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Neurokinin B is a preferred agonist for a neuronal substance P receptor and its action is antagonized by enkephalin

(tachykinins/opiate receptor/acetylcholine/guinea pig ileum/receptor subtypes)

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ABSTRACT **Receptor specificity of the substance P-related peptides neurokinin A and neurokinin B was studied in the isolated guinea pig ileum. Substance P and the recently discovered neurokinins elicit contraction of the ileum both directly through action on a muscle cell receptor and indirectly through stimulation of a neuronal receptor, leading to release of acetylcholine, which causes muscle contraction via muscarinic receptors. Two specific assay procedures for the function of the neuronal receptor were developed. The muscular receptor was inactivated either by desensitization with the selective agonist substance P methyl ester or by receptor blockade with the selective antagonist [Arg^6, D-Trp^{7,9}, Me-Phe^8]substance P-(6–11) hexapeptide. Both procedures revealed that the neuronal receptor is clearly distinct from the muscular receptor, since it exhibits different agonist specificity and is insensitive to antagonists of the muscular receptor. Neurokinin B was found to be the most potent agonist (EC_{50} = 1 nM) for the neuronal receptor. Furthermore, [D-Ala^2, Met^5]enkephalinamide inhibited in a naloxone-sensitive manner the effect of neurokinin B mediated via the neuronal receptor. These results suggest that the different mammalian tachykinins can play specific physiological roles by virtue of their distinct receptor specificities.**

The tachykinins are a family of biologically active peptides that share the common COOH-terminal sequence Phe-Xaa-Gly-Leu-Met-NH_2 (1). For a long time, substance P, a putative neurotransmitter or neuromodulator (2, 3), was the only tachykinin known to occur in the mammalian nervous system. Substance P elicits a variety of pharmacological responses (3). Of particular interest is its role in the transmission of pain stimuli, as well as its interaction with the analgesic enkephalin peptides (2, 4–6). Recently, two additional mammalian tachykinins, designated neurokinin A and neurokinin B, were discovered in the porcine spinal cord (7, 8) (Fig. 1).

The existence of a substance P-related family of neuropeptides emphasizes the question of the physiological significance of this multiplicity of peptides. One hypothesis that could explain different physiological roles played by specific substance P-related peptides is the existence of tachykinin receptor subtypes, each responsible for a specific physiological response. The concept of two types of substance P receptors was suggested by Iversen and coworkers (9). It was based on a different order of relative potencies of a series of nonmammalian tachykinins in eliciting contraction of various smooth muscle preparations (9–11). The currently accepted classification describes one receptor subtype (the SP-P receptor) on which physalaemin is moderately more potent than the other tachykinins and a second receptor subtype (SP-E receptor) for which eledoisin and kassinin are considerably more potent than substance P or physalaemin (11).

In the present work we have taken advantage of the finding that the substance P response of the isolated guinea pig ileum also has a significant cholinergic component (12–14). In this preparation, the substance P-induced contraction is mediated in part by a direct action of the peptide on the muscle cells and in part indirectly via release of acetylcholine (13, 14). Thus, the guinea pig ileum offers the opportunity to study, in the same preparation, both a neuronal and a muscular receptor for substance P. Furthermore, the guinea pig ileum has the advantage that its neuronal circuitry is much simpler than that of the central nervous system and yet it is sufficiently developed to allow a functional assay of the effect of enkephalins (15).

Characterization of the neuronal substance P receptor in the guinea pig ileum preparation revealed that neurokinin B is a preferred agonist for this receptor and that the activity of neurokinin B can be inhibited by an enkephalin analog.

MATERIALS AND METHODS

Substance P, substance P methyl ester, physalaemin, atropine, and tetrodotoxin were obtained from Sigma. Eledoisin and kassinin were purchased from Novabiochem (Läufelfingen, Switzerland) and neurokinin A and neurokinin B, from Cambridge Research Biochemicals (Harston, U.K.). Physalaemin and eledoisin were also obtained from Farmitalia, and neurokinin A, from Peninsula Laboratories. [D-Pro^2, D-Trp^{7,9}]-substance P and [D-Ala^2, Met^5]enkephalinamide were purchased from the Peptide Institute (Osaka, Japan).

The substance P antagonist [Arg^6, D-Trp^{7,9}, Me-Phe^8]substance P-(6–11) hexapeptide (SP-Ant) (16, 17) was prepared by solid-phase synthesis. In the isolated guinea pig ileum assay (in the presence of 1 μM atropine), 0.2 μM of the antagonist reduces the effect of a double dose of substance P to that of a single dose (pA_2 = 6.7).

Isolated smooth muscle preparations (11) were suspended in a 10-ml organ bath containing Tyrode's solution (composition in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5, NaH_2PO_4, 1.0; $NaHCO_3$, 25; and glucose, 11), gassed with a 95% O_2/5% CO_2 mixture and maintained at 34°C. Contractions were recorded isotonically under a resting load of 0.3–0.8 g. Before the experiments were started, tissues were equilibrated for 30–60 min. Peptides were applied at 2- to 3-min intervals with less than 30 sec of contact time (guinea pig ileum) or at 15- to 30-min intervals with a contact time of 1–3 min (urinary bladder preparations). The effects of various

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Abbreviation: SP-Ant, [Arg^6, D-Trp^{7,9}, Me-Phe^8]substance P-(6–11) hexapeptide.

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Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

FIG. 1. Structures of the mammalian tachykinins. Residues identical in all three peptides are underlined.

drugs and antagonists on the contractile response elicited by an agonist were determined by applying these compounds 1–2 min prior to addition of agonist.

At the start of a desensitization experiment, the tissue was incubated with a high dose (0.1 μM) of substance P methyl ester until the response had faded to the baseline level (2–4 min). The tissue was then washed and immediately reincubated with substance P methyl ester (0.1 μM) for 2 min. A test dose of a given agonist was then added, the contraction was recorded (less than 30 sec of contact), and the tissue was washed. This cycle (substance P methyl ester for 2 min, agonist test dose, washout) was repeated for each test dose of agonist.

The results for each agonist are expressed as a percentage of the maximal contraction that could be obtained with that particular compound. All tachykinins produced similar maximal contractions in a given test preparation. Relative potencies were calculated from EC_{50} values (concentration of agonist producing 50% of the maximal contraction).

RESULTS

The potencies of agonists of the muscular SP-P receptor were determined by performing experiments in the presence of the muscarinic blocker atropine (1 μM) (9) and are summarized below. To determine the pharmacological profile of the neuronal receptor, it was first necessary to eliminate the component of the contractile response mediated through direct action of tachykinin agonists on the muscular receptor. This was achieved by two different methods, specific desensitization or blockade with an antagonist of the muscular SP-P receptor.

Substance P methyl ester is a specific SP-P receptor agonist equipotent to substance P (11). When applied to the ileum at a high concentration (0.1 μM), it produced a large contraction that faded to baseline after a contact time of 2–4 min. In the continuous presence of substance P methyl ester (0.1 μM), the tissue became insensitive to further test doses of that peptide (Table 1). The EC_{50} of substance P methyl ester was increased by greater than 300-fold, indicating strong inactivation of the SP-P receptor-mediated contractile response. Substance P, however, was still active in the ileum pretreated with substance P methyl ester, although its dose-response curve was significantly (20- to 30-fold) shifted to the right (Fig. 2*A*). On the other hand, the response to neurokinin B was not significantly affected by desensitization with substance P methyl ester (Table 1 and Fig. 2*A*).

Table 1. Activity of substance P-like peptides in the guinea pig ileum after inactivation of the SP-P receptor

	EC_{50}, nM		
		After pretreatment	
Test peptide	Before inactivation	Substance P methyl ester	SP-Ant
Substance P methyl ester	3.4 ± 0.5 (5)	>1000 (4)	270 ± 32 (2)
Substance P	2.7 ± 0.1 (5)	60 ± 18 (8)	41 ± 11 (5)
Neurokinin B	1.0 ± 0.3 (5)	1.5 ± 0.3 (9)	0.4 ± 0.03 (3)

Tissues were pretreated with either substance P methyl ester (0.1 μM, 2 min) or SP-Ant (5 μM, 1–2 min) and not washed before application of a test dose of peptide. The EC_{50} values were derived from complete dose–response curves obtained with several concentrations of test peptide. The number of experiments, performed in independent preparations, is indicated in parentheses. Each value is the mean ± SEM.

Similar results were obtained when the ileum was pretreated with SP-Ant. In the presence of antagonist (5 μM), the sensitivity of the tissue to substance P methyl ester was reduced by a factor of about 80 (see Table 1). Thus, this treatment also led to inactivation of the SP-P receptor, although to a lesser extent than desensitization with substance P methyl ester. We did not, however, use higher concentrations of the antagonist, since preliminary results had indicated that this compound, like other substance P antagonists (18), was toxic at high concentrations. The dose–response curve of substance P was shifted 10- to 20-fold to the right (Fig. 2*B*), and the response of the tissue to neurokinin B was not reduced by pretreatment with SP-Ant (Table 1 and Fig. 2*B*). Similar results were obtained after pretreatment of the ileum with the substance P antagonist (17) [D-Pro^2, D-Trp^{7,9}]substance P (1 μM; results not shown). It should be noted that neither SP-Ant (5 μM) nor [D-Pro^2, D-Trp^{7,9}]substance P (1 μM) had any effect on the contractile activities of substance P and neurokinin B in an ileum preparation desensitized with substance P methyl ester (results not shown).

Since inactivation of the SP-P receptor did not reduce the potency of neurokinin B, it appears that this peptide also potently stimulates a distinct receptor subtype that does not interact with substance P methyl ester and the substance P

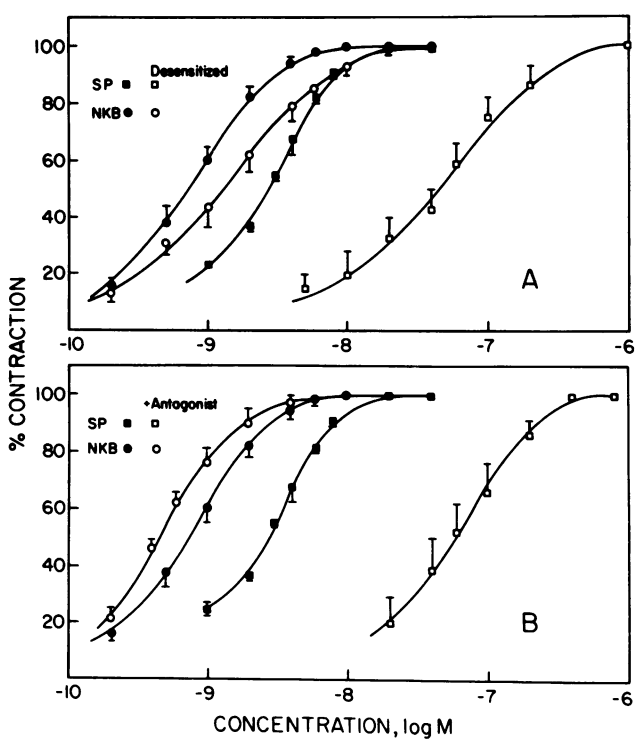

FIG. 2. Contractile effects of substance P (SP) and neurokinin B (NKB) in the guinea pig ileum, before and after inactivation of the SP-P receptor. The tissues were pretreated with either substance P methyl ester (0.1 μM, *A*) or SP-Ant (5 μM, *B*) as described in the legend to Table 1. Dose–response curves were determined before (closed symbols) and after (open symbols) either treatment. Results are mean ± SEM from three to eight experiments.

antagonist. On the other hand, the shift in the dose–response curve of substance P after inactivation of the SP-P receptor indicates that although substance P also stimulates the second receptor subtype, it interacts much more potently with the SP-P receptor.

In the ileum desensitized with substance P methyl ester, atropine antagonized the contraction caused by neurokinin B in a concentration-dependent and noncompetitive manner (see Fig. 3). In this preparation, atropine (1 μM) also abolished the effect of 0.1 μM substance P and strongly reduced that of 1 μM substance P (less than 50% contraction). Also in the presence of SP-Ant, atropine (1 μM) totally inhibited the contractile effect of substance P (0.1 μM) and neurokinin B (1 μM). These results provide definitive evidence for the inactivation of more than 99% of the SP-P receptors and also show that after desensitization or blockade of the muscular receptor, virtually all of the activity of the tachykinins is mediated through the release of acetylcholine. It should be emphasized that neither desensitization nor pretreatment with the antagonist had any significant effect on the response of the ileum to carbamoylcholine (results not shown).

[D-Ala^2, Met^5]Enkephalinamide inhibited the contractile effect of neurokinin B in the desensitized ileum (Fig. 4). This inhibition was completely prevented by naloxone, a specific antagonist of opiate receptors. Tetrodotoxin also inhibited the effect of neurokinin B in this preparation, indicating that nerve conduction was involved in mediating the response. These data are in agreement with the observation of Holzer and Lembeck (12) that the remaining contraction of the guinea pig ileum after prolonged exposure to substance P is decreased by morphine and tetrodotoxin.

To determine whether activation of the neuronal tachykinin receptor led to the release of neurotransmitters other than acetylcholine, the effects of various antagonists on the contractile potency of neurokinin B were studied in the desensitized ileum. The following compounds did not produce any significant reduction of the potency of neurokinin B: chlorpheniramine (1.5 μM), methysergide (10 μM), metiamide (1 μM), propranolol (2.5 μM), phentolamine (1 μM), theophylline (0.5 mM), and indomethacin (10 μM). Moreover, pretreatment of the tissue with desensitizing doses of serotonin (10 μM) or bradykinin (10 μM) did not affect the response to neurokinin B (results not shown). It is therefore unlikely that histamine, serotonin, norepinephrine, adenosine, prostaglandins, or bradykinin are involved in the neuronal response to neurokinin B.

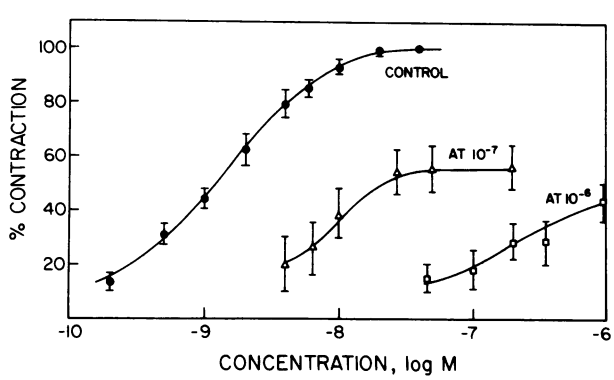

FIG. 3. Atropine inhibits the contractile effect of neurokinin B in the guinea pig ileum desensitized with substance P methyl ester. The tissues were pretreated with substance P methyl ester (0.1 μM, 2 min) as described in the legend to Table 1, and a dose–response curve for neurokinin B ("control") was constructed. The experiment was then performed in the presence of atropine (10^{-7} M or 10^{-6} M) added to the organ bath 1–2 min prior to a test dose of neurokinin B. Each point represents the mean ± SEM from three experiments.

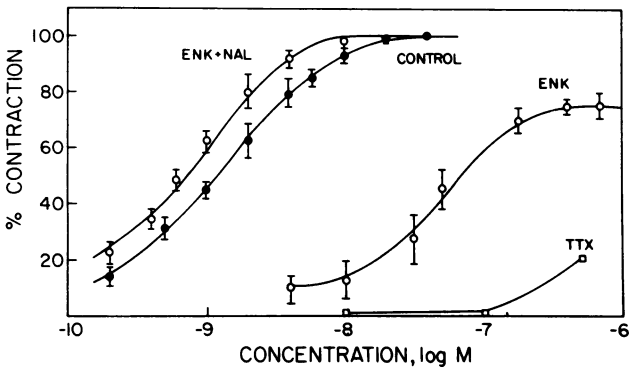

FIG. 4. [D-Ala^2, Met^5]Enkephalinamide and tetrodotoxin inhibit the contractile effect of neurokinin B in the guinea pig ileum desensitized with substance P methyl ester. The control curve for neurokinin B was determined as described in the legend to Fig. 2. A dose–response curve for neurokinin B was then established in the presence of 0.2 μM [D-Ala^2, Met^5]enkephalinamide (ENK), added 1–2 min prior to a test dose of neurokinin B. The experiment was then repeated, using 1 μM [D-Ala^2, Met^5]enkephalinamide, in the presence of 1 μM naloxone (ENK + NAL). Tetrodotoxin (TTX, 0.5 μM) was applied to the organ bath 2 min prior to a test dose of neurokinin B. Tetrodotoxin did not inhibit the contractile response to carbamoylcholine (not shown). Each point represents the mean ± SEM from two or three experiments.

The potencies of tachykinins relative to substance P on the contraction of the guinea pig ileum, mediated through either the muscular or the neuronal receptor, are summarized in Table 2. The potencies for stimulation of the muscular receptor were determined in the presence of atropine, and those for the neuronal receptor were determined on either desensitized or antagonist-treated tissues. In agreement with previous reports (9, 19, 20), all tachykinins tested had comparable potencies on the muscular SP-P receptor. The rank order of potencies was physalaemin > substance P ≈ neurokinin B ≈ kassinin > eledoisin > neurokinin A. However, the rank order of tachykinin potencies on the neuronal receptor was significantly different, neurokinin B being 30–70 times more potent than substance P: neurokinin B > eledoisin > kassinin > physalaemin > neurokinin A ≈ substance P. There was a good correlation between the results obtained in desensitized or antagonist-treated tissues, indicating again that either of these procedures reliably measured the neuronal response to tachykinins.

The potencies of tachykinins were also determined on two classical SP-E systems, hamster and mouse urinary bladders. Neurokinins A and B were found to be approximately equipotent with eledoisin, and about 100 times more potent than substance P in these systems. Table 2 summarizes these results, together with those obtained by other workers, using classical SP-E systems (11, 19).

DISCUSSION

The present results show that neurokinin B is a preferred agonist (50-fold more potent than substance P and neurokinin A) for a neuronal receptor in the guinea pig ileum that mediates the release of acetylcholine from cholinergic neurons. The neuronal receptor is clearly distinct from the muscular SP-P receptor, since both desensitization with a specific SP-P agonist and blockade with specific antagonists decreased by more than two orders of magnitude the response mediated by the muscular receptor, and yet it had little or no effect on the neuronal receptor. Previous studies had suggested heterogeneity of substance P receptors in the guinea pig ileum (9, 14, 21, 22). None of these studies, however, used a specific agonist and antagonist to differen-

Table 2. Relative tachykinin potencies on the contraction mediated through the muscular and the neuronal tachykinin receptors in the guinea pig ileum, and on classical SP-E systems

	Guinea pig ileum			
	Neuronal receptor		Muscular	
Peptide	A	B	receptor	SP-E systems
Substance P	1.0	1.0	1.0	1.0
Neurokinin A	1.3 ± 0.1 (5)	1.6 ± 0.3 (3)	0.12 ± 0.02 (3)	100 -340
Neurokinin B	31.5 ± 3.6 (7)	74 ± 29 (3)	0.94 ± 0.32 (3)	50 -100
Physalaemin	2.8 ± 0.1 (2)	1.6 ± 0.3 (2)	1.4 ± 0.1 (3)	0.6- 1.1
Eledoisin	8.1 ± 1.6 (6)	5.6 ± 1.0 (3)	0.55 ± 0.22 (2)	90 -120
Kassinin	5.0 ± 0.5 (2)	3.1 (1)	0.90 ± 0.20 (2)	160 -500

Relative potency (substance P = 1) is defined as the ratio of EC_{50} of substance P to EC_{50} of peptides tested. Potencies on the neuronal receptor were determined either in the presence of substance P methyl ester (A) or substance P antagonist (B) as described in the legend to Table 1. Potencies on the muscular receptor were determined in the presence of atropine (1 μM). The results for the guinea pig ileum system are means ± SEM of the relative potencies calculated for each experiment (number of experiments in parentheses). The results for SP-E systems represents extreme ranges of relative potencies measured in several assay systems (contraction of mouse and hamster urinary bladder, potentiation of electrically evoked contractions of rat vas deferens). These data were pooled from our own results (with urinary bladder preparations) and those reported by other workers (11, 19), except those for neurokinin B, which were from our laboratory only. In the guinea pig ileum, the EC_{50} of substance P for stimulation of the muscular receptor was 2.5 ± 0.1 nM (n = 3), and for the neuronal receptor it was 40–60 nM (see Table 1). In SP-E systems, the EC_{50} was 8–21 μM (11).

tiate between receptor subtypes, and the action of the mammalian tachykinins on these receptors was not studied. The order of potencies of the tachykinins on the contraction of the guinea pig ileum induced through the neuronal receptor is neurokinin B > eledoisin > kassinin > physalaemin > neurokinin A ≈ substance P. These results are in agreement with those of Fosbraey *et al.* (14), who reported the potencies of eledoisin, kassinin, physalaemin, and substance P in evoking release of acetylcholine from the guinea pig myenteric plexus.

It should be noted that the specificity of the neuronal receptor in the guinea pig ileum differs significantly from that of classical SP-E systems. Thus, while kassinin and neurokinin A are about equipotent with neurokinin B, and up to 500-fold more potent than substance P in SP-E systems (11, 19), these peptides are less potent than neurokinin B, and only slightly more active than substance P, in stimulating the neuronal receptor. Moreover, the EC_{50} value of substance P on the neuronal receptor is 40–60 nM, while on SP-E systems it is about 100-fold higher (11). We propose therefore to designate the neuronal, neurokinin B-specific receptor "SP-N" in accord with the existing nomenclature of tachykinin receptors (9). Interestingly, the recently reported (23–25) tachykinin potencies in competing with a radiolabeled eledoisin analog for binding to rat brain preparations are similar to those we have determined for the ileal SP-N receptor. A growing body of evidence (24–26) thus points to the existence of three different tachykinin receptors in the mammalian nervous system, the SP-P, SP-E, and SP-N receptors. The most potent agonists for these receptors are substance P and neurokinin B, neurokinin A and B, and neurokinin B, respectively.

The question about the possible specific role of the recently discovered substance P-like peptides can now be answered in part by the demonstration of multiple receptor subtypes for which different tachykinins act as preferred agonists. To assess the physiological significance of these findings, more information will be needed on the distribution of the substance P receptor subtypes in the central nervous system, as well as on local concentrations of each one of the endogenous tachykinins. Work in this direction has been reported recently (27).

The current view on the interaction between substance P and enkephalins suggests that enkephalins inhibit the release of substance P via interaction with an opiate receptor located on substance P-containing neurons. Such an inhibition was found in rat trigeminal slices (5) and in isolated sensory ganglia cells in culture (6). Our findings suggest an additional mode of interaction between enkephalin and neurokinin B, in which each peptide, through interaction with a specific receptor, inhibits or induces, respectively, the release of a neurotransmitter from neuronal cells. The guinea pig ileum is a convenient model system in which functional assays of these receptors can be performed. Indeed, this system is used extensively for the evaluation of opiate analgesic drugs (15, 28). Whether the naloxone-sensitive inhibition by [D-Ala^2, Met^5]enkephalinamide of the SP-N receptor-mediated effect of neurokinin B in the guinea pig ileum might reflect a similar interaction in the central nervous system remains to be established. It is noteworthy, however, that the highest concentration of neurokinin B is found in the dorsal horn of the spinal cord (27), which is a major site of action of opiate analgesics. Thus, it is likely that the functional counteraction between neurokinin B and enkephalin found in the guinea pig ileum will be useful for evaluation of analgesic drugs. Furthermore, the development of antagonists for the SP-N receptor might lead to a new class of analgesic agents different from the opiates and perhaps devoid of the adverse effects of addiction, tolerance, and respiratory depression.

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