparent dissociation constant of agonist-receptor complex, K_d, with dimensions of (A) and q is the fraction of initial concentration of total receptors for the given agonist remaining active after irreversible inactivation. The K_d and q values were estimated from pooled data of individual experiments under each condition and tabulated as mean ± standard error.

The fractional receptor occupancy for each agonist at each bath concentration (A) was calculated by using the equilibrium dissociation constant (K_d) as determined by Furchgott's method (14) and the following relationship (17):

$$(RA)/(Rt) = (A)/K_d + (A)$$

where (RA) is the concentration of the receptor-agonist complex and (Rt) is the total receptor concentration. The control response for each agonist is then replotted as a function of the negative logarithm of the percentage of receptor occupation $[(RA)/(Rt) \times 100]$. To estimate the relative amount of "spare receptor" of each agonist, their EC_{50} values as estimated from the dose response curves were compared with their pharmacologically estimated fractional receptor occupation, i.e., $(RA)/(Rt) \times 100$.

For [^{3}H]SP binding assays, rabbit eyes were obtained from a local source and transported on ice to the laboratory within 2 hr. The iris sphincter muscle strips were prepared as described above and were placed in ice-cold buffer A (50 mM Tris-HCl, pH 7.4). The strips (1.46 g, from a total of about 100 eyes) were then homogenized in 30 ml of ice-cold buffer B (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl) with an Ultraturrax homogenizer for 10–15 sec at maximal setting and then preincubated on ice in the presence of 300 mM KCl and 10 mM EDTA for 30 min. The homogenate was then centrifuged at 50,000 × g for 10 min. The pellet was resuspended in 30 ml of ice-cold buffer A (mid-setting, 10 sec) and subsequently centrifuged at 50,000 × g for 10 min. The final pellet was resuspended in 30 ml of buffer A and used for equilibrium receptor binding assay. The binding assay was initiated by the addition of 0.36 ml of the membrane suspension in triplicate to biovials containing 50 μl of varying concentrations of labeled [^{3}H]SP, with or without 1 μM unlabeled SP and a cocktail of peptidase inhibitors: 10 μl of bacitracin (2 mg/ml), 10 μl of leupeptin (0.2 mg/ml), 10 μl of bovine serum albumin (2 mg/ml), and 10 μl of chymostatin (0.1 mg/ml).

The assays were terminated after 30 min by the addition of 5 ml of ice-cold buffer A and immediately filtered through Whatman GF/C glass filters (previously pretreated with 0.1% polyethyleimine/0.02% bovine serum albumin for at least 3 hr at 4°). The filters were then rinsed three times with ice-cold buffer B and subsequently dried in air. Radioactivity on the filters was measured by liquid scintillation counting.

Specific binding was defined as that displaced by 1 μM unlabeled SP. All dilutions were done in buffer A and the incubations were performed at room temperature.

Materials. All peptides were purchased from Peninsula Laboratories (San Carlos, CA). PBZ hydrochloride (Dibenyline), cimetidine, and methysergide were gifts from Smith, Kline & French. CCh, yohimbine, propranolol, atropine sulfate and indomethacin were purchased from Sigma Chemical Co. Mepyramine was a gift from May & Baker. [^{3}H]SP was synthesized and characterized as described previously (18).

Results

The tachykinins were capable of producing as large a contraction as a maximally effective concentration of CCh. Tachyphylaxis was not observed with the concentrations employed and individual responses were reproducibly obtained to a given dose during the whole course of the experiment. The relative rank order of potencies of tachykinins and CCh was: Ele > SP = Ph = NK_b > Kass > NK_a ≫ CCh (Fig. 1). The estimated EC_{50} values of the tachykinins are given in Table 1. The onset of contractions was fast, usually between 5 and 10 sec on application to the bath. NK_a was notably faster in onset than any other agonist tested. However, the offset of contractions after washout differed significantly depending on the peptides studied. Both Ph- and SP-induced contractions persisted even after multiple washouts (Fig. 2). NK_b contractions were less

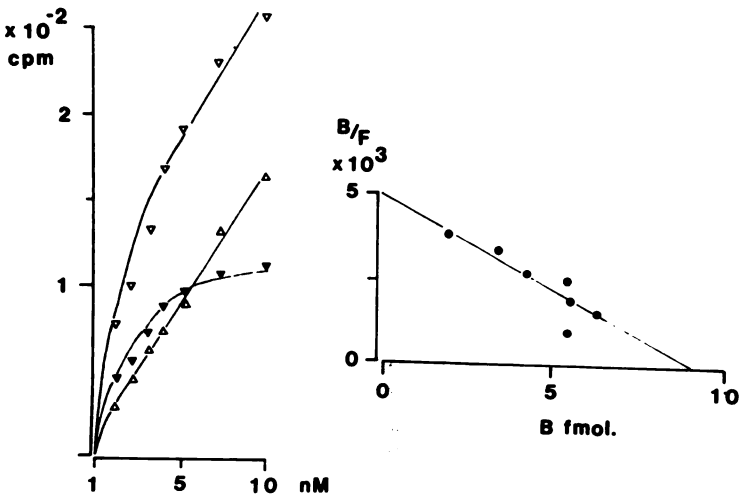

Fig. 6. Binding of [^{3}H]SP to the rabbit iris sphincter muscle homogenate. Specific binding (▼) is defined by the difference between total binding (◇) and the nonspecific binding (△) in the presence of 1 μM unlabeled SP. Each *point* is the mean of quaduplicates and the standard deviations were less than 10% of the means. *Inset*: Scatchard plot. The apparent dissociation constant was estimated to be 3.7 nM and the B_{max} was 0.52 pmol·g^{-1} (wet weight).

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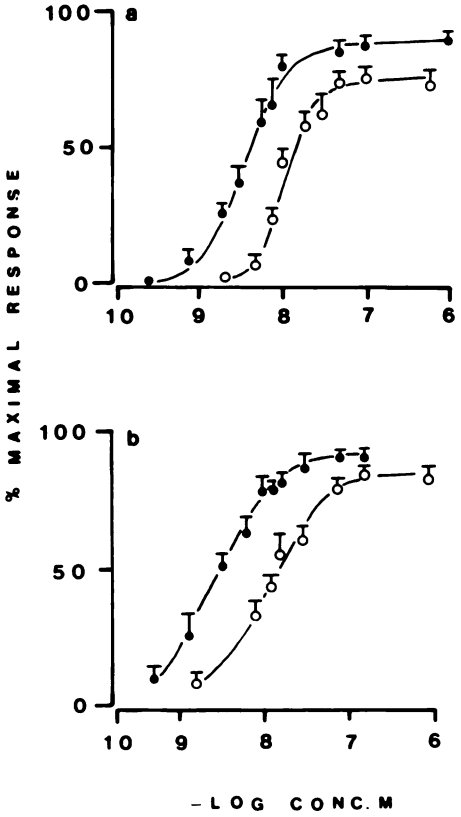

Fig. 7. Protection of the contractile effects of Kass (a) and Ele (b) from inactivation by 200 μM PBZ. Ele (1 μM) protected the Kass- and Ele-induced contractions. ○, "inactivated;" ●, protected by 1 μM Ele. Results are expressed as means ± standard errors (N = 10).

TABLE 3

Partial protection from PBZ inactivation by 1 μM Ele

The changes in the EC_{50} values (means ± standard errors) of the tachykinin concentration-response curves were analyzed by the Student's two-tailed unpaired t test. p values greater than 0.05 are considered insignificant. Before inactivation, the EC_{50} values were: SP, 1.6 nM; Phy, 1.3 nM; Kass, 3.0 nM; Ele, 0.5 nM.

Tachykinins	EC_{50} values		p Value
	200 μM PBZ	"Protected"	
	nM		
SP	13.12 ± 3.72	7.98 ± 0.13	0.05 $< p <$ 0.1
Phy	3.87 ± 1.44	1.44 ± 0.35	0.005 $< p <$ 0.01
Kass	17.12 ± 1.64	4.37 ± 0.61	0.000 $< p <$ 0.0005
Ele	15.17 ± 3.17	2.14 ± 0.32	0.0005 $< p <$ 0.0025

persistent than SP or Ph but were significantly more than that of either Ele, Kass, or NK_a. This slow relaxation after washout was found to be lengthened at higher concentrations. With a 1 μM concentration of either SP or Ph, the tissue could remain contracted for as long as 5 hr, showing little sign of relaxation even with multiple washings. However, Ele (1 μM) or Kass (1μM) contractions relaxed back to baseline in less than ½ hr after washout. Iris sphincter muscles from older animals showed greater delay in relaxation after washout for the same dose of Ph or SP than when compared to tissues from younger animals. These slow offset profiles of both Ph- and SP-induced contractions were not abolished even with Iris sphincter muscles prepared from isolated eyes which were stored for 2 days at 4°.

(D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP appeared to be more potent in inhibiting the contractions induced by either Ele,

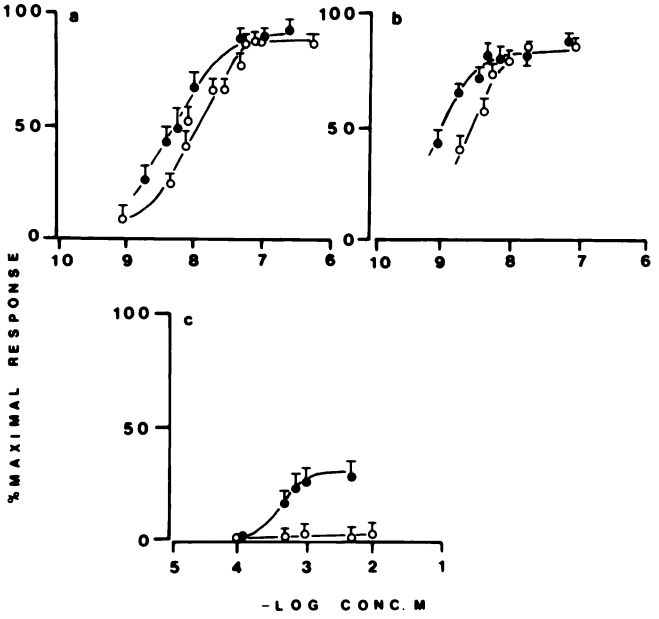

Fig. 8. Lack of protection of SP (a)-induced contractions from 200 μM PBZ inactivation by 1 μM Ele. Contractions induced by Ph (b) and CCh (c) were partially protected by 1 μM Ele. ○, "inactivated;" ●, protected by Ele. The EC_{50} value of CCh prior to PBZ inactivation was 0.74 ± 0.01 μM. Results are expressed as means ± standard errors (N = 10).

Kass, or NK_a than was SP or Ph (Fig. 3). NK_b contractions were not inhibited by this analogue at concentrations as high as 10 μM. No agonist actions of the analogue were observed even at concentrations as high as 1.65×10^{-4} M. An equilibration period of 5 min was sufficient for the maximal inhibition of the tachykinins. The antagonism could be reversed within 20 min of removal of the antagonist. With multiple applications of the antagonist, the time course of contraction persisting after washout of SP, Ph, and NK_b was shortened (data not shown). In a series of parallel experiments, control strips were exposed to the vehicle in which the antagonist was dissolved and there were no observable changes in the persistence of contractions of any of the three peptides. CCh-induced contractions were not affected by this antagonist even at concentrations as high as 10 μM. Another SP antagonist, (D-Pro[2], D-Trp[7,9]) SP (a gift from Dr. K. Folkers), showed a slight agonistic effect when used at a concentration of 6.6×10^{-5} M.

Neither the contractions induced by tachykinins nor the slow offset profiles of Ph, NK_b, and SP contractions after washout were affected by yohimbine (10 μM), propranolol (10 μM), mepyramine (10 μM), cimetidine (10 μM), methysergide (10 μM), dPTyr(Me)AVP (1 μM), atropine (10 μM), indomethacin (1 μM), or tetrodotoxin (1 μM).

PBZ was found to dissolve in alcohol with greater ease than in 0.05 M HCl. However, PBZ dissolved in 0.05 M HCl showed slightly greater potency in inhibiting tachykinin-induced contractions than when it was dissolved in alcohol.

Although there were no significant changes in the dose response curves of both Ph (Fig. 4A) and SP (Fig. 4B) after treatment with *20 μM* PBZ dissolved in alcohol, the relaxation profiles of Ph and SP after washout were changed (Fig. 5). This was not due to temporal changes in contractility as in a series of parallel studies; the control strips were exposed to the same conditions but with 20 μl of alcohol instead of PBZ, and no subsequent changes in the contractility and relaxation profiles

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TABLE 4

Amino acid sequences of some naturally occurring tachykinins

Neurokinin a, neurokinin b, and substance P are mammalian tachykinins. Pyr, pyroglutamic acid residue.

Neurokinin a			His-	Lys-	Thr-	Asp-	Ser-	Phe-	Val-	Gly-	Leu-	Met-	NH_2
Kassinin	Asp-	Val-	Pro-	Lys-	Ser-	Asp-	Gln-	Phe-	Val-	Gly-	Leu-	Met-	NH_2
Neurokinin b			Asp-	Met-	His-	Asp-	Phe-	Phe-	Val-	Gly-	Leu-	Met-	NH_2
Eledoisin		Pyr-	Pro-	Ser-	Lys-	Asp-	Ala-	Phe-	Ile-	Gly-	Leu-	Met-	NH_2
Substance P		Arg-	Pro-	Lys-	Pro-	Gln-	Gln-	Phe-	Phe-	Gly-	Leu-	Met-	NH_2
Physalaemin		Pyr-	Ala-	Asp-	Pro-	Asn-	Lys-	Phe-	Tyr-	Gly-	Leu-	Met-	NH_2

after washout of Ph and SP were observed. Both the Ele and Kass dose response curves were shifted to the right with no change in the maximal response to high concentrations of the peptides (Fig. 4, C and D). However, the CCh dose response curve was shifted by more than 1000-fold to the right, and the maximal contractions even at very high CCh concentrations were reduced to as much as 50% of the control (data not shown).

At an order of magnitude higher PBZ concentration (*200 μM* in alcohol), Kass-, Ele-, and SP-induced contractions were inhibited to a greater extent than that of Ph (Table 2). The maximal contractions to all tachykinins were reduced (Fig. 4). As with the effect of 20 μM PBZ, the slow relaxation after washout of contracted tissues of Ph or SP was accelerated.

A comparison of the q and K_d values as determined from Furchgott's plot are given in Table 2. Ph contractions were more resistant to inactivation by PBZ than Kass, Ele, and SP. The K_d values of an agonist as determined from inactivation by different concentrations of PBZ were very similar. The rank order of potency with respect to the K_d values are as follows: Ele = SP > Ph > Kass ≫ CCh. Although Ph and SP were about equipotent in contracting the sphincter muscle, their K_d values differed (6.82–7.32 nM for SP and 20.8–37.8 nM for Ph, Table 2).

We have evaluated the predicted K_d value for SP by a direct determination of K_d using saturation binding with [^{3}H]SP. Employing this independent approach, a single population of apparently homogeneous sites, with K_d = 3.7 nM (B_{max} = 0.52 pmol/g of wet weight; Fig. 6) was identified, in good agreement with Furchgott's analysis (Table 2).

A μM concentration of Ele protected the tachykinin-induced responses differently (Figs. 7 and 8). It appeared that Ele had greater preference in protecting the contractile effects induced by Ele, Kass, and Ph than those induced by SP (Table 3). However, the protection was only partial, in that the tachykinins were less potent in contracting the iris sphincter muscle when compared to controls. Ele (1 μM) also protected CCh contractions slightly (Fig. 8) from PBZ inactivation. It was *not* possible to study the effects of 1 μM SP in protecting against alkylation by PBZ as 1 μM SP caused a prolonged contraction, even with multiple washouts, and the application of PBZ 30 sec after the addition of SP could not abolish this persistence in contraction. To ensure that high concentrations of Ele did not have adverse effects on the responses of the tissue to the agonists, a series of experiments was carried out in which the tissue received exactly the same treatments except that 20 μl of alcohol was used instead of PBZ and the subsequently established dose response curves for SP, Ph, and CCh were exactly the same as that before the exposure for 1 μM Ele. In contrast, the dose response curves of Kass and Ele were slightly shifted to the right but with no change in the maximal response to high doses of these peptides. This is probably due to desensitization or tachyphylaxis of the tissue to these peptides. To rule out the possibility of a physical occlusion of PBZ in contracted tissues, thereby falsely resulting in the protection of the tachykinin contractions, tissues were exposed to 1 mM CCh instead of 1 μM Ele and dose response curves of the agonists were subsequently established. No protection of Ele, SP, or Kass contractions against the inactivation by PBZ was observed (data not shown), but Ph contractions appeared to be slightly protected under such conditions.

Discussion

The observation that all of the tachykinins appeared to act on the rabbit iris sphincter muscle directly (15), without the involvement of nerve-mediated release of secondary myotropic substances (which could complicate the interpretation of the results), has enabled us to investigate the occurrence of multiple tachykinin receptors within a single tissue.

Based on the persistence of contractions after washout, the tachykinins can be divided into three basic subgroups. The first subgroup, consisting of Kass, NK_a and Ele, possesses characteristically fast onset and fast offset after washout. The second subgroup is made up of SP and Ph, which have fast onset but significantly slower offset after washout. The third subgroup is made up of NK_b, which showed offset characteristics intermediate to the above two (Fig. 2). Interestingly, the converse pattern exists in the rat duodenum and urinary bladder, where Ele and Kass but not Ph showed slow relaxation of contraction after washout (19). It is interesting to note that the three functional subgroups share certain sequences (Table 4): 1) an aromatic (Phe/Tyr) versus a non-aromatic hydrophic residue (Ile/Val) in positions 4 from the C-terminus; 2) a neutral amino acid (Gln or Asn) versus an acidic amino acid (Asp) at position 7 from the C-terminus; 3) proline at position 8 from the C-terminus versus a variable residue; and 4) a conserved C-terminal amino acid sequence: Phe-*X*-Gly-Leu-Met-NH_2.

It appeared that the most important region for tachykinin activity is the C-terminal pentapeptide (20). However, the residue substitutions noted above undoubtably account for the pharmacological differences. In accordance with the nomenclature proposed by Iversen *et al.* (21), the former subgroup has been called the "SP-E" type (Kass, Ele, and NK_a) and the latter as the "SP-P" type (SP and Ph). Although NK_b had many structural similarities to the "SP-E"-type ligand, it displayed pharmacological effects that appeared not to fit into either of the two types.

The SP antagonist, (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) SP, has been reported to antagonize the effects induced by various tachykinins (22). However, its specificity may not be absolute, as it has been reported to antagonize the actions of bombesin and vasopressin (23). The possibility of an endogenous bombesin- and vasopressin-like peptide released by the tachykinins

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contributing to the observed responses was ruled out because bombesin as high as 1 μM did not affect the rabbit iris sphincter muscle and because the potent vasopressin antagonist, dPTyr(Me)AVP, was found not to antagonize the effects induced by the tachykinins (15). The lack of contractile effects of the antagonist would rule out the possibility of cross-tachyphylaxis between the antagonist and the tachykinins. Moreover, a noncumulative approach was used for this study to avoid the possibility of cross-tachyphylaxis. The antagonist inhibited the "SP-E"-type ligands somewhat more than the "SP-P"-type ligands and, surprisingly, did not inhibit NK_b contractions at concentrations as high as 10 μM (Fig. 3). The difference in the degree of antagonism of the two groups ("SP-E" and "SP-P") was not very large, only about 10 times when comparing their IC_{50} values. Earlier findings using the same antagonist suggested a greater selectivity for the "SP-E"-type responses than for the "SP-P" type (24), but subsequent studies by Watson (22) suggested a lack of selectivity in inhibiting the two types of responses.

The results obtained in this study on the inactivation of tachykinin receptors with PBZ were comparable to that reported by Lin and Musacchio (25) for the guinea pig ileum, with similar K_d values for SP, Ph, and Kass. However, the q values differed from those reported herein and may be due to the longer exposure time to PBZ in their experiments.

The validity of this technique for the determination of both the K_d and q values has recently been challenged (26, 27). However, not all of the alkylation studies showed discrepancies in the K_d values as compared to *in vitro* radioligand binding studies (28–30). The K_d for CCH (252 μM), as determined herein on the rabbit iris sphincter muscle, was very similar to that reported for the low affinity site (290 μM) on the longitudinal muscle strips from the guinea pig ileum as determined from agonist inhibition curves of [^{3}H]PrBCM binding to intact tissue (31). The estimated K_d for SP was approximately 6 nM (Table 2) as compared to 3.7 nM as determined by [^{3}H]SP (Fig. 6). The K_d values estimated after exposure to different concentrations of PBZ did not differ significantly for any of the tachykinins (Table 2). Similarly, the K_d values estimated after exposure to different concentrations of dibenamine did not differ significantly for a variety of α-adrenergic agonists (30). These observations may lend support to the validity of this technique for the determination of K_d for some receptors.

Evidence for a separate type of interaction of PBZ and dibenamine with calcium channels is accumulating (26, 32). This observation may be related to the slow relaxation of SP and Ph contractions after washout, which is attenuated by PBZ. SP and Ph responses may thus involve events resulting in prolonged calcium channel activation which is unlike the Kass or Ele responses.

Employing the K_d values as determined by this technique, calculations using the relationship:

$$(RA)/(Rt) = (A)/K_d + (A)$$

to determine the fractional receptor occupation (17), it was found that an occupation of about 14% of the total receptors by SP is sufficient to elicit 50% of the response [similar to the finding reported by Lin and Musacchio (25)] as compared to 6.8% for Ele and 2% for Kass. If both Ele and Kass were to act on the same receptor and SP were to act through another, then the former receptor type would appear to have a greater "receptor reserve." It must be emphasized that this does not necessarily imply that the absolute number of receptors of the former is greater than that of the latter type (29). In relation to ocular injury response, an endogenous ligand preferring the former receptor type with the apparent receptor reserve and rapid onset of contraction would be ideal for inducing rapid miosis.

Protection experiments using 1 μM Ele suggest the presence of two tachykinin receptor types. Protection of Ele- and Kass-, but not SP-induced responses, from PBZ inactivation by Ele pretreatment suggests that a common receptor site or mechanism may mediate the actions of Ele and Kass. Although Ph has structures similar to SP and showed similar characteristics in both the persistence in contractions after washout and the lack of inhibition of the analogue, (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP (Fig. 3), there is an important pharmacological difference between the two peptides. High concentrations of Ele (Fig. 8b) and CCh (data not shown) appeared to protect responses to Ph but not SP from alkylation. It may be that Ph acts in part through another site which is less susceptible to alkylation, especially when the tissue is contracted by high concentrations of either Ele or CCh. In contrast to the results here, earlier work (25) reported that CCh could cross-protect SP against PBZ inactivation but not the reverse, whereas the observations herein showed cross-protection of Ph by CCh but not for the other tachykinins. It is unclear what the origin of the differences may be.

In conclusion, the evidence as summarized below suggests the presence of at least two tachykinin receptors on the rabbit iris sphincter muscle: (i) similar durations of action of related tachykinins, (ii) differential inhibition with (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})SP, (iii) differential inactivation by PBZ, and, importantly, (iv) highly selective protection against PBZ inactivation by related tachykinins. Consequently, the treatment of smooth muscles as "SP-P" and "SP-E" type is likely to be an oversimplification, and both receptor types may be found on the same tissue in some instances. The existence of possibly three different tachykinin receptor subtypes has recently been suggested (12, 13). This naturally raises the question as to the physiological roles of these multiple tachykinin receptor subtypes in the rabbit iris sphincter muscle.

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