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Neurotensin receptors in canine intestinal smooth muscle: preparation of plasma membranes and characterization of (*Tyr*^{3}-^{125}I)-labelled neurotensin binding

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To study the binding of (*Tyr*^{3}-^{125}I)-labelled neurotensin to intestinal muscle, plasma membranes have been purified from dog intestinal circular smooth muscle. Purification was done by differential centrifugation followed by separation on a sucrose gradient. Electron microscopic study revealed that the dissected circular muscles used as the source of membranes were free of myenteric plexus and that the plasma membrane fraction obtained was free of any mitochondria or synaptosomes. The fraction used was obtained at the interface of 14%–33% sucrose density on the gradient and was 25-times enriched in the plasma membrane marker enzyme 5′-nucleotidase activity as compared to post-nuclear supernatant. This fraction contained negligible activity of mitochondrial membrane marker enzyme cytochrome *c* oxidase and low activity of a putative endoplasmic reticulum marker enzyme NADPH-cytochrome-*c* reductase. This membrane fraction contained a high density of neurotensin binding sites. This binding was studied by kinetic and by saturation approaches. Analysis of data from saturation binding studies by the computer programs (EBDA and LIGAND) suggested the presence of a two-site model ($K_{d1} = 0.118$ nM, $K_{d2} = 3.18$ nM, $B_{max1} = 9.73$ fmol/mg and $B_{max2} = 129.8$ fmol/mg). A part of specifically bound neurotensin was rapidly dissociated. No cooperativity between the two receptor types could be detected. A kinetic analysis of binding gave the K_d value equal to 0.107 nM. Carboxy terminal amino acid residues 8–13 were found to be essential for the binding activity and replacement of Tyr^{11} by tryptophan reduced the affinity of the peptide by 10 times in displacement studies. Binding was modulated by sodium ions and a guanine nucleotide Gpp[NH]p. $MgCl_2$, $CaCl_2$ and KCl were also found to reduce the specific binding. Evidence was found of a high specific binding to another membrane fraction poor in plasma membranes and rich in synaptosomes. We concluded that plasma membrane of canine intestinal circular muscle contains neurotensin receptors with recognition properties distinct from those obtained in previous studies of neurotensin binding sites in murine tissues. Another neurotensin binding site may be present on neuronal membranes.

Introduction

A number of studies in vivo and in vitro have been carried out to define the sites of neuropeptide actions on smooth muscles of the gastrointestinal tract [1–8]. Also, the distribution of nerves containing neuropeptides which innervate this muscle has been studied [9–11]. One of these peptides, neurotensin, has been demonstrated in a number of in vivo and in vitro studies, to have a profound effect on the gastrointestinal motility in a variety of laboratory animals and in man

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[4,5,12–17] via both indirect (through other neurotransmitters) and direct effects on the smooth muscle cells of gastrointestinal tract. Neurotensin is a tridecapeptide which was first isolated from bovine hypothalamic extracts [18] and later its mino acid composition was established by Carraway and Leeman [19,20]. In the periphery, the highest concentration of neurotensin immunoreactivity has been found in the ileum, where it is present in endocrine-like cells (N-cells) and reported by some [9,10,21–23] but not others [11] to be in nerve fibers. In dogs, too, neurotensin has been shown to have both nerve-mediated as well as direct effects on the smooth muscle cells of the gastrointestinal tract [4,5].

Specific neurotensin receptors are shown to be present in a number of animals by radioligand binding and autoradiographic studies on central as well as peripheral structures, both neuronal and non-neuronal [16,24–28]. Recently, Goedert et al. [16] and Kitabgi et al. [29] have described specific neurotensin binding sites in membranes of different smooth muscles of rats and guinea pigs. The recognition sites in guinea pigs appeared to differ from those in rats in that they failed to recognize neurotensin with high affinity in contrast to the rat. To our knowledge, no direct demonstration of neurotensin specific binding sites in any smooth muscle (or in any tissue of dog) has been made by radioligand binding studies. In preliminary studies we found [Trp^{11}]-neurotensin to be a weak functional activator of neurotensin receptors in smooth muscle of canine intestine or stomach and its iodinated derivative did not demonstrate good binding to the membranes from these tissues.

We developed purified plasma membrane fractions from the canine intestinal smooth muscle cells (from circular muscle) since the receptors (for neurotensin as well as for other neuropeptides) appear to be present on both neuronal and non-neuronal structures [24–28]. In these highly purified fractions for radioligand binding the non-specific binding to contaminants was reduced and the signal to noise ratio improved. Among many methods [31–36] for isolation of purified plasma membrnes from various smooth muscles, one has been very useful in our laboratory [33,34]. In this method, homogenization with a Polytron is followed by appropriate separation of mitochondria and microsomes by differential centrifugation and by separation of microsomal membranes on a sucrose density gradient.

In the present paper, we report the method for the isolation of a fraction highly enriched in plasma membranes from the circular muscle of dog small intestine and describe the binding characteristics of Tyr^3-^{125}I)-labelled neurotensin to these membranes.

Materials and Methods

A. Purification of plasma membranes

1A. Tissue-handling and membrane fractionation. All the steps, unless otherwise indicated, were carried out in the cold at 4°C. Leaving behind about six inches from the ileo-caecal junction, the whole intestine was removed from adult mongrel dogs after they had been killed with pentobarbital (100 mg/kg i.v.) and was immediatley placed in ice-cold sucrose-Mops buffer (25 mM Mops/10 mM $MgCl_2$/250 mM sucrose (pH 7.4)). Approximately one inch pieces of tissue were cut and cleared of the mesenteric arcade, fat and connective tissues, opened along the mesenteric line and pinned mucosal surface down on a dissecting dish put on ice. Longitudinal muscle layers were carefully peeled off with a pair of forceps in such a way so that a thin layer of circular muscles also came along with it. The remaining circular muscles were then also peeled off in a similar fashion and placed in cold sucrose-Mops buffer after rinsing with the same buffer. The circular muscle peels were blotted dry on a filter paper, weighed, resuspended in buffer (10 ml/g tissue wet weight) and finely minced with a pair of scissors. In some cases the tissues were frozen at −70°C for later use, a procedure which did not affect binding. The tissue was homogenized in Polytron PT20 homogenizer for 3 × 7 seconds bursts at approximately 15000 rpm allowing some cooling time between the bursts. This procedure was evolved after preliminary studies designed to develop a procedure of homogenization which minimized the contamination of microsomes with mitochondrial membranes and maximized the protein yield. The crude homogenate was spun at $1000 \times g$ for 10 min. The nuclear debris was washed with the same buffer, spun again at $1000 \times g$ for 10 min and

both superantants combined. This postnuclear supernatant (PNS) was spun at $10000 \times g$ for 10 min. The pellet thus obtained was resuspended in the same buffer and centrifuged again at $10000 \times g$ for 10 min to yield a MIT-I pellet. Both the supernatants were combined and centrifuged at a high speed of $170000 \times g$ for 60 min in a fixed angle (60 Ti) rotor of Beckman LS50 ultracentrifuge to obtain the microsomal pellet (MIC-I) and soluble fraction (SOL). The microsomal pellet was resuspended in the buffer using Teflon-coated hand-held homogenizer. The suspension (MIC-I) was centrifuged at $10000 \times g$ for 10 min to obtain MIT-II pellet and MIC-II supernatant which was loaded onto a discontinuous sucrose density gradient (14%, 33%, 40% and 48% w/v sucrose, 2.5 ml each) constructed according to the earlier results of distribution of proteins and marker enzyme activities on a continuous sucrose density gradient of 8–60% sucrose run in a swinging bucket (SW 40) rotor of Beckman LS50 at 30000 rpm for 100 min.

2A. Biochemical assays. Determination of protein concentration in various fractions was done according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Various fractions were tested for the specific activities of marker enzymes 5′-nucleotidase (EC 3.1.3.5), NADPH-cytochrome-*c* reductase (EC 1.6.99.3), cytochrome-*c* oxidase (EC 1.9.3.1), rotenone-insensitive cytochrome-*c* reductase and alkaline phosphatase (EC 3.1.3.2) as described elsewhere [33,34].

3A. Morphological techniques. For morphological studies, transmission electron microscopy of sections of tissues and various fractions were done as previously described [5,6]. In studying each fraction, multiple sections from all parts of each pellet were examined.

B. Characterization of neurotensin binding

1B. Iodination. (Tyr^3-^{125}I)-labelled neurotensin was prepared according to the procedure described by Sadoul et al. [37]. Briefly, 2 equivalents of neurotensin were incubated at room temperature with 1 equivalent of ^{125}INa and 0.1 mg lactoperoxidase in 5 mM sodium phosphate buffer at pH 7.4. Four aliquots (0.25 equiv.) of H_2O_2 were added at 30-s intervals after which the reaction was terminated by the addition of 1 equivalent sodium metabisulfite and 1 mg bovine serum albumin was added to bind any remaining free iodine in the reaction mixture. The iodinated product was separated over a SP-Sephadex C-25 column. The radioactive peak corresponding to the monoiodinated product was screened for its binding activity and the fractions having the highest binding activity were pooled and quick frozen in small aliquots. The specific activity of the monoiodinated peptide was essentially that of iodine. When required, these fractions were diluted with uniodinated neurotensin to quench the specific activity, and in those cases new specific activities were defined.

2B. Binding assays. The binding of the radioactively labelled neurotensin to the purified plasma membranes was performed by the slight modification of the method of Sadoul et al. [24] using 70–80 pM radioactively labelled neurotensin (spec. act. 2000 Ci/mmol). The incubation buffer consisted of 50 mM Tris-HCl (pH 7.4) containing 0.2% bovine serum albumin and 1 mM 1,10-phenanthroline. The reaction was carried out at 37°C. The separation of the bound ligand from free was done by a filtration technique using a Millipore filtration apparatus and Gelman GA-8 (0.2 μm pore size) filters. The filters were presoaked in the incubation buffer for at least 4 h before use. The reaction, unless otherwise specified, was always terminated by dilution of the incubation sample (0.25 ml) with 2 ml ice-cold incubation buffer, and immediately filtering under reduced pressure (20 μm). The tube and the filters were washed twice with 2 ml incubation buffer. The radioactivity on the filters was counted on a gamma counter (Searle model 1285) with counting efficiency of approx. 80%. Nonspecific binding was defined as that not displaced in the presence of excess (1 μM) unlabelled neurotensin. The specific binding was described as the difference between the total binding and nonspecific binding. No specific binding on the filters was observed; i.e., the binding on the filters in the absence or in the presence of excess of unlabelled neurotensin was equal.

3B. Protein binding profile. Varying amounts of protein (0–180 μg per assay tube) were incubated with 70–80 pM neurotensin in the incubation

mixture. At 25 min the incubation mixture was diluted with cold buffer and filtered immediately as described earlier.

4B. Association and dissociation kinetics. Plasma membranes (20–50 μg per assay tube) were incubated at 37°C in the incubation buffer containing 1 mM 1,10-phenanthroline. The association was started by the addition of labelled neurotensin (2000 Ci/mmol). At various time intervals, 0.25 ml aliquots were withdrawn, diluted in 2 ml cold incubation buffer, filtered and tubes and filters washed twice with the buffer.

After 25 min of association, the dissociation was initiated by (a) addition of excess (1 μM) unlabelled neurotensin; (b) dilution of the incubation medium to 40-folds or (c) dilution of the incubation medium in the presence of 1 μM of unlabelled neurotensin. In the case of dilution, 10-ml aliquots were filtered and the filters were washed twice with 2 ml cold incubation buffer. In other cases, 0.25 ml aliquots were taken and filtered as usual.

5B. Equilibrium binding experiments. The membranes (20–50 μg protein per assay tube) were incubated with increasing concentration of the radioactively labelled neurotensin (spec. act. 2000 Ci/mmol or serially reduced specific activity by dilution with unlabelled neurotensin) in a total volume of 0.25 ml containing 1 mM 1,10-phenanthroline at 37°C for 25 min. The reaction medium was then filtered as described before.

6B. Competition experiments and the effects of various ions. The membranes were incubated at 37°C in the presence 70–80 pM radioactively labelled neurotensin and varying concentrations of the competing peptides and fragments or various ions or Gpp[NH]p. After 25 min, the reaction medium was filtered as usual.

7B. Computer analysis. Ligand binding data were analyzed using an IBM-PC/XT and the program LIGAND prepared by Munson and Rodbard [38] and adapted by G.A. McPherson [39].

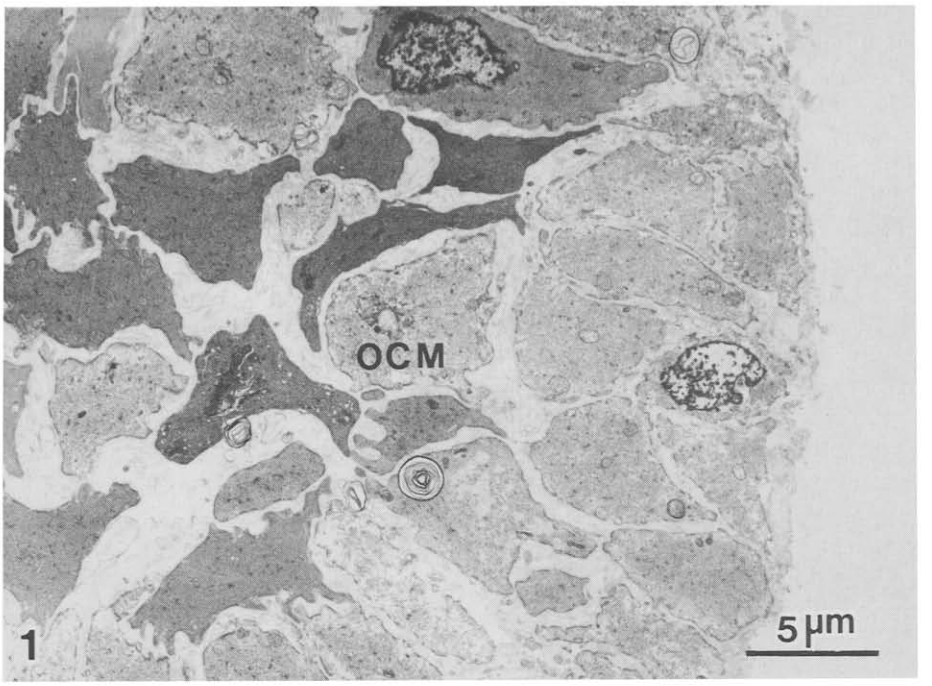
Results

A. Plasma membrane preparation

The electron microscopy of the dissected tissue showed that the dissected circular muscles were free of myenteric plexus (Figs. 1, 2) but contained (not shown) some bundles of nerves between muscle bundles and in the deep muscular plexus. The myenteric plexus plus a thin layer of circular muscle remained with the layer of longitudinal muscle (Fig. 3). Various fractions obtained during the purification procedure were also examined under the electron microscope. The mitochondrial fractions (MIT-I and MIT-II) contained some intact synaptosomes as well as mitochondria (Fig. 4). As shown in Figs. 5 and 6, microsomal fraction (MIC-II) and F3 fraction from the gradient were free of synaptosomes. Any synaptosomes were apparently removed in mitochondrial fractions MIT-I and MIT-II.

Various fractions were obtained by differential centrifugation of the tissue homogenate as described in Methods. The microsomal fraction (MIC-I) was layered on top of continuous sucrose density gradient. After centrifugation, 0.5 ml fractions were withdrawn and assayed for various marker enzyme activities and protein. The results are shown in Fig. 7. 5′-Nucleotidase activity migrated with a peak at 23.5% (w/v) sucrose. There were two protein peaks. One peak (probably corresponding to contractile proteins) was observed at low sucrose density at 13% (w/v) sucrose associated with no marker enzyme activity. One major protein peak was observed at 38% (w/v) sucrose concentration which corresponded to the peaks of NADPH-cytochrome-*c* reductase and cytochrome-*c* oxidase. However, when the MIC-II fraction, instead of MIC-I, was loaded, the protein peak corresponding to a higher buoyant density was reduced considerably (results not shown). In subsequent experiments, MIC-II fraction was loaded on to the gradient.

The summary of the results of the biochemical marker enzyme activities obtained during differential centrifugation and discontinuous sucrose density gradient is given in Tables I and II. The mitochondrial fraction MIT-I contained the specific activity of cytochrome-*c* oxidase equivalent to 312 nmol/mg per min as compared to 45 nmol/mg per min in postnuclear supernatant (PNS), an enrichment of about 7-fold. Fractions F7 and F8 were also enriched in this particular marker enzyme activity. The MIC-I fraction was nevertheless still contaminated by mitochondrial membranes as demonstrated by the specific activ-



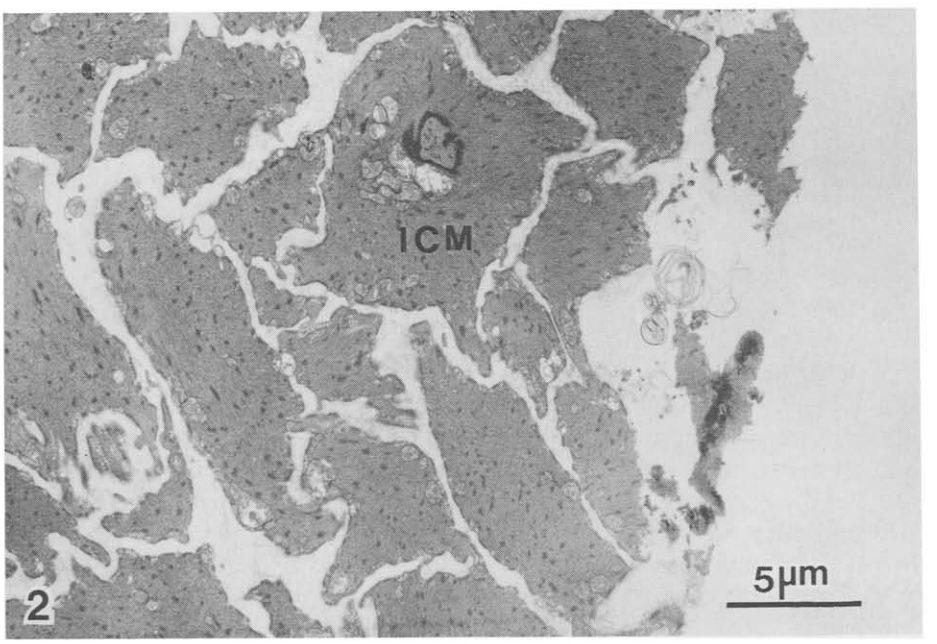
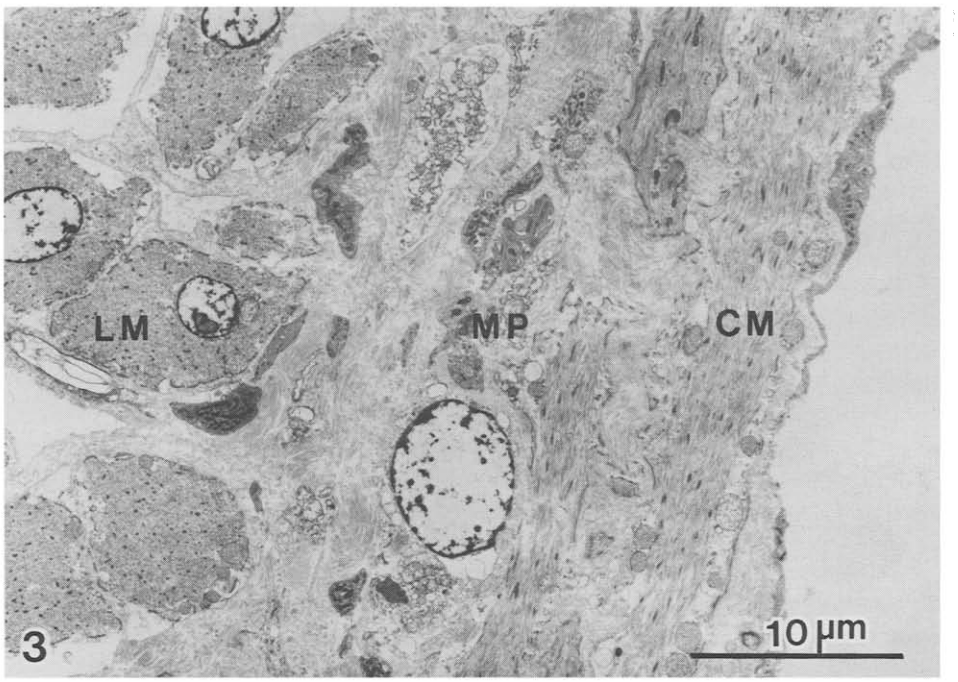


Fig. 1. Electron micrograph of the outer border of dissected circular muscles. Low-power electron micrograph showing the outer border of circular muscles (OCM) near the submucous plexus after cleaning.

Fig. 2. Electron micrograph of the inner border of dissected circular muscles. Low-power electron micrograph showing the inner border of circular muscles (ICM) near the myenteric plexus after cleaning. Note that the myenteric plexus has been completely removed.



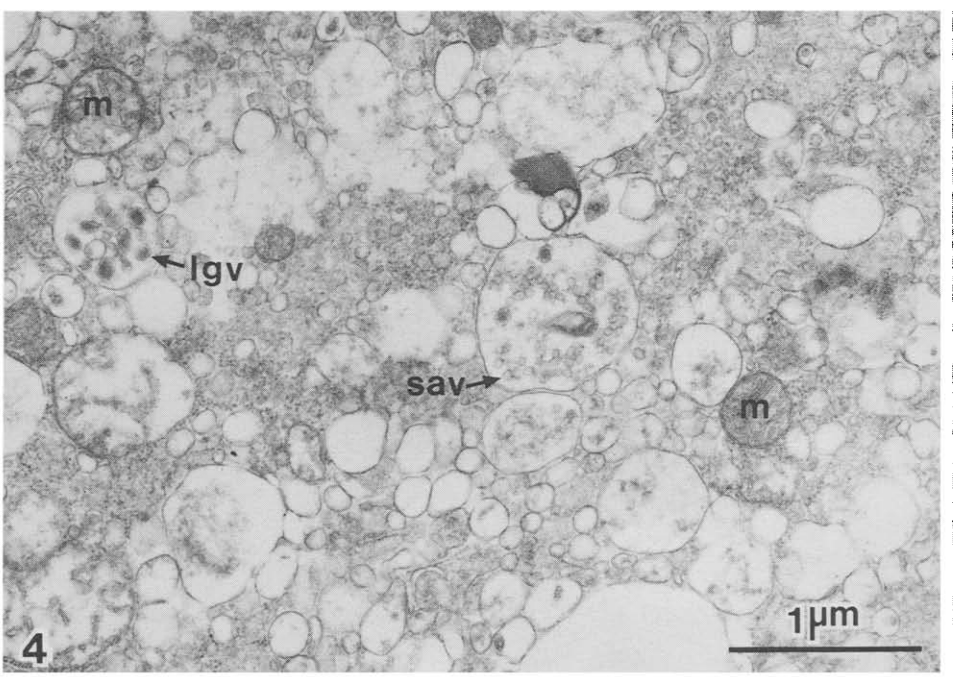
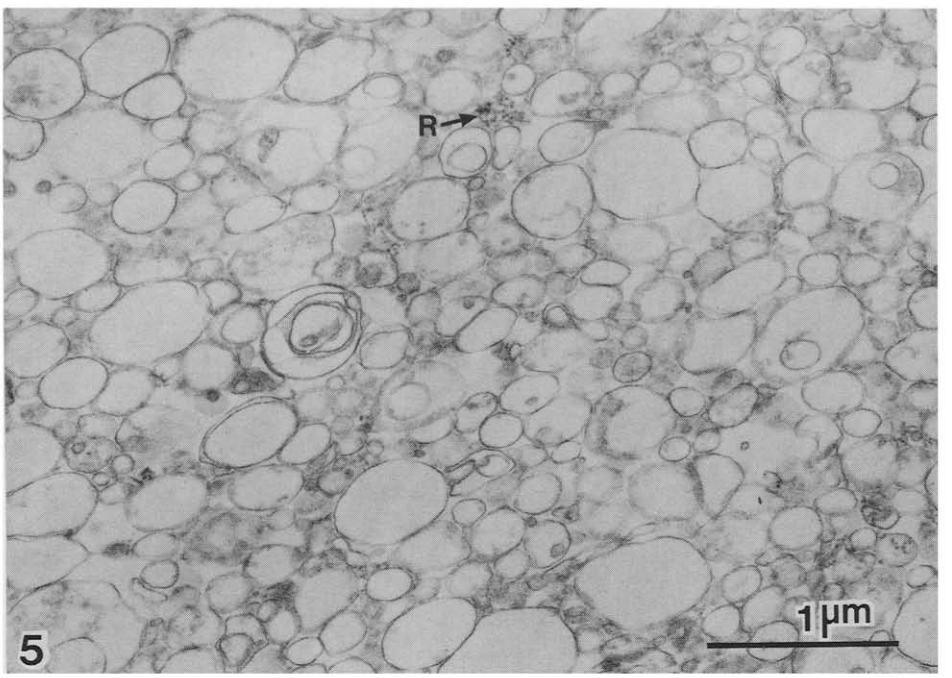


Fig. 3. Electron micrograph of the separated longitudinal muscles showing myenteric plexus. Low-magnification electron micrograph of the cross-section of the inner border of the longitudinal muscle (LM) after cleaning. The myenteric plexus (MP) and several layers of circular muscle (CM) have been always left during cleaning.

Fig. 4. Electron micrograph of the MIT-II fraction. High magnification electron micrograph of the mitochondrial fraction (MIT-II). Note the nerve varicosities containing large granular vesicles (lgv) and small agranular vesicles (sav) present in this fraction. m, mitochondria.



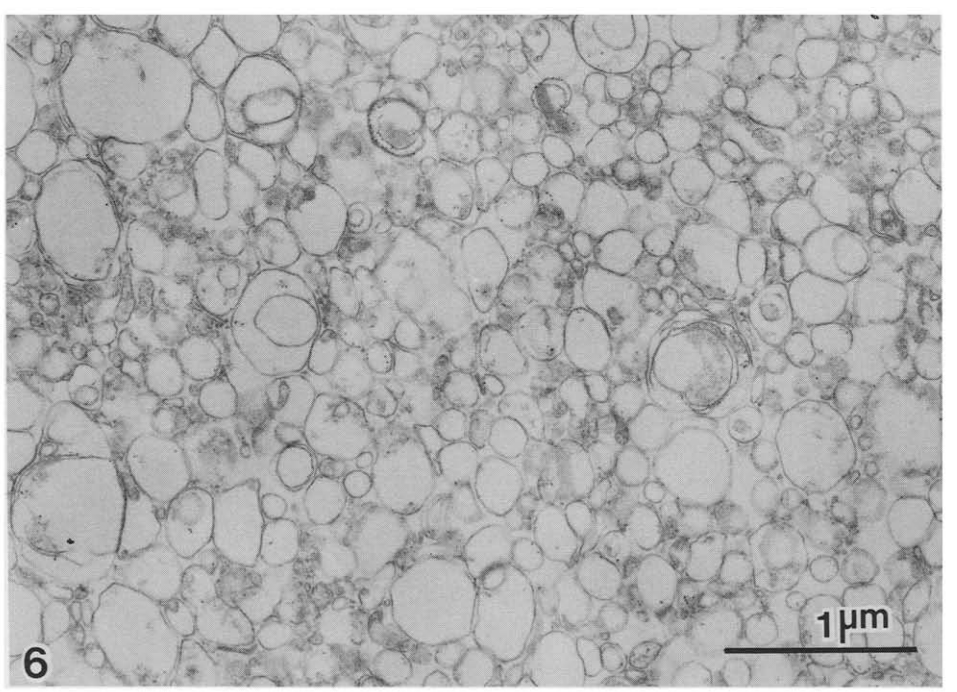


Fig. 5. Electron micrograph of the MIC fraction. High-power electron micrograph of the MIC-II fraction. Small amounts of ribosomes (R) are present in this fraction.

Fig. 6. Electron micrograph of the purified plasma membranes. High-power electron micrograph of the purified plasma membranes.

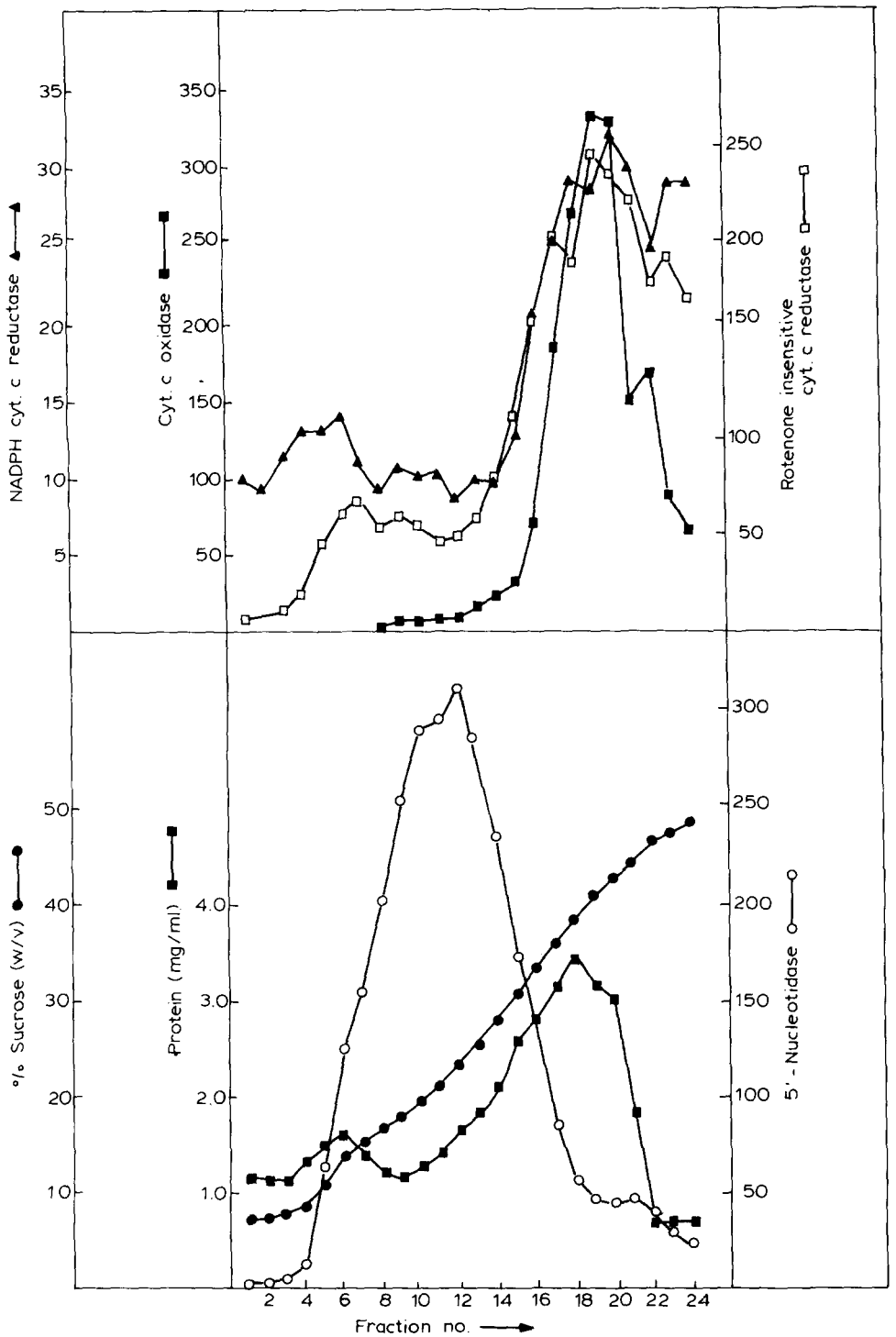


Fig. 7. Distribution of marker enzyme activities after continuous sucrose density gradient. Microsomes were prepared as given in Methods and run over 8–60% sucrose density gradient at 30000 rpm for 100 min. 0.5-ml fractions were collected and assayed for enzyme activities. The figure is the representative of five such experiments.

TABLE I

SPECIFIC ACTIVITIES OF THE MARKER ENZYMES

Fractions were obtained as given in Methods and marker enzyme activities were determined. The values represent mean ± S.D. of the number of experiments given in parentheses. n.d., not detectable. Units of the enzyme activities are as follows; 5′-nucleotidase, μmol P_i/h per mg protein; alkaline phosphatase, μmol/h per mg protein; NADPH-cytochrome-*c* reductase, rotenone-insensitive cytochrome-*c* reductase and cytochrome-*c* oxidase, nmol/min per mg protein. Cyt. *c*, cytochrome *c*

	PNS	MIT-I	MIT-II	MIC-I	MIC-II	SOL
5′-Nucleotidase (8)	9.5 ± 1.2	14.1 ± 3.5	47.9 ± 5.7	58.7 ± 4.7	84.0 ± 6.7	3.6 ± 2.6
NADPH-cyt. *c* reductase (8)	2.4 ± 0.4	3.4 ± 0.6	10.1 ± 0.8	7.2 ± 0.9	4.4 ± 0.5	1.0 ± 0.3
Cyt. *c* oxidase (8)	45.3 ± 8.3	311.9 ± 58.3	325.8 ± 24.8	91.1 ± 16.1	n.d.	n.d.
Rotenone-insensitive cyt. *c* reductase (1)	36.1	133.0	276.8	259.7	111.7	3.8
Alkaline phosphatase (1)	0.63	1.9	5.0	4.3	1.8	0.07

TABLE II

SPECIFIC ACTIVITIES OF THE MARKER ENZYMES

Fractions were obtained as given in Methods and marker enzyme activities were determined. The values represent mean ± S.D. of the number of experiments given in parentheses. n.d., not detectable. Units of the enzyme activities are as follows; 5′-nucleotidase, μmol P_i/h per mg protein; alkaline phosphatase, μmol/h per mg protein; NADPH-cytochrome-*c* reductase, rotenone-insensitive cytochrome-*c* reductase and cytochrome-*c* oxidase, nmol/min per mg protein. Cyt. *c*, cytochrome *c*.

	F1	F2	F3	F4	F5	F6	F7	F8
5′-Nucleotidase (8)	8.6 ± 2.6	40.4 ± 6.8	229 ± 30.6	73.7 ± 21.5	54.2 ± 7.8	33.6 ± 11.6	26.5 ± 3.8	17.0 ± 2.2
NADPH-cyt. *c* reductase (8)	4.5 ± 0.2	5.6 ± 0.4	2.9 ± 0.1	6.3 ± 0.8	8.6 ± 1.2	29.3 ± 7.3	9.4 ± 0.7	8.6 ± 0.8
Cyt. *c* oxidase (8)	n.d.	n.d.	4.1 ± 1.3	17.8 ± 2.8	40.6 ± 2.9	86.3 ± 5.6	324.3 ± 71.7	278 ± 48.3
Rotenone-insensitive cyt.-*c* reductase (1)	30.8	88.1	174.2	201.6	281.4	214.8	305.1	231.3
Alkaline phosphatase (1)	0.42	0.63	1.52	2.94	4.63	7.59	8.96	4.2

ity of 91 nmol/mg per min of cytochrome-*c* oxidase enzyme while this enzyme was undetectable in the MIC-II fraction. The sucrose gradient fractions enriched in cytochrome-*c* oxidase activity were also enriched in the enzymes rotenone-insensitive NADH-cytochrome-*c* reductase and alkaline phosphatase. Fraction F3 contained the highest activity of the plasma membrane marker enzyme 5′-nucleotidase (229 μmol of P_i/mg per h) which was about 25-fold higher as that in PNS. This fraction contained negligible activity of cytochrome-*c* oxidase (4.05 nmol/mg per min) and NADPH cytochrome-*c* reductase (2.87 nmol/mg per min). The highest activity of NADPH-cytochrome-*c* reductase a putative endoplasmic reticulum marker enzyme, was found in fraction F6 (29.32 nmol/mg per min).

B. Neurotensin binding characteristics

1B. Binding to various fractions. Binding of (Tyr^3-^{125}I)-labelled neurotensin to various fractions obtained during the purification procedure is given in Table III. There is approx. 20-fold more binding in the plasma membrane fraction as compared to the postnuclear supernatant (8.74 ± 2.9 vs. 0.449 ± 0.17). Other than plasma membrane fraction, the fractions enriched in mitochondrial membrane marker enzyme showed quite high binding; for example, MIT-II had 6.93 ± 1.71 fmol of specific binding per mg protein.

2B. Protein binding profile. The amount of specifically bound label increased linearly with the increase in the content of membrane protein in the incubation medium up to 100 μg per assay tube (Fig. 8). Nonspecific binding to the plasma mem-

TABLE III

(Tyr^3-^{125}I)-LABELLED NEUROTENSIN BINDING TO FRACTIONS OBTAINED DURING THE PURIFICATION

70–80 pM labelled peptide (2000 Ci/mmol) was incubated with 20–80 μg protein from each fraction at 37°C for 25 min in 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% bovine serum albumin and 1 mM 1,10-phenanthroline. The reaction was terminated and bound ligand was separated as described in Methods. Non-specific binding was determined in parallel experiments in the presence of 1 μM unlabelled neurotensin. Data are expressed as mean ± S.D. with the number of experiments given in parentheses.

Fraction	Bound (fmol/mg)
PNS	0.449 ± 0.17 (7)
MIT-I	2.38 ± 1.1 (5)
MIT-II	6.93 ± 1.71 (6)
MIC-I	4.07 ± 1.06 (4)
MIC-II	2.85 ± 0.91 (7)
PM	8.74 ± 2.9 (11)

branes was found to be negligible at this concentration (80 pM) of the label; all the nonspecific binding observed was on the filters. The non-specific binding did not increase by increasing the concentration of protein in the incubation medium.

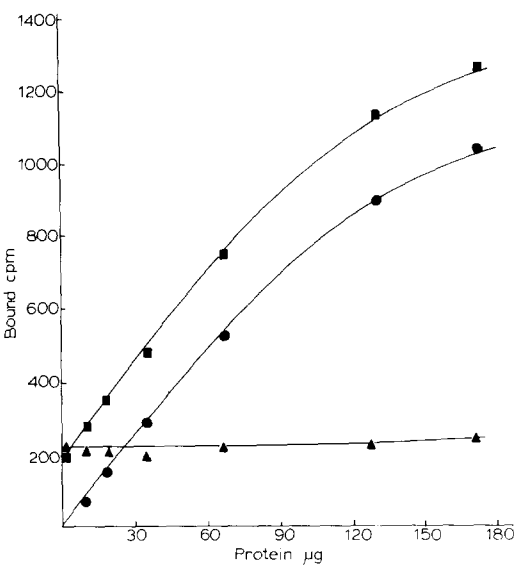


Fig. 8. Protein binding profile. Labelled neurotensin (2000 Ci/mmol; 70–80 pM) was incubated at 37°C in the presence of increasing concentration of plasma membranes (0–180 μg) under the conditions described in Methods. The results are plotted as total (■———■), non-specific (▲———▲) and the specific (●———●) cpm bound.

3B. Time-course of association and dissociation. The specific binding of (Tyr^3-^{125}I)-labelled neurotensin increased with time and reached equilibrium by 20 min (Fig. 9). The half-time of association ($T_{1/2}$) was found to be 3.5 min, about 90% of the total binding at equilibrium (B_{eq}) was achieved by 15 min. The levels of nonspecific binding and the specific binding at equilibrium did not change for up to 80 min of incubation. At equilibrium, bound neurotensin represented only 2% of the total neurotensin label added in the incubation medium, hence at equilibrium, free ligand can be considered to be equal to the total amount of ligand added. For this reasons, the association kinetics could be analyzed as pseudo-first order reaction according to the equation

$$\ln([B_{eq}]/([B_{eq}]-[B])) = (k_1[L]+k_{-1})t$$

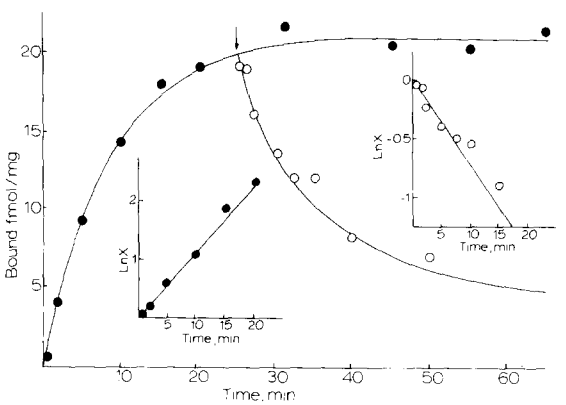


Fig. 9. Time-course of the binding of (Tyr^3-^{125}I)-labelled neurotensin to dog ileum plasma membranes. Labelled peptide (2000 Ci/mmol; 0.07 nM) was incubated with the plasma membranes. At indicated times 0.25-ml aliquots were withdrawn and filtered as described in Methods. Dissociation was initiated by the addition of excess (1 μM) unlabelled neurotensin at the time indicated by an arrow. Non-specific binding was measured throughout the experiment in a different set which contained 1 μM unlabelled neurotensin from the beginning. The data are plotted in terms of specific binding at various times. The insets show the same data plotted according to the pseudo first-order reaction for association and first-order reaction for dissociation. $X = [B_{eq}]/([B_{eq}]-[B])$, where $[B_{eq}]$ is the concentration of the bound ligand at equilibrium and [B] is the concentration of the bound ligand at time t. $Y = [B]/[B_0]$, where [B] and $[B_0]$ are the concentration of bound ligand at time t and time zero of dissociation, respectively.

where $[B_{eq}]$ is the concentration of the bound ligand at equilibrium, [B] is the concentration of the bound ligand at the time t, [L] is the concentration of the ligand added, k_1 is the rate constant of association and k_{-1} is the rate constant of dissociation. The plot $\ln([B_{eq}]/([B_{eq}] - [B]))$ vs. time (Fig. 9 inset) gave a straight line with a slope value $k = k_1[L] + k_{-1} = 0.1175$ min^{-1} where [L] = 69 pM.

The reaction was reversible. Addition of excess (1 μM) of unlabelled neurotensin induced rapid dissociation approaching the value of nonspecific binding (Fig. 9). The dissociation proceeded quickly for about 10 min and slowly after that. Such a biphasic dissociation might be attributed to the two classes of binding sites, since at the concentration of the label used the majority of the ligand bound to the high affinity sites, the dissociation would essentially be from the high-affinity sites (descirbed below). Only the initial rate of dissociation has been taken into account and the data are represented according to the first order kinetics. The plot of $\ln(B/B_0)$ vs. time gave a straight line. A logarithmic representation of the data is given in the inset Fig. 9, the slope of this line represents the rate constant of dissociation which was found to be 0.0715 min^{-1}.

Introduction of k_{-1} into the expression for k (see above) gave a value of $5.8 \cdot 10^8$ for k_1. The dissociation constant was calculated from these values of the rate constants and was 0.107 nM.

The dissociation rate was also assessed by excessively diluting the incubation medium (40-fold) by the incubation buffer at 37°C and diluting the medium in the presence of excess of unlabelled neurotensin to check for the presence/absence of cooperativity between the receptor sites. The results are given in Fig. 12. As is evident from the figure, the rate as well as extent of dissociation is the same in both the cases.

4B. Equilibrium binding characteristics. With increasing concentration of the radioactively labelled peptide, the specific binding to the plasma membranes also increased. The specific binding was saturable at approx. 10 nM and did not increase further (Fig. 10). The nonspecific binding increased linearly as a function of ligand concentration. Analysis of the binding data by the computer program EBDA (McPherson, 1984) gave a curvi-

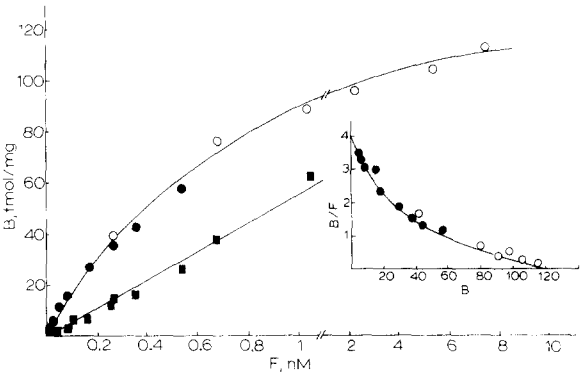


Fig. 10. Binding of (Tyr^3-^{125}I)-labelled neurotensin as a function of increasing concentration of the ligand. Plasma membranes were incubated at 37°C with the increasing concentration of the ligand. After 25 min the reaction was stopped and the bound ligand was separated from the free by filtration. ■———■ denotes the non-specific binding and ●———● and ○———○, the specific binding with the specific radioactivities 2000 Ci/mmol (●———●) or with serially decreasing radioactivities (○———○). The inset is the representation of the same data according to the Scatchard analysis.

linear plot indicative of two binding sites. Further analysis of these binding data with the program LIGAND (Munson and Rodbard, 1980) gave a significantly better fit for a two-site model against a single-site model ($p < 0.05$). The high-affinity site component of the binding was calculated to have the dissociation constant (K_{d1}) value equal to 0.118 nM and the binding capacity (B_{max1}) of this site was 9.73 fmol/mg protein. The corresponding values for the low-affinity sites were $K_{d2} = 3.176$ nM and $B_{max2} = 129.8$ fmol/mg protein. The dissociation constant value obtained by the saturation binding data corresponds reasonably well to that obtained by the reaction kinetics (0.107 nM), which represents the high-affinity sites.

5B. Competition for the neurotensin binding sites. Various fragments of neurotensin and other structurally related peptides were used to see the specificity and the structural requirements for the binding of (Tyr^3-^{125}I)-labelled neurotensin. The competitive inhibition of the binding by unlabelled neurotensin, partial sequences and other related peptides is given in Fig. 11. The IC_{50} values are given in the legend. These values have been obtained by the analysis of competition curves by EBDA program. Neurotensin(8–13) has

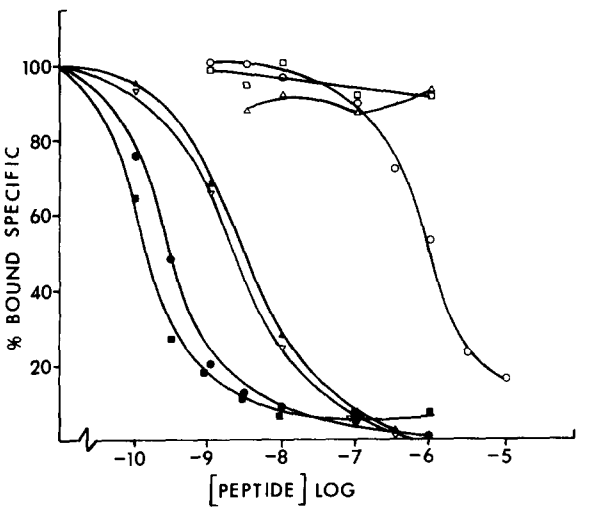


Fig. 11. Competitive inhibition of (Tyr^3-^{125}I)-labelled neurotensin binding by neurotensin analogs and fragments. The membranes were incubated with 70–80 pM labelled peptide (2000 Ci/mmol) with increasing concentration of the competitors under the usual incubations and filteration conditions and described in Methods. The results are plotted as the percentage of the specific binding in the absence of any competitor. ●———●, Neurotensin; ■———■, neurotensin (8–13); □———□, neurotensin (1–11): ▽———▽, [Phe^{11}]-neurotensin; △———△, neurotensin (1–8); ▲———▲, [L-Trp^{11}]-neurotensin; ○———○, [D-Trp^{11}]-neurotensin. The IC_{50} (concentration of unlabelled peptide required to inhibit the specific binding of labelled neurotensin by 50%) values obtained by the computer analysis of data and potency are presented. Potencies were calculated with the potency of the unlabelled neurotensin taken as 100.

Peptide	IC_{50} (nM)	Potency
Neurotensin	0.5	100
Neurotensin (8–13)	0.15	333
[L-Trp^{11}]-Neurotensin	4.5	11.1
[Phe^{11}]-Neurotensin	4.2	11.9
[D-Trp^{11}]-Neurotensin	1250	0.04
Neurotensin (1–11)	> 20000	< 0.0025
Neurotensin (1–8)	> 20000	< 0.0025

the lowest IC_{50} values and is about three times more potent as compared to unlabelled neurotensin itself. [Phe^{11}]-Neurotensin and [L-Trp^{11}]-neurotensin are about equipotent; about 9-times less potent as compared to unlabelled neurotensin. [D-Trp^{11}]-Neurotensin is much less active and neurotensin(1–11) and neurotensin(1–8) fragments are completely inactive up to 1 μM.

6B. Effects of ions and GTP analogue. The results for the effect of ions on the binding of (Tyr^3-^{125}I)-labelled neurotensin are given in Table IV. The specific binding of the peptide is modulated by all the ions tested, and the maximum inhibition of binding was by $CaCl_2$ (residual binding only 6% at 75 mM of $CaCl_2$). At 50 mM concentration in the incubation medium, NaCl inhibited the binding by about 70%, KCl by 52%, $MgCl_2$ by 41% and $CaCl_2$ by 88%.

TABLE IV

EFFECT OF IONS ON THE SPECIFIC BINDING OF MONOIODO (Tyr^3-^{125}I)-LABELLED NEUROTENSIN TO DOG ILEUM PLASMA MEMBRANES

Plasma membranes (20–50 μg) were incubated with labelled neurotensin (2000 Ci/mmol; 70–80 pM) in the presence of varying concentrations of ions as indicated. The data are expressed as percent of specific binding in the absence of any cation.

	Percent binding at			
	10 mM	25 mM	50 mM	75 mM
NaCl	64.8 ± 3.6	32.9 ± 9.4	30.0 ± 4.4	17.4 ± 2.5
$MgCl_2$	70.9 ± 2.8	64.1 ± 3.5	52.8 ± 3.1	29.8 ± 3.8
KCl	60.3 ± 9.3	52.6 ± 2.5	48.4 ± 1.9	37.6 ± 1.8
$CaCl_2$	39.9 ± 6.7	14.8 ± 5.8	12.2 ± 2.8	6.1 ± 2.7

The guanine triphosphate analogue, Gpp[NH]p modulated the specific binding of the peptide. At 10^{-8} M of Gpp[NH]p, 98% of the specific binding was observed. This binding decreased with increasing concentration of the nucleotide; at 10^{-7} M, 88% binding was observed, at 10^{-6} M, 83%; 10^{-5} M, 74% and at 10^{-4} M, only 58% specific binding remained.

Discussion

A. Membrane preparation

The ultimate aim of these studies was to demonstrate the presence of receptors for various neuropeptides (neurotensin, opiates, substance P, etc.) on the smooth muscle plasma membranes. It was necessary to avoid the contamination of smooth muscle plasma membranes by synaptic membranes or synaptosomes, since the presence of a number of neuropeptide receptors on myenteric plexus and other neural structures of various mammalian intestinal tissues has been well estab-

lished [7–11]. We removed the myenteric plexus completely by dissection, but rsidual nerve elements were present in the muscle layer. The various fractions were examined for the presence of synaptosomes of intrinsic nerves which innervated this smooth muslce. Some synaptosomes were observed in MIT-I and MIT-II fractions and the plasma membranes were free of synaptosomes. Although, membranes derived from broken synaptosomes or nerves may have passed into MIC-II and plasma membrane fractions, we feel this occurs only minimally because of studies to be reported elsewhere using various nerve and muscle membrane markers. Moreover, the majority of the contamination by dissociated mitochondrial membranes was eliminated in the MIT-II fraction. The F3 fraction from the gradient, corresponding to the plasma membrane marker enzyme activity had very low mitochondrial membrne marker enzyme activity. The major contamination of this fraction was with the membranes containing rotenone-insensitive NADH-cytochrome-*c* reductase as in a previous isolation of highly purified plasma membrane fraction from smooth muscle [33]. However, over 24-fold enrichment in the plasma membrane marker enzyme activity was achieved as compared to the postnuclear supernatant. This outcome is better than achieved with most other comparable procedures [36]. We believe this fraction is suitable for ligand binding studies to muscle plasma membranes.

B. Neurotensin binding characteristics

In the intestine of dogs, neurotensin is proposed to interact in lowest concentrations with adrenergic transmission by releasing norepinephrine which inhibits the intestinal motility by the action on α_2-adrenergic receptors apparently on post-synaptic cholinergic neurons [4,5,12]. Histamine from mast cells has also been suggested to be a mediator of neurotensin action [26]. In other species and in dogs neurotensin also acts on intestinal smooth muscle directly [4,5,12,16,29]. In dogs, this action is inhibitory [4,5,12]. Neurotensin-immunoreactive nerves were not found in this tissue [11] so neurotensin may act as a hormone. The present study demonstrates the binding of radioactively labelled neurotensin to specific binding sites on a highly purified fraction of plasma membranes prepared from dog intestinal smooth muscle.

The comparison of various fractions, obtained during the purification, for their binding activity revealed the presence of a large number of binding sites in the fraction enriched in plasma membranes. MIT-II fraction which lacks a high-density of markers for smoth muscle plasma membranes also contained a large number of neurotensin binding sites. Two observations, (i) neurotensin inhibits (possibly indirectly) the release of acetylcholine from nerve terminals in the ileum [4,5,12], and (ii) electron microscopic studies of the fractions revealed the presence of intact synaptosomes in MIT-II fraction, suggested to us that the binding observed in this fraction may be to these synaptosomes and synaptic membranes of these nerve terminals which become concentrated in this fraction. This requires further study.

The binding sites on muscle plasma membranes could be resolved in high affinity and low capacity ($K_{d1} = 0.118$ nM; $B_{max1} = 9.3$ fmol/mg), and low affinity and high capacity ($K_{d2} = 3.18$ nM; $B_{max2} = 129.8$ fmol/mg). The binding properties to different preparations are described in Table V. The binding parameters obtained in the present study are similar to those for rat brain and human brain synaptic membranes. The binding sites in rat fundus smooth muscles are of somewhat higher affinity and lower capacity than those for dog ileum.

The high-affinity binding sites observed in our study pose a problem in our understanding of functioning of neurotensin receptors. Sodium ions and the guanine nucleotides are known to regulate the receptors by converting the high-affinity sites to the low-affinity ones. Since receptors normally operate in the presence of 150 mM Na^+ concentration as well as other ions and guanine nucleotides are likely to be present inside the cells, it seems likely that only the low-affinity recpetors are operative in vivo. This is consistent with the observation that the inhibition of intestinal motility in vivo occurs in nanomolar range. The effect of these two modulators to reduce the specific binding of the labelled peptide suggests that the two types of binding sites are two forms of a single type of receptor. The possibility of the negative cooperativity has been studied by observ-

TABLE V

NEUROTENSIN RECEPTORS IN DIFFERENT PREPARATIONS

Comparison of neurotensin receptors on various preparations as present in the literature. The values are taken from the references given in the brackets.

Source	K_{d1} (nM)	K_{d2} (nM)	B_{max1} (fmol/mg)	B_{max2} (fmol/mg)
Dog ileum	0.118 ± 0.042	3.18 ± 1.37	9.73 ± 2.2	129.8 ± 48.3
Rat brain [40]	0.1	4.7	17	126
Rat fundus [29]	0.056	1.92	6.6	11.4
Human brain [24]	0.26	4.3	26	89
Neuroblastoma cells [25]	0.17		9.0	

ing the dissociation achieved by diluting the incubation mixture in the absence or presence of the excess of unlabelled neurotensin. The rate as well as the extent of dissociation was found to be equal in both cases (Fig. 12). However, only about 50% dissociation was achieved in both the cases, the reason for this is unclear. In the case of negative cooperativity between the receptors as opposed to the two-site model one would expect more rapid dissociation in the presence of excess of unlabelled peptide since occupation of more receptors would cause a decrease in the affinity. Since no such difference was observed, we propose that in isolated membranes under our conditions there are two classes of receptors rather than negative cooperativity among the receptors.

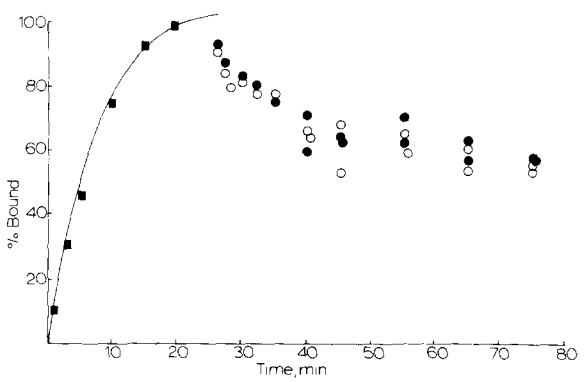


Fig. 12. Dissociation of the bound labelled neurotensin from the plasma membranes. The association was performed as usual. At 25 min, dissociation was induced by diluting the incubation mixture 40-fold by the incubation buffer (●) or, by diluting 40-fold in the presence of 1 μM unlabelled neurotensin (○). The data are plotted as percent of binding at equilibrium.

In murine species, the receptors are different from those in dog. Neurotensin and [Trp^{11}]-neurotensin are of the same potency and [D-Trp^{11}]-neurotensin only slightly less potent [28,29]. In human brain [Trp^{11}]-neurotensin is about 10-times less potent as compared to the native neurotensin [24]. Canine smooth muscle receptors, like those in human brain, discriminate between neurotensin and [L-Trp^{11}]- and [D-Trp^{11}]-neurotensin. The activity of [L-Trp^{11}]-neurotensin was 1/10 that of neurotensin and that of [D-Trp^{11}]-neurotensin was 200-fold less. Functional studies of these receptors show a corresponding lack of potency of [L-Trp^{11}]-neurotensin (1/10 of neurotensin) (Fox, J.E.T. and Daniel, E.E., unpublished data). Neurotensin (8–13) was found to be the most potent peptide tested. In some other species too, this peptide is found to be more potent than neurotensin, but this is not a general observation [24–28].

An observation deserving further study was the high levels of binding in MIT-II fraction. The presence of synaptosomes in this fraction suggests that these synaptosomes may be responsible for this binding activity. Furthermore, we have confirmed by target size analysis by radiation inactivation (paper in preparation) that receptor populations from plasma membranes and MIT-II fractions are different from each other in terms of their molecular size (M_r 200 000 on plasma membranes; 100 000 in MIT-II). We suggest that the receptors in the MIT-II fraction represent those on adrenergic and probably on some other nerve terminals and that they modulate the release of other neurotransmitters. We are at present in the process of studying the properties of the binding sites observed in the MIT-II fraction.

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