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^{3}H-Neurokinin A Labels a Specific Tachykinin-Binding Site in the Rat Duodenal Smooth Muscle

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SUMMARY

^{3}H-Neurokinin A (^{3}H-NKA) with high specific activity (75 Ci/mmol) was synthesized to study NKA (NK-2)-binding sites on membrane preparations of various tissues in the rat, including brain, spinal cord, duodenum, vas deferens, and ileum. The binding capacity of ^{3}H-NKA (0.9 nM) was very low in membrane preparations of different central nervous system regions and the ileum smooth muscle (0.2–2 fmol/mg of protein). In contrast, relatively high specific binding was found in membrane suspensions of the rat duodenal smooth muscle (18 fmol/mg of protein) and the vas deferens (8 fmol/mg of protein). ^{3}H-NKA-binding sites were further characterized on the rat duodenal smooth muscle. The specific binding of ^{3}H-NKA was shown to be temperature dependent, saturable, reversible, and increased in parallel with the protein concentration. Scatchard analyses and Hill plots of equilibrium binding studies in the concentration range of 0.40–30 nM revealed that ^{3}H-NKA bound to a single class of noninteracting binding sites (B_{max} = 270 fmol/mg of protein, K_D = 13.3 nM). Displacement of ^{3}H-NKA with different tachykinin-related peptides gave the following rank order of potencies: NKA > NKA (4-10) > kassinin > eledoisin > NKB ≫ substance P > physalaemin, which suggests that the binding site labeled by ^{3}H-NKA is different from substance P (NK-1)- and NKB (NK-3)-binding sites. The biological activities of tachykinins and related peptides were tested by measuring their contractile effects on the rat duodenum and rabbit pulmonary artery, two tissues known to be sensitive for NKA. K_i values were correlated with the EC_{50} obtained in biological assays. The results revealed a significant correlation (r = 0.86, $p < 0.01$) between K_i and EC_{50} values obtained in the isolated rabbit pulmonary artery, whereas there was no significant correlation between binding affinities and biological responses on the rat duodenum (r = 0.62, $p > 0.05$).

The tachykinins consist of a family of peptides sharing a common C-terminal sequence, Phe-X-Gly-Leu-MetNH_2. The nonmammalian tachykinins have been isolated from amphibian skin, for instance, KAS and PHY, or from octopus salivary gland (ELE) (1). The well known neuropeptide, SP, was considered the only tachykinin present in mammalian tissues until the discovery of two novel tachykinins, NKA and NKB (2, 3). Measurements of tachykinin immunoreactivities in tissue extracts have revealed a close similarity in the distribution of NKA and SP (4), whereas the distribution of NKB differed from that of the two other tachykinins in certain regions of the brain (4) and in the spinal cord (5, 6). These findings are supported by the recent identification of three different SP precursors: α-, β-, and γ-preprotachykinin A, the first of which contains one SP sequence, whereas β- and γ-preprotachykinin contain a copy of both SP and NKA (7, 8). NKB appears to be processed by a separate precursor, preprotachykinin B, the gene of which was discovered recently (9).

Although the pharmacological actions of the tachykinins are similar, differences in potencies between the various peptides have been demonstrated in isolated organs. Early observations by Lee *et al.* (10) and Watson *et al.* (11) revealed that SP, PHY, ELE, and KAS were equipotent as stimulants of the guinea pig ileum, rat bladder, guinea pig bladder, and guinea pig vas deferens, whereas KAS and ELE were found to be much more potent than SP and PHY in the mouse bladder, rat vas deferens, and rat duodenum. Further studies by Regoli and co-workers (12–14) have indicated that the mammalian peptides SP, NKA, and NKB show different orders of potencies in certain isolated

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ABBREVIATIONS: KAS, kassinin; PHY, physalaemin; ELE, eledoisin; SP, substance P; NKA, neurokinin A; NKB, neurokinin B; CNS, central nervous system; ^{125}I-BHSP, ^{125}I-labeled Bolton-Hunter derivative of substance P; ^{125}I-BHELE, ^{125}I-labeled Bolton-Hunter derivative of eledoisin; ^{125}I-BHNKA, ^{125}I-labeled Bolton-Hunter derivative of neurokinin A; (Hcy^{10})NKA, neurokinin A homocysteine thiolactone; HPLC, high pressure liquid chromatography; EDTA, ethylenediaminetetraacetate.

tissues. For instance the dog carotid artery was found to be particularly sensitive to SP, whereas NKA was more potent than SP and NKB in contracting the rabbit pulmonary artery and the rat duodenum. NKB was, on the other hand, the most potent stimulant of the rat portal vein. Based on these pharmacological studies it was concluded that at least three different tachykinin receptor subtypes are present in the peripheral organs, namely, the NK-1 (SP), NK-2 (NKA), and NK-3 (NKB) types.

In parallel with the pharmacological evidence of multiple receptors, *in vitro* binding studies, performed with labeled tachykinins, demonstrated the presence of different classes of tachykinin-binding sites in both the CNS and peripheral organs. Thus, the occurrence of two types of binding sites was demonstrated in the CNS by using ^{125}I-BHSP (15, 16) and ^{125}I-BHELE (17, 18). The two binding sites showed considerable differences in binding characteristics and topographical localization (19–24). SP and PHY had the highest affinities for the ^{125}I-BHSP site, whereas ^{125}I-BHELE was displaced preferentially by NKB and KAS, suggesting that two sites specific for SP and NKB are present in the brain (25). Comparative binding studies made with ^{125}I-BHELE and ^{3}H-NKB demonstrated that, indeed, the two ligands labeled identical sites in the CNS (26): it was thereby concluded that ^{125}I-BHELE labels a specific NK-3 site in the brain. Studies intended to demonstrate an NK-2 site have been performed previously with ^{125}I-BHNKA (27, 28). Thus, Quirion and Dam (29) have reported that NKA had the highest affinity for the ^{125}I-BHNKA site of synaptosomal membrane preparations of the guinea pig brain. In the same study, it was also shown that the substantia nigra contains a relatively high density of ^{125}I-BHNKA sites. However, in another study (26), it was observed that the ^{127}I-labeled Bolton-Hunter derivatization of NKA increases the affinity for the ^{3}H-NKB site of the cerebral cortex. It thus appears likely that ^{125}I-BHNKA labels not only an NK-2 site but, at least partly, also an NK-3 site.

Binding studies performed with ^{125}I-BHSP, ^{125}I-BHNKA, and ^{125}I-BHELE have revealed differences between the tachykinin-binding sites in the CNS and at the periphery (30, 31). Although the pattern of inhibition of the ^{125}I-BHSP binding was similar in the brain and in peripheral tissues, there were considerable differences in the K_i values of the tachykinins. The most conspicuous discrepancy was observed with ^{125}I-BHELE, which appeared to label the same site as ^{125}I-BHNKA in the rat duodenum and the mouse bladder (30, 31). In these organs, NKA showed the highest affinity for the ^{125}I-BHELE-binding site, while in the brain this label detects NKB-binding sites. However, in the guinea pig intestine longitudinal muscle, ^{125}I-BHELE labeled a site showing characteristics similar to those of the CNS.

The present study was undertaken to avoid possible inaccuracies due to the poor selectivity of Bolton-Hunter-derivatized ligands. A tritiated NKA (^{3}H-NKA) with high specific activity (75 Ci/mmol) was synthesized (32) and the binding of ^{3}H-NKA was studied on membrane preparations from various tissues of the rat, including the brain, spinal cord, duodenum, ileum, and vas deferens. Moreover, to examine whether a correlation could be found between binding affinities and biological activities, the contractile effects of the tachykinins and some related peptides were measured on two isolated organs, the rat duodenum and the rabbit pulmonary artery.

Materials and Methods

Chemicals. ^{3}H-NKA was synthesized by reacting ^{3}H-methyl-4-tolylsulfonate with NKA homocysteine thiolactone [Hcy^{10}]NKA thiolactone (33). In brief, a mixture of [Hcy^{10}]NKA thiolactone (2 μmol) and ^{3}H-methyl-4-tolylsulfonate (1 μmol, specific activity 75 Ci/mmol) was stirred at −60° in dry liquid ammonia (100 μl) for 1 hr. After evaporation of the liquid ammonia, the crude product was purified by reverse phase HPLC eluted isocratically from a μBondapak C_{18} column at a flow rate of 1.5 ml/min with 21.6% acetonitrile in 0.1% (v/v) trifluoroacetic acid in water (retention time, 20 min). After removal of the acetonitrile and lyophilization, ^{3}H-NKA was diluted in water in the presence of mercaptoethanol (0.2%, v/v) and stored in liquid nitrogen.

The synthesis of NKA, NKB, SP, [D-Pro^{10}]SP, [L-Pro^9]SP, [D-Pro^9]SP, [MePhe^8]SP, [L-Ala^9]SP, and [Cys^{2,6}]NKB has been described elsewhere (34–36).

ELE, KAS, pGlu-SP (6–11), PHYS, and SP free acid were purchased from Peninsula Laboratories (San Carlos, CA). SP-Methyl ester was purchased from Cambridge Research Biochemicals (Cambridge, England). Kelatorphan was kindly provided by Dr. B. Roques. All other chemicals were purchased from appropriate suppliers and were of analytical grade.

Preparation of membrane suspensions. Sprague-Dawley rats (200–300 g, Charles River, France) were sacrificed, and the various organs were dissected rapidly and put on ice at 4°. The CNS was dissected into cortex, cerebellum, striatum, hypothalamus, hippocampus, mesencephalon, pons-medulla, and the cervical part of the spinal cord. Segments of the rat duodenum and ileum (3 cm long) were removed, and the mucosa was scraped off with a blunted scalpel. The CNS tissues, the remaining smooth muscles of duodenum and ileum, and the vas deferens were homogenized with a polytron apparatus in 15 volume (w/v) of Tris-HCl buffer, 50 mM, pH 7.4, containing 120 mM NaCl and 5 mM KCl. The homogenates were centrifuged at 30,000 × *g* for 30 min and then resuspended in 30 volumes of ice-cold Tris-HCl, 50 mM, containing 10 mM EDTA and 300 mM KCl, and incubated 60 min at 4°. After centrifugation (30,000 × *g*, 30 min), the membrane suspensions were washed twice with ice-cold Tris-HCl, 50 mM, pH 7.4. The washed pellets were then resuspended in the incubation buffer consisting of Tris-HCl buffer, 50 mM, pH 7.4, containing bovine serum albumin (0.4 mg/ml), bacitracin (0.04 mg/ml), leupeptin (4 μg/ml), chymostatin (4 μg/ml), kelatorphan (10^{-6} M), and $MnCl_2$ (3 mM).

^{3}H-NKA binding assay. Stock solutions of various peptides were dissolved in redistilled water except for KAS, NKB, and pGlu-SP (6–11), which were dissolved in 1–2% dimethyl sulfoxide, and aliquots were kept at −20° until use. Dilutions of peptides and the radioactive ligand were made in the incubation medium. Binding assays were performed in 1.5-ml Eppendorf tubes precoated with 5 mg/ml bovine serum albumin in Tris/HCl, 50 mM, pH 7.4. Routinely, 20 μl of the membrane suspension, corresponding to approximately 300 μg of proteins, were incubated with ^{3}H-NKA (30,000 dpm, 0.9 mM), with or without unlabeled ligands in a final volume of 200 μl. After incubation for 25 min at 22°, the tubes were centrifuged in an Eppendorf Microfuge (30 sec, 10,000 × *g*). The supernatants were discarded and the pellets were washed once with 1 ml of ice-cold incubation medium. After a second centrifugation the pellets were dissolved in 10 ml of Aquasol-2 (New England Nuclear Research Products, Boston, MA), and the tissue-bound radioactivity was measured in an LKB-Wallace liquid spectrometer. All assays were run in triplicate and non-specific binding was defined as the amount of labeled ligand bound in the presence of 10^{-6} M NKA. Protein content was measured by the method of Lowry *et al.* (37).

Biological assays. Albino rabbits and rats of both sexes were killed by stunning and exsanguination; the organs (rabbit pulmonary artery and rat duodenum) were taken out rapidly and suspended as stripes (the artery) or segments (the duodenum) in organ baths containing Krebs solution at 37° (the artery) or Tyrode's solution at 32° (the duodenum), according to the method of Regoli et al. (12) and D'Orleans-

Juste *et al.* (14). Changes of tension produced by contractile agents were recorded immediately with Grass force transducers (FTO3C) or physiographs (Grass model 7D). Concentration response curves of neurokinins and related peptides were measured in order to evaluate the apparent affinity of agonists in terms of EC_{50} expressed in nM. The biological activities of the various peptides could therefore be compared with the binding affinities.

HPLC analysis. HPLC analysis was performed for determination of whether ^{3}H-NKA was degraded during incubation with the rat duodenal smooth muscle preparation. ^{3}H-NKA was incubated with the membrane suspension for 25 min at 22° and an aliquot of the supernatant was applied to a C_{18} μBondapak column (Waters Associates, Milford, MA). The peptide and the minute amounts of degradation products were eluted with acetonitrile (18%)/triethylamine phosphate (0.25 M), pH 3.0, as solvent.

Results

Binding capacity of ^{3}H-NKA in different tissues. The binding capacity (fmol/mg of protein) of ^{3}H-NKA was estimated in different tissues from rat, including brain, spinal cord, intestinal smooth muscle (duodenum and ileum), and vas deferens, using a concentration of 0.9 nM. The specific binding of ^{3}H-NKA (0.9 nM) was very low in membrane preparations of different brain regions (cerebral cortex, striatum, hippocampus, hypothalamus, mesencephalon, pons-medulla, and cerebellum), and the cervical part of the spinal cord (0.2–1.6 fmol/mg of protein). The apparently low specific binding of ^{3}H-NKA (0.2–1.0% of total binding) on membrane preparations of CNS tissues was further confirmed by binding studies on synaptosomal preparations and by autoradiography, which gave equivalent results (data not shown). In peripheral tissues the highest specific binding was obtained in the rat duodenal smooth muscle (18 fmol/mg of protein), which was approximately twice the level estimated in membrane suspensions of the rat vas deferens (8 fmol/mg of protein). In contrast, in the rat ileum smooth muscle, the specific binding was low, only 2 fmol/mg of protein, which was comparable to the binding capacity found in the CNS. In further experiments, the membrane suspension from the rat duodenal smooth muscle was used to study in more detail the characteristics of the ^{3}H-NKA-binding site.

Binding of ^{3}H-NKA to rat duodenal membrane suspension. The total and nonspecific binding of ^{3}H-NKA increased linearly with protein concentrations up to 7.5 mg/ml. A protein concentration of 1.5 mg/ml was used routinely, at which the specific binding was 70% of total binding and approximately 3% of the total radioactivity added. HPLC analysis of ^{3}H-NKA after incubation with the membrane suspension revealed that only 10% of the added ^{3}H-NKA was degraded in the presence of kelatorphan (10^{-6} M) (Fig. 1). In the absence of inhibitor, NKA was degraded by almost 50%. Studies at 4°, 22°, and 37° demonstrated that the ^{3}H-NKA specific binding was temperature dependent (Fig. 2). The specific binding at 4° was only 20% of that obtained at 22° after 25 min incubation. At 37° equilibrium was reached in 7 min, and the rapid decline of specific binding observed after 10 min may be due to degradation of the peptide by peptidases (Fig. 2).

Kinetic characteristics of ^{3}H-NKA binding. The association rate of ^{3}H-NKA to the duodenal membrane preparation is shown in Fig. 2. Specific binding reached equilibrium at 15 min and remained stable for at least 45 min. The nonspecific binding was maximal after only 2.5 min (data not shown). The association rate constant was determined according to the

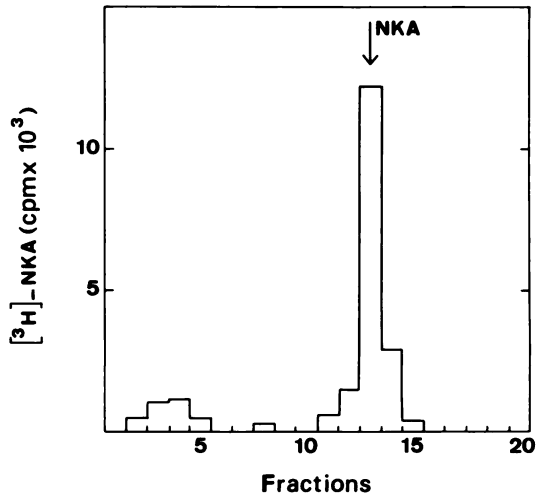

Fig. 1. Identification by HPLC of ^{3}H-NKA after incubation with rat duodenal smooth muscle membranes. ^{3}H-NKA (0.9 nM) was incubated with rat duodenal smooth muscle suspension for 25 min at 22° as described under Materials and Methods. An aliquot of the supernatant was applied at a C_{18} μBondapak column and the products were eluted with acetonitrile (18%)/triethylamine phosphate (0.25 M), pH 3.0, as solvent. The radioactivity present in an aliquot of each 1-min fraction was determined.

method of Frost and Pearson (38), using the formula $\ln [B_{max} (L_0 - B_t)/L_0 (B_{max} - B_t)] = k_{+1} (L_0 - B_{max})t$, where B_{max} is the concentration of ligand bound at equilibrium, B_t is the concentration of ligand bound at time t, L_0 is the total concentration of added ligand, and k_{+1} is the association rate constant. A plot of $\ln [B_{max} (L_0 - B_t)/L_0 (B_{max} - B_t)]$ versus t is shown in Fig. 2. From the association plot a k_{+1} value of $1.9 \pm 0.2\ 10^{+8}$ M^{-1} min^{-1} ($n = 3$) was calculated.

Specific binding of ^{3}H-NKA was reversible because dissociation of the ligand was observed when a 1 μM concentration of unlabeled NKA was added after 25 min of incubation (Fig. 3). The dissociation curve was plotted as a first order reaction according to the formula $\ln (B/B_0) = -k_{-1}t$, where B_0 represents the amount of ligand bound at equilibrium and B is the amount of ligand bound at different times, t. Two distinct phases were obtained from the dissociation plot (Fig. 3). The first phase was very rapid with a rate of dissociation (k_{-1}) of $1.5 \pm 0.06\ 10^{-1}$ min^{-1}, giving a half-life of 4.6 ± 0.19 min. The second phase was much slower, having a k_{-1} of $6.5 \pm 0.77\ 10^{-3}$ min^{-1} and half-life of 108 ± 6.9 min ($n = 2$). The rapid phase of dissociation was calculated after substracting the contribution of the slow phase. Since the major part of ^{3}H-NKA was dissociated after 15 min, this rapid phase of the curve was used arbitrarily for the estimation of the apparent equilibrium dissociation constant ($k_D = k_{-1}/k_{+1}$). In this condition, when k_{-1} was $1.5\ 10^{-1}$ min^{-1}, the K_d value obtained was 0.8 nM.

Equilibrium binding studies. The smooth muscle membrane preparation of the rat duodenum was incubated with increasing concentrations (0.4–30 nM) of ^{3}H-NKA alone or together with unlabeled NKA (10^{-6} M). Due to technical difficulties, concentrations higher than 30 nM could not be used. Binding data demonstrated that the specific binding was saturable, while the nonspecific binding increased linearly with increasing concentrations of ^{3}H-NKA (Fig. 4). Scatchard plots were linear (Fig. 4), indicating a single class of binding sites in the concentration range used (0.4 nM–30 nM). The equilibrium constant (K_D) and the number of binding sites (B_{max}) were 13.3 ± 0.38 nM and 270 ± 23 fmol/mg of protein, respectively. The

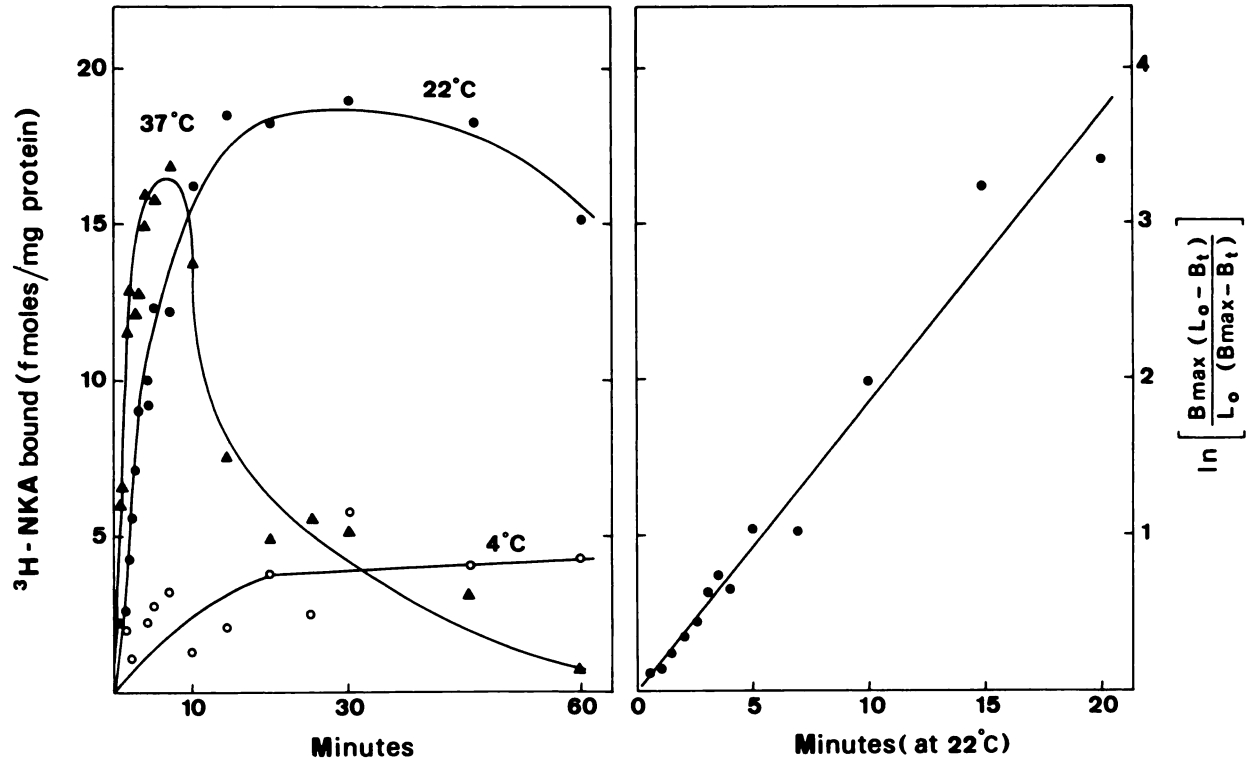

Fig. 2. Association of ^{3}H-NKA to rat duodenal smooth muscle membranes. *Left*: Association time course of specific ^{3}H-NKA binding to rat duodenal smooth muscle membranes at three different temperatures: 4°, 22°, and 37°. ^{3}H-NKA (0.9 nM) was incubated with the membrane suspension for different intervals as indicated on the *abscissa*. Total and nonspecific bindings were estimated as described under Materials and Methods. Each *point* represents the mean of data obtained in three different experiments. *Right*: Linearization of the association time course using the pseudo-first order equation for association: $\ln [B_{max}(L_0 - B_t)/L_0 (B_{max} - B_t)] = k_{+1}(L_0 - B_{max})\, t$, where B_{max} represents the maximal amount of ligand bound at equilibrium, B_t is the amount of ligand specifically bound at time, t, and L_0 is the initial ^{3}H-NKA concentration in the incubation medium.

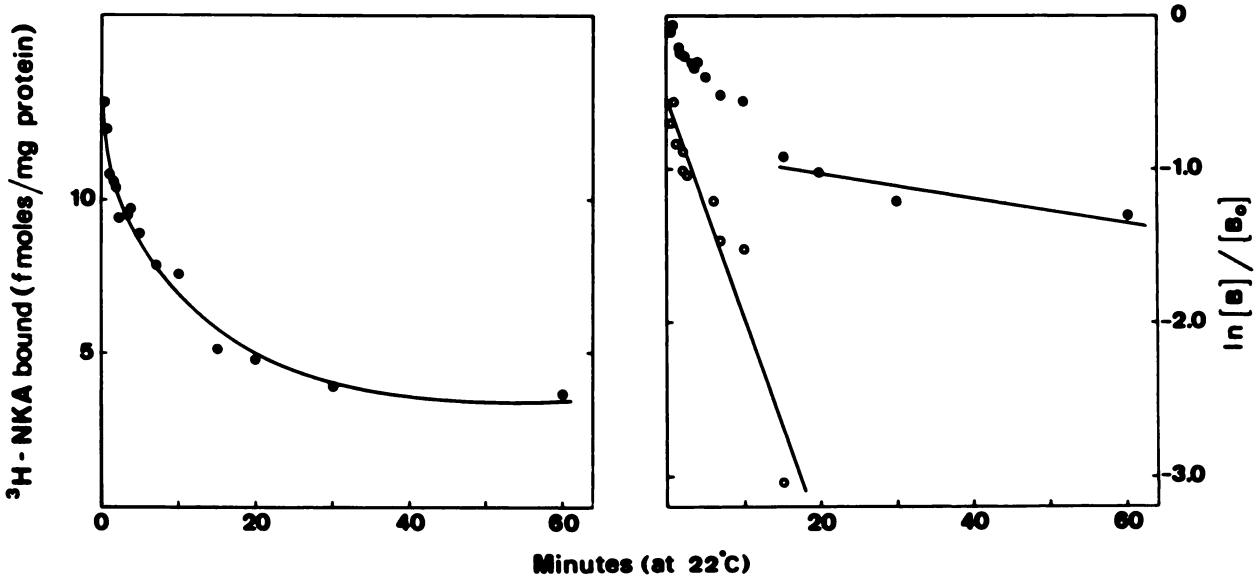

Fig. 3. Dissociation of ^{3}H-NKA from rat duodenal smooth muscle membranes. *Left*: ^{3}H-NKA (0.9 nM) was incubated with rat duodenal smooth muscle membranes at 22° for 25 min as described under Materials and Methods. Dissociation was initiated by addition of NKA (10^{-6} M), and the bound ^{3}H-NKA was measured at different intervals as indicated on the *abscissa*. *Right*: Linearization of the dissociation using the first order rate equation for calculation of the dissociation rate constant, as described under Results. The dissociation curve was resolved in two different phases by curve-peeling, with the fast one (○) being obtained by subtraction of the contribution of the slow process (●).

Hill plot of these data gave a slope, n_H, of 1.06, a result indicating that the ligand bound to a single population of noninteracting sites.

Displacement of specific ^{3}H-NKA binding by different tachykinins and related peptides. The affinities of different tachykinins and related peptides for the ^{3}H-NKA-binding site on membrane suspension of rat duodenal smooth muscle were determined. The K_i values and relative potencies are given in Table 1. NKA (K_i = 7.4 nM) and the C-terminal fragment NKA (4–10) (K_i = 9.5 nM) had the highest affinity for the site. KAS,

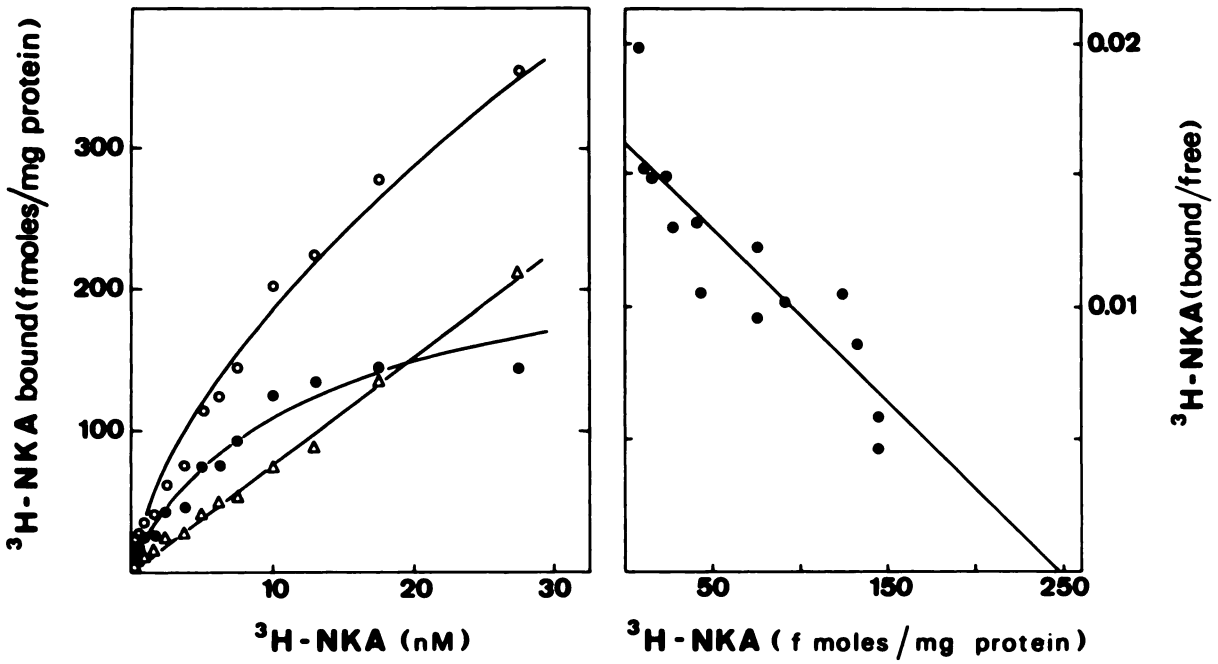

Fig. 4. Saturation of ^{3}H-NKA binding to rat duodenal smooth muscle membranes. *Left*: Different concentrations of ^{3}H-NKA were incubated with rat duodenal smooth muscle membrane suspension for 25 min at 22°. Total (○), nonspecific (△), and specific (●) bindings were estimated as described under Materials and Methods. Each *point* represents the mean of triplicate determinations obtained in a single representative experiment. *Right*: Scatchard transformation of the same data.

TABLE 1

Displacement of ^{3}H-NKA binding to rat duodenal smooth muscle membrane suspension by tachykinins and tachykinin analogues

Inhibition constants (K_i) were obtained using the Cheng-Prussoff equation: $K_i = IC_{50}/[1 + (L_o/K_D)]$, where L_o represents the labeled ligand concentration in the incubation medium and K_D the apparent dissociation constant calculated from saturation curves obtained at equilibrium. The IC_{50} values were derived from the orginal data plotted according to log $[(B_o - B)/B]$ versus log peptide concentration, where B_o represents the amount of ligand specifically bound in the absence of competitor and B the amount of ligand specifically bound in the presence of competitive agents. The data are mean values obtained from 3 to 12 independent experiments. The standard deviation did not exceed 60% of the mean.

Compounds	K_i	Relative potency
	nM	
NKA	7.4	1
NKA (4-10)	9.5	0.78
KAS	25	0.30
ELE	27	0.27
NKB	33	0.22
[D-Pro^9]SP	140	0.05
SP	206	0.04
PHY	936	0.008
[L-Ala^9]SP	1,200	0.006
[Cys^{2,5}]NKB	1,500	0.005
[MePhe^8]SP	2,600	0.003
pGlu-SP (6-11)	4,670	0.002
DiMe-C7	4,900	0.001
[L-Pro^9]SP	>10,000	<0.0001
SP Free acid	>10,000	<0.0001
SP-Methyl ester	>10,000	<0.0001

ELE, and NKB were approximately 4 times less potent, and the affinities of SP, PHY, and pGlu-SP (6–11) were much lower by 1–2 orders of magnitude. The SP analogues SP free acid, SP methyl ester, and DiMe-C7 were completely inactive. Four tachykinin analogues which, earlier, have been shown to have a high affinity for the ^{125}I-BHSP and the ^{3}H-NKB site, respectively, were tested in order to verify their selectivity. [MePhe^8]SP and [Cys^2, Cys^5]NKB, which have a good affinity for the ^{3}H-NKB site (35, 36), had a very low affinity for the ^{3}H-NKA-binding site. The same applied to [L-Pro^9]SP and [L-Ala^9]SP, which are highly potent inhibitors of ^{125}I-BHSP binding (36) but were found to be very weak competitors of ^{3}H-NKA binding.

Potencies of different tachykinins and tachykinin analogues in isolated preparations of the rat duodenum and rabbit pulmonary artery. The potencies of tachykinins and some synthetic analogues were tested in isolated preparations of the rat duodenum and the rabbit pulmonary artery. As it appears from the results summarized in Table 2, NKA was equipotent in both assays with EC_{50} values of 6.0 and 6.1 nM, respectively. NKB was also equally potent in both assays and about 5 times less active than NKA. The EC_{50} value of ELE was similar to that of NKA in the rabbit pulmonary artery, but only one-third as active as NKA in the rat duodenal preparation. The opposite was observed with KAS, which was more active in contracting the rat duodenum than the rabbit pulmonary artery. Although SP, PHY, and pGlu-SP (6–11) were much less potent than NKA in both assays, there was a larger difference in activity in the rabbit pulmonary artery preparation. Also, SP-methyl ester and DiMe-C7, which were completely inactive in contracting the rabbit pulmonary artery, showed some potency in the rat duodenal assay. However, the most striking difference was seen when comparing (D-Pro^9)SP, [L-Pro^9]SP, and [Cys^{2,5}]NKB, which were inactive or had a very low activity in the rabbit pulmonary artery preparation, but which, in contrast, had a relatively high potency in contracting the rat duodenum.

Comparison between the potencies and affinities of the tachykinins. The K_i values of the peptides obtained in the ^{3}H-NKA binding assay were correlated with their estimated EC_{50} values in contracting the rat duodenum and the rabbit

TABLE 2

EC_{50} values (expressed in nM) obtained with various tachykinins and related peptides in *in vivo* pharmacological preparations sensitive to NKA

Compound	EC_{50} value	
	Rabbit pulmonary artery	Rat duodenum
	nM	
NKA (4-10)	3.8	ND[a]
NKA	6.0	6.1
ELE	6.0	20
KAS	22	10
NKB	35	28
SP	741	316
[$Cys^{2,5}$]NKB	750	11
PHY	955	302
[MePhe^8]SP	1,310	ND
SP(6-11)	1,950	180
[L-Pro^9]SP	>10,000	30
[D-Pro^9]SP	>10,000	43
SP-Methylester	>10,000	130
DiMe-C7	>10,000	590
SP Free acid	>10,000	ND
[D-Pro^{10}]SP	>10,000	ND

[a] ND, not determined.

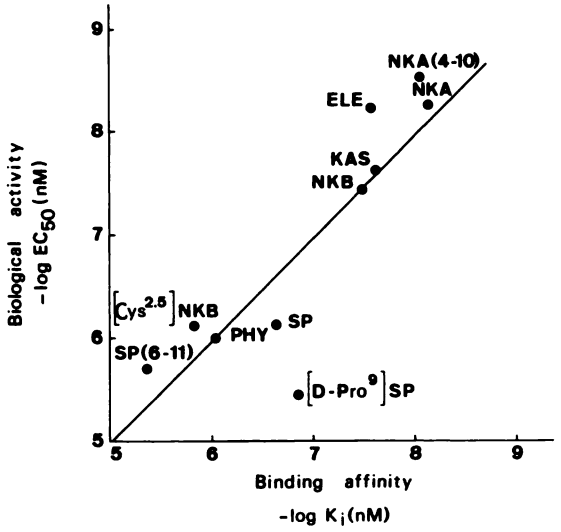

Fig. 5. Correlation between binding affinity and biological activity in the isolated rabbit pulmonary artery preparation. The K_i values of different tachykinins and tachykinin-related peptides obtained in the ^{3}H-NKA binding assay were correlated with their EC_{50} values obtained when measuring the contraction of the isolated rabbit pulmonary artery as described under Materials and Methods.

pulmonary artery (Figs. 5 and 6). A significant correlation ($r = 0.86$, $p < 0.01$) was obtained when correlating the K_i with the EC_{50} values measured in the rabbit pulmonary artery. The slope was 1.05, suggesting an excellent correlation not only in rank order of potencies but also in absolute values of K_i and EC_{50}. In contrast, the correlation with the K_i values and the EC_{50} values, obtained in the rat duodenal preparation, was found to be nonsignificant ($r = 0.62$, $p > 0.05$); moreover, as it appears from Fig. 6, the slope deviated markedly from unity.

Discussion

The characterization of different subtypes of tachykinin-binding sites in the CNS and in peripheral organs has been

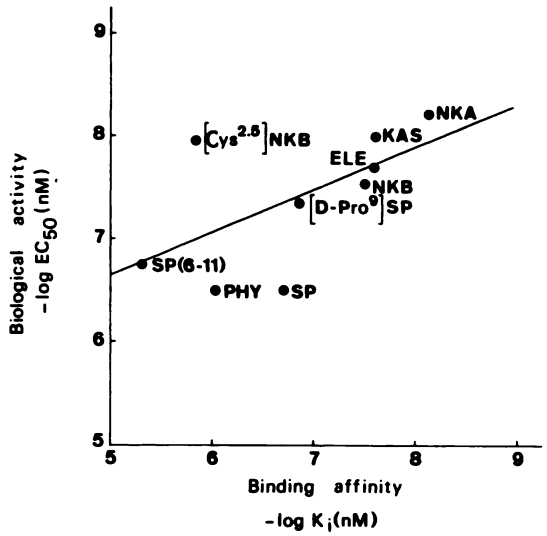

Fig. 6. Correlation between binding affinity and biological activity in the isolated rat duodenal preparation. The K_i values of different tachykinins and tachykinin-related peptides obtained in the ^{3}H-NKA binding assay were correlated with their EC_{50} values obtained when measuring the contraction of the isolated rat duodenum as described under Materials and Methods.

performed generally with ^{125}I-Bolton-Hunter-derivatized ligands (15–18, 29). There are, however, indications that insertion of a ^{125}I-BH group in the peptide changes both the affinity and the selectivity of some tachykinins. Previous studies in our laboratory and others have shown that ^{127}I-BHSP and SP have identical affinities for the ^{125}I-BHSP site (15, 16), whereas ^{127}I-BHELE is slightly more potent in inhibiting the ^{125}I-BHELE (or ^{3}H-NKB) binding than ELE in the rat CNS (19). However, the most striking change in affinity was observed after ^{127}I-BH derivatization of NKA, which led to a 50-fold increase in displacement of ^{3}H-NKB (26). It therefore appears that ^{125}I-BHNKA does not label only a tachykinin site of the NKA type but also the NKB site, thus explaining the close similarity observed between the distribution of binding sites for ^{125}I-BHNKA and ^{3}H-NKB (or ^{125}I-BHELE) in the rat CNS (20, 23, 26–28). An opposite situation appears to occur in some peripheral tissues, where ^{125}I-BHNKA and ^{125}I-BHELE label an identical site which, based on displacement studies, appears to be of the NK-2 type (30, 31). From these studies, it was suggested that ^{125}I-BHNKA may be an unselective ligand, particularly when investigating NK-2-binding sites in the CNS. The present study, therefore, was undertaken to characterize a tachykinin-binding site of the NK-2 type by using ^{3}H-NKA, a labeling which does not change the chemical structure of the peptide.

A specific ^{3}H-NKA binding was found only in a peripheral organ, the rat duodenum. This was consistent with earlier studies in which high densities of ^{125}I-BHNKA-binding sites were demonstrated in the rat duodenal muscle layer (31). In the present experiment the binding of ^{3}H-NKA was measured with a slightly modified protocol with respect to that described by Buck *et al.* (30) for ^{125}I-BHNKA. The binding equilibrium with ^{3}H-NKA was reached after 15–20 min at room temperature, such that the incubation time could be shortened to 25 min. The peptidase inhibitor, kelatorphan (10^{-6} M), was needed to avoid degradation of the labeled ligand during the incubation with the duodenal membrane suspension, which apparently contains considerable amounts of peptidases. Under the con-

ditions utilized in the present study, the specific binding of ^{3}H-NKA was saturable, reversible, and temperature dependent, and it increased with increasing concentrations of membrane proteins. Results from Scatchard and Hill analyses of binding data obtained in the concentration range, 0.4–30 nM, demonstrated that ^{3}H-NKA bound to a single population of noninteracting binding sites, similar to ^{125}I-BHNKA in previous studies (31). The equilibrium dissociation constant, K_D, calculated from the Scatchard analyses, was 13.3 ± 0.38 nM and in the same range as the K_i value for NKA (7.5 nM), the slightly higher value reported by Burcher *et al.* (31) for ^{125}I-BHNKA. The number of binding sites, B_{max}, was 270 ± 23 fmol/mg of protein. A direct comparison of the B_{max} values for ^{3}H-NKA and ^{125}I-BHNKA on rat duodenal smooth muscle membranes was not possible because the B_{max} for the ^{125}I-BHNKA was expressed in fmol/mg of wet weight (31). However, an approximate recalculation of our data revealed that the obtained B_{max} values of the two ligands appear to be in the same range. The binding was reversible in a biphasic manner, with a first rapid phase (k_{-1} = 1.5 ± 0.06 10^{-1} min^{-1} and a second slower phase (k_{-1} = 6.5 ± 0.77 10^{-3} min^{-1}). Similar biphasic dissociations have been reported previously for ^{125}I-BHNKA binding on membrane suspensions of the rat duodenal smooth muscle (31) and ^{125}I-BHELE and ^{3}H-NKB binding of rat cortical synaptosomes (17, 26). The biphasic dissociations may provide an explanation for the deviation in the K_D values obtained with Scatchard (13.3 nM) and kinetic analysis (0.8 nM). We cannot exclude the possibility that the slow phase of dissociation reveals a dissociation of ^{3}H-NKA from a distinct population of sites exhibiting different characteristics. However, such sites could not been demonstrated by our Scatchard analysis.

Displacement of ^{3}H-NKA from rat duodenal membranes revealed a rank order of potencies of various tachykinins and related peptides that clearly distinguished the ^{3}H-NKA-binding site from the binding sites for ^{3}H-NKB (26) and ^{125}I-BHSP in the CNS (16). NKA showed the highest affinity for the site, followed by ELE, KAS, and NKB, which were approximately 4 times less potent. SP, PHY, and pGlu-SP (6–11) were much weaker competitors, showing K_i values 30–600 times higher than that of NKA. SP free acid and SP-methyl ester were completely inactive in inhibiting ^{3}H-NKA. It is noteworthy to mention that the C-terminal fragment NKA (4–10) was almost as potent as NKA. Some synthetic tachykinin analogues, known to have high affinities for either the ^{125}I-BHSP or the ^{3}H-NKB site, were included in the binding experiments to verify their selectivities. [L-Ala^9]SP and [L-Pro^9]SP, which are potent inhibitors of ^{125}I-BHSP binding in the rat brain (36), were weak competitors of ^{3}H-NKA binding. Likewise, [MePhe^8]SP and [Cys^2-Cys^5]NKB, which both have a high affinity for the NK-3 site (35, 36), displaced ^{3}H-NKA in concentrations higher than 10^{-6} M, indicating that the cyclic analogue [Cys^2-Cys^5]NKB is a more selective ligand for the NK-3 site than the endogenous ligand NKB.

When binding affinities are measured, it is important to find out whether the obtained K_i values have any physiological relevance, i.e., whether they are in the same range and have the same rank order of potencies as EC_{50} values obtained in an appropriate biological test. Recent pharmacological studies have demonstrated that the isolated rabbit pulmonary artery and the rat duodenum are more sensitive to NKA than to other tachykinins (13, 14). EC_{50} values of tachykinins and related peptides were therefore measured in the two assays, in order to compare the biological responses and binding affinities. The results revealed that the K_i values correlated very well (r = 0.86, $p < 0.01$) with the EC_{50} values obtained in the rabbit pulmonary artery. To our knowledge, *in vitro* binding studies have not been performed in the rabbit pulmonary artery, and routine binding assays appear to be difficult in this organ because of the scarcity of available tissue. The good correlation between the K_i and EC_{50} values further suggests, however, that the rabbit pulmonary artery contains exclusively receptors of the NK-2 type.

The nonsignificant correlation between the K_i and EC_{50} values obtained in the rat duodenum is likely explained by the fact that this organ contains more than one type of tachykinin-binding site. Indeed, previous *in vitro* binding studies with ^{125}I-BHSP and ^{125}I-BHNKA have revealed sites of both the NK-1 and the NK-2 types (31), the distribution and densities of which appeared to be different. NK-2 binding sites have been observed both in the longitudinal and the circular muscle layers, whereas NK-1 sites only occur in deep muscle plexa and in some myenteric plexa (31). The higher density of NK-2-binding sites observed in the rat duodenal smooth muscle probably explains why NKA is much more efficient in contracting the tissue preparation. So far, there are no reports on the distribution and density of specific NK-3 sites in the rat duodenum. However, previous binding studies with ^{125}I-BHNKA and ^{125}I-BHELE in this tissue have demonstrated certain differences in the binding of the two ligands (31). The specific binding of ^{125}I-BHELE was found to be much lower than that of ^{125}I-BHNKA, and the absolute K_i values of the tachykinins were markedly different, even if the rank order of potencies of peptides was identical. In our study there were two tachykinins, in particular, [Cys^{2,5}]NKB and SP (6–11), whose K_i and EC_{50} values deviated significantly. These two ligands have been demonstrated earlier to bind preferentially to NK-3-binding sites in rat cortical synaptosomes (17, 35). Their comparatively high effects on the rat duodenum suggest, therefore, that, in addition to NK-1 and NK-2 receptors, these tissues also contain NK-3 receptors. The observation that [Cys^{2,5}] NKB and SP (6–11) were more potent in contracting the rat duodenum than NKB may be explained by higher stability against enzymatic degradation.

In a previous study it has been demonstrated that a specific ^{125}I-BHNKA-binding site may be present in the guinea pig brain (29). NKA was shown to have the highest affinity to this site, a finding which left out the possibility that the site could be of the NK-3 type. Moreover, a relatively high density of ^{125}I-BHNKA labeling was found in the substantia nigra, both in the guinea pig and the rat (29). To ascertain the presence of specific NK-2 sites in the rat brain, the binding capacity of ^{3}H-NKA was estimated in several different regions of the CNS. The specific binding of ^{3}H-NKA was found to be very low (0.2–2 fmol/mg of protein) in all the studied regions, which makes any further characterization unfeasible. A similar low specific binding was obtained with several experimental protocols, earlier used for the estimation of ^{125}I-BHSP, ^{125}I-BHELE, and ^{3}H-NKB binding, including binding on intact synaptosomes (16, 17, 26) and autoradiography (20). The discrepancy between our present results and those previously obtained with ^{125}I-BHNKA in the guinea pig brain might suggest that there are differences between species. The absence of a specific NK-2-binding site in the rat CNS remains problematic, considering several dem-

onstrated effects of NKA, for instance, dopamine liberation in the striatum after NKA injection in the substantia nigra (39) and locomotor hyperactivity after NKA injection in the ventral tegmental area (40).

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