PEPTIDE RECEPTORS IN CANINE SMALL INTESTINE

By

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ABSTRACT

Localization and subtype distribution of the receptors for neurotensin and opioids in canine small intestine was studied by the radioligand binding technique.

Extensive dissection procedure was developed to separate a) longitudinal muscle (LM) layer containing myenteric plexus (MP), b) the circular muscle (CM) layer containing the deep muscular plexus (DMP) and c) the submucosa containing the submucous plexus (SMP).

Purified membranes were prepared from the LM, MP, CM, DMP and SMP by differential and the density gradient centrifugations and using the markers 5'-nucleotidase (for smooth muscle plasma membranes), NADPH cytochrome C reductase (for endoplasmaic reticulum), cytochrome C oxidase (for mitochondrial membranes), specific binding of [^{3}H]saxitoxin for the neuronal membranes and the content of the vasoactive intestinal polypeptide immunoreactive material as a measure of intact synaptosomes.

The fractions enriched in the membranes from LM, CM, DMP, MP and SMP were used to study the distribution and properties of neurotensin receptors using [^{125}I]Tyr^{3}-neurotensin and of opioid receptors using [^{3}H]diprenorphine, [^{3}H]etorphine and [^{3}H]ethylketocyclazocine.

Neurotensin receptors were confined to the CM, DMP and SMP. These receptors had the similar affinity and recognition properties at their high affinity sites (Kd 0.1 - 0.2 nM). The low affinity receptors on the DMP were of lower affinity than their CM counterparts (Kd 40 nM vs 3 nM). The receptors on the CM differed from those on the DMP in their radiation target size (mw ~ 190,000 da. on the CM and ~120,000 da. on the DMP). Reduced disulfide bridges were required for the binding to both the CM and DMP neurotensin receptors. However, *in vitro*, the excitation, but not the inhibition, of the circular muscle strips to added neurotensin was abolished on reduction of the disulfide bonds.

Opioid receptors were present on the DMP, MP and SMP but not on any smooth muscle. The receptors on all the three plexuses had similar affinity for the non-selective opioid ligand [^{3}H]diprenorphine (Kd ~0.1 - 0.2 nM). Both the DMP and MP contained ~40-45% of μ- and δ-subtypes of opioid receptors as assessed in competition studies using μ-selective PLO17, and δ-selective DPDPE. SMP contained a higher proportion of μ- over δ-subtype (64% μ and 24% δ). All three plexuses contained similar proportion of k-subtype (10-15%), assessed by competition studies with dynorphin[1-13] and U-50488H, and confirmed by saturation experiments with [^{3}H]ethylketocyclazocine with or without sheilded μ- and δ-receptors. Ionic regulation of these receptors was similar to those observed for the opioid receptors in other systems.

Therefore the action of neurotensin on the motility of the canine small intestine may be the combined result of its action on the smooth muscle and on the modulation of the release of other mediators presynaptically at the DMP level. The action of opioids on the motility is probably through the modulation of the release of other mediatorsat the DMP and MP level: direct action of opioids on the smooth muscle is not supported by the present studies.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
PNS	Post nuclear supernatant
Mit.	Mitochondrial
Mic.	Microsomal
Sol.	Soluble fraction
DMP	Deep muscular plexus
MP	Myenteric plexus
SMP	Submucous plexus
MOPS	3-(N-morpholino)propanesulfonic acid
Tris	Tris(hydroxymethyl)aminomethane hydrochloride
DPDPE	[D-Pen^2-D-Pen^5]enkephalin
PLO17	[N-Me-Phe^3-D-Pro^4]morphiceptin
STX	saxitoxin
TTX	Tetrodotoxin
DPR	Diprenorphine
NT	Neurotensin
5'-ND	5'-nucleotidase
DTT	1,4 dithiothreitol

dedicated to my parents

CHAPTER 1

PRESENT STUDIES : RATIONALE

Functions of the gastrointestinal tract are under neuronal and hormonal influences as well as myogenic ones. Several functional approaches have been used to elucidate how various mediators alter the function of the gastrointestinal tract. Immunohistochemical methods, radioimmunoassays and direct measurements have localized a number of mediators to intrinsic or extrinsic neurons, or to the endocrine cells within the gastrointestinal tract. Beside classical neurotransmitters, peptides have also been identified within nerves or endocrine cells of the gastrointestinal tract of several species. As an attempt to establish their physiological roles, functional, ligand binding, and other biochemical studies have been carried out to determine the nature of the responses elicited by classical or peptide neurotransmitters or hormones, the type of receptors which are involved and their coupling to the response. These have not been adequately complemented by studies of the biochemical characterization of these receptors or their coupling to the response, mainly because of the difficulty to separate various cell types which may contain a particular receptor.

The present work was undertaken to study the receptors for two such peptides, neurotensin and opioids, chosen for several reasons. Both of these peptides have been shown to be present within the gastrointestinal tract. Functional studies have demonstrated their action on motility as well on secretion/absorption. The actions of both of these peptides on contractility of small intestine, it has been suggested, may result from their direct action on the

smooth muscle and indirect actions through the modulation of the release of other mediator/s. One of them (opioids) is present primarily in enteric neurons, while the other (neurotensin) is present predominantly in endocrine cells. Ligand binding and functional studies have established the presence of opioid receptors on enteric nerves, but their presence on the smooth muscle cells of gastrointestinal tract is still controversial. Neurotensin receptors on the other hand have been demonstrated on the smooth muscle of gastrointestinal tract, no attempt has been made to study them on enteric neurons. No ligand binding studies with either of these peptides had been performed on the canine gastrointestinal tract in which different responses to these peptides have been observed compared to other species and in which immunohistochemical differences from other species have also been observed. Further interest in opioid receptors arose from the recent availability of ligands, highly selective for opioid receptor subtypes. No binding studies in the gastrointestinal tract have been reported which have used these highly selective ligands to study the opioid receptor subtypes.

Radioligand binding studies were therefore undertaken to localize and characterize the neurotensin and opioid receptors in the canine small intestine. Attainment of these goals required development of techniques to separate cell types and characterize membranes from various cell types. In addition, some functional data about the actions of neurotensin were obtained to correlate with biochemical studies.

Since, extensive characterization of the receptors by the radioligand

binding techniques requires the membrane preparation, the purification of the relevant membranes becomes the first essential step for such experiments. Therefore, the first part of the present project was to establish the procedures for separating, purifying and characterizing membranes from various plexuses and smooth muscle layers of canine small intestine; this will be described and discussed in Chapter 2. Chapter 3 will deal with the previous and present studies on neurotensin and neurotensin receptors in the gastrointestinal tract and Chapter 4 will deal similarly with opioid receptors. Concluding remarks are presented in the Chapter 5 followed by an appendix describing the technique and principle of ligand binding studies and the structure of the gut. Some publications of the work described here and some other relevent publications from our and our collaborators laboratories are listed in the appendix for the reference.

CHAPTER 2

MEMBRANE PURIFICATION

2.1. INTRODUCTION

Plasma membranes of living cells function not only as a structural component contributing to the integrity and shape of the cells, but also as a physical barrier in the form of a semipermeable membrane to protect the intracellular environment and separate and maintain intracellular components from extracelluar components. The plasma membrane plays a very important role in regulating intracellular milieu of the cell for various physiological functions as a consequence of the presence of various ion selective pores (channels), as well as pumps and exchangers which regulate the electrochemical gradients of charged species. Furthermore, plasma membranes provide the site of action for a majority of chemical messengers, and are instrumental in delivering the extracelluar signal to the internal milieu of the cell for the proper integration of functions in multicellular organisms. The properties of the plasma membrane reflect, to some extent, the function of the cell it surrounds.

Many tissues contain several different cell types. As discussed earlier, the gastrointestinal tract, besides containing smooth muscle cells, contains several other cell types, with each potentially having unique properties regarding its plasma membrane. Furthermore, several intracellular organelles are also surrounded by membranes again each having some unique properties. It becomes imperative, therefore, to obtain reasonably pure membranes from the desired cell type or

organelle if biochemical studies including radioligand binding or transport studies, are to be performed on a particular receptor in a membrane preparation from a particular cell type.

The importance of pure membrane preparations for such studies is two fold. One is to remove the contaminating macromolecules and organelles (nucleus, endoplasmic reticulum, mitochondria etc) from the preparation, so that a better signal to background ratio is obtained, yielding reproducible and more trustworthy results. The second is to obtain the preparation of the relevant membranes from a complex homogenate, which, even after careful dissection, may contain membranes from several different cell types (eg. smooth muscle, neuronal elements, glial cells and interstitial cells), so that appropriate conclusions regarding the actual locus and density of the binding sites can be reached.

The concept of the membrane purification from the smooth muscle is not new. A number of studies, describing the purification of plasma membranes from various smooth muscles are present in literature (Kidwai,1971; Wie,1976; Kwan,1979; Matlib,1979; Grover,1980; Maeda,1983). In general, the membrane purification procedure involves dissection of the tissue to limit the number of cell types present, homogenization of the tissue and a series of centrifugation steps to separate the membranes and particles. This is usually followed by further purification according to the buoyant density of individual membranes with density gradient centrifugation. Various fractions obtained during this procedure are,

therefore, evaluated as to the content of specific markers for different membrane types. Although the general schema for the purification of the plasma membranes from various sources is similar, modifications in methods are required for individual tissues.

Before the homogenization, it is important to dissect the tissue as precisely as possible in order to begin by reducing the number of different cell types present, and enhancing the proportion of the desired cell type. For each tissue, appropriate dissection procedure needs to be established. Careful dissection was even more important for the present studies, since the aim of was to establish the locus of peptide receptors in various layers within the wall of small intestine. Therefore, an attempt was made to separate various layers by dissection, in order to obtain appropriate membrane fractions. The following layers were separated by dissection;

a) Longitudinal muscle/myenteric plexus - to purify the plasma membranes from longitudinal smooth muscle and from the myenteric plexus.

b) Circular muscle layer - to obtain circular smooth muscle plasma membranes and the neuronal membranes from the deep muscular plexus.

c) The submucosa - to purify submucosal plexus neuronal membranes for further studies on peptide receptors.

The membrane purification procedure relies heavily on the use of the marker enzymes for different membrane types. Excellent reviews have appeared

in recent years focusing on the validity of markers and procedures for membrane purification, the reader is referred to those for detailed description (Daniel,1982; Kwan,1987). The following is a brief description of the markers that have been used in the present studies.

Reasonably good markers are available for the smooth muscle plasma membranes. Routinely used markers for the smooth muscle plasma membranes are 5'-nucleotidase (EC 3.1.3.5), ouabain-sensitive, K^+-activated, p-nitrophenyl phosphatase, and phosphodiesterase ((EC 3.1.4.1) (Daniel,1982; Kwan,1987). 5'-nucleotidase and phosphodiesterase, however, remain the most widely used marker enzymes for smooth muscle plasma membranes since Na-pump components are present in all cell types. A minor limitation for the use of 5'-nucleotidase is its presence (40-50% of the total enzyme activity) in the soluble fractions (Kidwai,1971; Wie,1976; Kwan,1979; Matlib,1979; Grover,1980; also see Daniel,1982), but in all the studies performed this enzyme shows a unimodal distribution in particulate fraction, parallel with the activities of other smooth muscle plasma membrane marker enzymes. Low activities of this enzyme are present in neurons as well; this aspect will be discussed later. The advantage of using this enzyme as a marker is that it is easily measurable and smooth muscles usually contain high specific activity of this enzyme. Phosphodiesterase activity, however, may be quite low in some tissues (Kidwai,1970).

Two classes of markers enzymes have been used to localize the

mitochondrial membranes, ones that are present on the inner mitochondrial membranes and the others that are present on the outer mitochondrial membranes. The enzyme rotenone-insensitive NADH cytochrome C:reductase (EC 1.6.2.1) has been used as a marker for outer mitochondrial membranes. However, it has been suggested (Crane,1976), that this enzyme may be present on the plasma membranes in liver or fat cells. Furthermore, Grover *et. al.* (1983) also found it to be highly enriched in fractions also enriched in the smooth muscle plasma membranes in canine trachealis. Kwan *et. al.* (1982) observed that the distribution of rotenone-insensitive NADH cytochrome C:reductase more closely resembles that of the endoplasmic reticulum rather than the mitochondrial membranes. Similar observations were also made by Matlib *et. al.* (1979). Therefore, the status of rotenone insensitive cytochrome C:reductase appears to be unclear at the moment and requires further studies. Cytochrome C oxidase (EC 1.9.3.1) is present on the inner mitochondrial membranes and is used more often than any other marker for the mitochondrial membranes. The high specific activity of cytochrome C oxidase and the ease of its measurement makes it a preferred enzyme as a marker for the mitochondrial membranes. Cytochrome c oxidase is not very stable upon storage of membranes, but this problem can be circumvented if measurement are performed on the fresh membranes. Succinate cytochrome C:reductase (EC 1.3.99.1) has also been used as a marker for outer mitochondrial membranes, but this assay appears to be less sensitive in some tissues (Kwan,1987). In canine

trachealis membrane fractionation studies, high specific activity of this enzyme was observed which was enriched by about 20 fold in the mitochondrial fraction (Grover,1980). Therefore, succinate cytochrome C:reductase also appears to be a good mitochondrial membrane marker enzyme, at least for some tissues.

The best marker enzyme for the smooth muscle endoplasmic reticulum (ER), NADPH cytochrome C:reductase (NCR, EC 1.6.99.3), is still known as a "putative" ER marker. However, data from many smooth muscles are consistent with it being associated with ER membranes (see Daniel,1982). Matlib *et. al.* (1979) found [^{3}H]leucine incorporation and RNA content enriched in gradient fractions, following a pattern similar to that of the NCR.

To summarize, reasonably good markers are available for the smooth muscle plasma membrane, the ER and also the mitochondrial membranes. A sensitive, specific and universal assay for the neuronal membranes innervating the gastrointestinal tract, and other smooth muscle organs in general was lacking at the initiation of this study. For the present studies on the localization and characterization of receptors for neuropeptides, it was essential to purify the plasma membranes from different smooth muscles (longitudinal and circular), to assess the contamination of smooth muscle by neuronal membranes, to purify the neuronal membranes from different plexuses (myenteric, deep muscular and submucosal plexuses), and to assess the contamination of the neuronal membranes by the smooth muscle plasma membranes. To achieve this goal, a good marker for

the neuronal membrane was required. Occluded lactate dehydrogenase and [^{3}H]choline uptake have been used for the preparation of synaptosomes from central nervous system and the guinea pig ileum (White,1982; Whittaker,1984), however, the drawbacks of using these markers is that their activity is dependent on the integrity of pinched-off nerve endings (synaptosomes) as closed vesicles. Moreover, the activity of these markers would be confined to the nerve endings, therefore, giving false negative results regarding the axonal membranes.

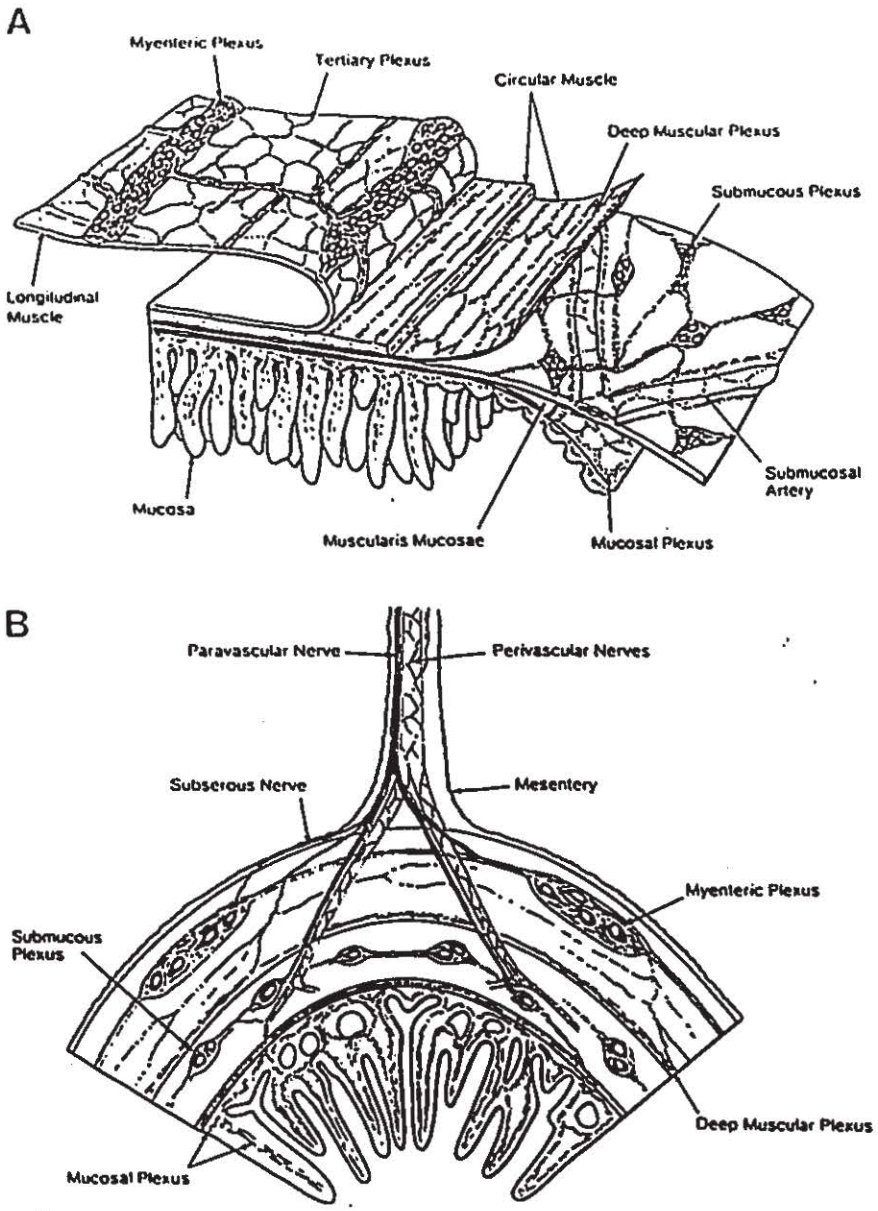
Most of the nerve-mediated actions within the gastrointestinal tract are tetrodotoxin-(TTX)sensitive. TTX and saxitoxin (STX) are guanidinium neurotoxins that bind to the voltage-sensitive sodium channels (VSSC), a channel absent from smooth muscle cells. The action of these toxins is to block the action potential in excitable tissues (nerves, cardiac and skeletal muscles) since in these tissues the inward depolarizing current for the action potential is generally carried by sodium ion through the VSSCs (Hodgkin,1952). In smooth muscles, on the other hand, VSSCs, are absent and the action potential current is carried by calcium ions (Bulbring,1963). We reasoned that since the peripheral nerves also contain VSSCs and smooth muscle cells lack these channels, a ligand for sodium channels may serve as a good marker for the enteric neurons, and perhaps, has a potential for use to either purify membranes of other peripheral nerves, or to assess the contamination of smooth muscle plasma membranes in general, by neuronal membranes. Both STX and TTX are available in the radioactive forms, and have

been shown to bind to the sodium channels in one-to-one fashion (Ritchie,1977). Rapid advances have been made in the study of VSSCs from central as well as peripheral nerves, and from cardiac and skeletal muscles. Using radioactively labelled STX and TTX as tools, VSCCs have been localized, purified, reconstituted, and even been cloned from various sources (Numa,1986; Agnew,1984; Lazdunski,1986; Rogart,1981). We decided to examine the potential of using radioactively labelled saxitoxin as a marker for enteric neurons.

To substantiate the validity of using STX as a neuronal marker, we decided to utilize the available facilities in our laboratory for the radioimmunoassay of vasoactive intestinal polypeptide (VIP) content. The advantage of measuring VIP-immunoreactivity (VIP-IR), against other peptides, as a marker for the synaptosomes, is that VIP-IR has been localised in cell bodies and varicosities throughout the gastrointestinal tract, including sphincteric and non-sphincteric regions of several mammalian species (Furness,1987). VIP is therefore, one of the most abundant and widely distributed neuropeptide in the mammalian gastrointestinal tract. Furthermore, VIP-IR is confined to the nerves and is not present in enteric endocrine cells and the concentration of VIP in small intestine (of at least guinea pig) appears to be constant in all the layers of the wall (Furness,1987). Therefore, VIP-IR also appeared to have potential as a marker for the assessment of the presence of intact synaptosomes.

The features of the small intestine are illustrated in the fig.1. This diagram was constructed on the basis of the observations in the guinea pig small intestine, but similar arrangement exists in the small inetstine of other mammals as well (Furness,1987)

Figure 1. Diagrammatic representation of the structure of the wall of the small intestine of guinea pig. Similar arrangement with minor differences exists in other mammalian species. Reproduced from Furness,1987.


A
Myenteric Plexus
Tertiary Plexus
Circular Muscle
Deep Muscular Plexus
Submucous Plexus
Longitudinal Muscle
Submucosal Artery
Mucosa
Muscularis Mucosae
Mucosal Plexus
B
Paravascular Nerve
Perivascular Nerves
Subserous Nerve
Mesentery
Myenteric Plexus
Submucous Plexus
Deep Muscular Plexus
Mucosal Plexus

2.2 MATERIALS

[11-^{3}H]saxitoxin (17-46 Ci/mmol) was purchased from Amesrham (Arlington Heights, IL), tetrodotoxin, 3-(N-morpholino)propanesulfonic acid (MOPS), bovine serum albumin, tris (hydroxymethyl)aminomethane hydrochloride (Tris.HCl), enzyme substrates etc. were from Sigma Chemical (St. Louis, MO). All other chemicals and salts were either from Sigma or BDH Chemicals. The VIP antiserum was kindly provided by Prof. N. Yanaihara.

2.3 METHODS

2.3.1 TISSUE HANDLING AND DISSECTION

Adult mongrel dogs were killed by intravenous injection of sodium pentobarbital (100 mg/kg). An incision was made in the abdomen and leaving behind about six inches from the ileocecal junction, the whole small intestine was removed and suspended in ice-cold sucrose-Mg-MOPS buffer (25 mM MOPS, 10 mM MgCl2, 8% w/v sucrose, pH 7.4; hereafter referred to as sucrose-MOPS buffer). All further steps, unless otherwise indicated, were carried out in the cold either on ice or at 4° C.

The intestine was cut into one to two inch lengths, cleaned of mesenteric arcade and fat, and opened along the mesenteric attachment line. These pieces were pinned with mucosal surface down on a dissecting plate put on ice. The longitudinal muscle layers were carefully peeled off in a way so that a thin layer of circular muscle (hence myenteric plexus) accompanied it. The remaining circular muscle layers were then pulled off in strips. The rest of the tissue containing mucosa, muscularis mucosa and the submucosa was then turned over such that the mucosa now faced upward. Using a razor blade, the mucosal layer and perhaps most of the muscularis mucosa was then scraped using firm pressure. The remaining layer was then a very thin film of connective tissue with the submucosal plexus present in it. All the layers dissected were put immediately in ice cold sucrose MOPS buffer. The tissues were then blotted dry on filter papers,

weighed, and either processed immediately, or stored frozen at -25°C until further processing.

2.3.2. MORPHOLOGICAL TECHNIQUES

For the purpose of the electron microscopy, the dissected tissue slices or the membrane pellets were immersed in 2% glutaralhyde (containing 4.5% sucrose + 1 mM $CaCl_2$ in 75 mM cacodylate buffer, pH 7.4) for 2 hrs. at room temperature. Following fixation the samples were washed overnight in cacodylate buffer, containing 6% sucrose and 1.25 mM $CaCl_2$, pH 7.4 at 4° C and postfixed in 2% osmium tetraoxide (in 50 mM cacodylate buffer, pH 7.4) at room temperature for 90 min. The samples were then stained *en bloc* with saturated uranyl acetate for 60 min. and dehydrated in graded ethanol and propylene oxide and embedded in spurr resin. Sections were cut on a Sorvall (MT2-B) ultramicrotome, stained for 3 min with lead citrate, and examined in a Phillips 301 electron microscope. Multiple sections from all parts of each sample were examined.

For the zinc-iodide osmium-tetraoxide staining of the tissue, the method of Maillet (1963) adapted by Rumessen *et. al.* (1982) was used. The tissue , using pins, was gently stretched on a dissecting plate and was incubated in the dark with the staining solution containing 0.4 % OsO_2 and 2.4 % ZnI_2. The tissue was examined periodically under a light microscope and at appropriate time it was rinsed and photographed.

2.3.2 MEMBRANE PURIFICATION

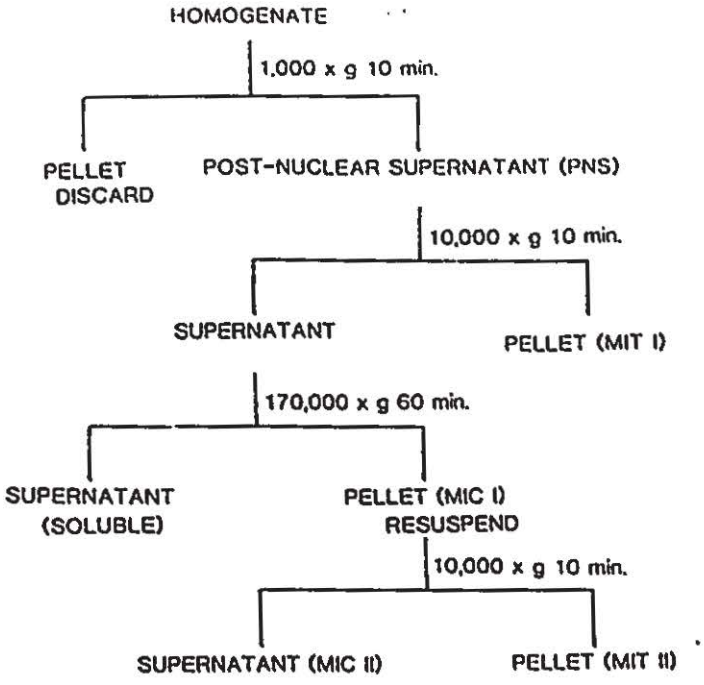
The circular muscle layers were resuspended in ten volumes of sucrose-MOPS buffer (usually about 20 gms of tissue was used per preparation) and finely minced with a pair of scissors. The minced tissue was transferred to chilled plastic 30 mL Sorwall tubes held on ice. The tissue was then homogenized in a Polytron PT-20 homogenizer for 3 X 7 seconds bursts at approximately 15,000 rpm, allowing some cooling time between the bursts.

The flow-chart for the preparation of the circular smooth muscle plasma membranes and the deep muscular plexus neuronal membranes is illustrated in Fig. 2. Briefly, the crude homogenate was centrifuged at 1,000 x g for 10 minutes, the pellet thus obtained was discarded and the supernatant, called post-nuclear supernatant (PNS), was centrifuged at 10,000 x g for 10 minutes. The pellet was the mitochondrial pellet (Mit I) and the supernatant was subjected to high speed centrifugation at 120,000 x g for 60 minutes in 60 Ti rotor of a Beckman LS50 or LS65 ultracentrifuge. The pellet at the bottom of the tubes was the crude microsomal pellet Mic I and the supernatant was the soluble fraction, Sol. The microsomal pellet was resuspended in the sucrose MOPS buffer using Teflon-coated hand-held homogenizer and was centrifuged at 10,000 x g for 10 minutes to obtained the purified microsomal supernatant (Mic II) and the second mitochondrial

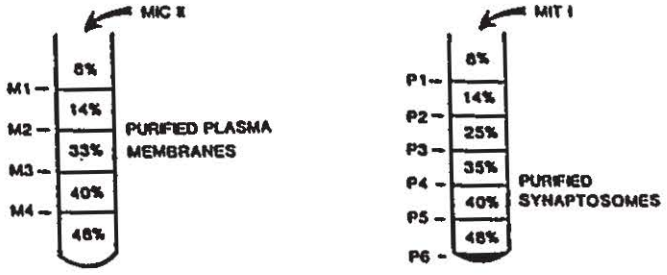
pellet Mit II. In initial experiments, the fractions Mic I and Mic II were loaded on the continuous sucrose density gradients (30,000 rpm for 100 min) in order to establish the focusing density for the smooth muscle plasma membranes. Based on the results of the continuous density gradients, step-wise gradient was constructed for the smooth muscle plasma membranes. The gradient consisted of 2.5 mL each of 48%, 40%, 33%, and 14% (w/v) sucrose. The gradients were centrifuged at 30,000 rpm for 100 minutes in a SW40 rotor in a Beckman LS50-B ultracentrifuge. The bands at each interface were collected and assayed for the enzyme and binding activities. To prepare synaptosomes form the deep muscular plexus, the fraction Mit I was loaded on a discontinuous gradient consisting of 2.0 mL each of 48%, 40%, 35%, 25%, and 14%. Again, bands at all interfaces and the pellet at the bottom were collected and assayed for the enzyme and binding activities.

The scheme for the processing of the longitudinal muscle/myenteric plexus, as described by Kostka *et. al.* (1987) is illustrated in the Fig. 3. A brief description is as follows. The tissue was suspended in ten volumes of sucrose MOPS imidazole buffer (0.25 M sucrose, 20 mM MOPS-imidazole, pH 7.4). The tissue was finely minced with the scissors and was homogenized in a Polytron for 20 seconds at 1,500 r.p.m. The homogenate was centrifuged at 800 x g for 10 min., the pellet was discarded, the supernatant was centrifuged again at 800 x g for 10 minutes and both the supernatants (post-nuclear supernatant, PNS) were combined. PNS was centrifuged at 2,500 x g for 10 min., the pellet thus obtained was P1 and the

Figure 2. The scheme for the processing of the circular muscle layer to obtain the purified circular smooth muscle plasma membranes and purified synaptosomes from the deep muscular plexus of canine small intestine. Mic I = crude microsomal fraction, Mic II = purified microsomal fraction, Mit I = first mitochondrial fraction, Mit II = second mitochondrial fraction, Sol = soluble fraction.



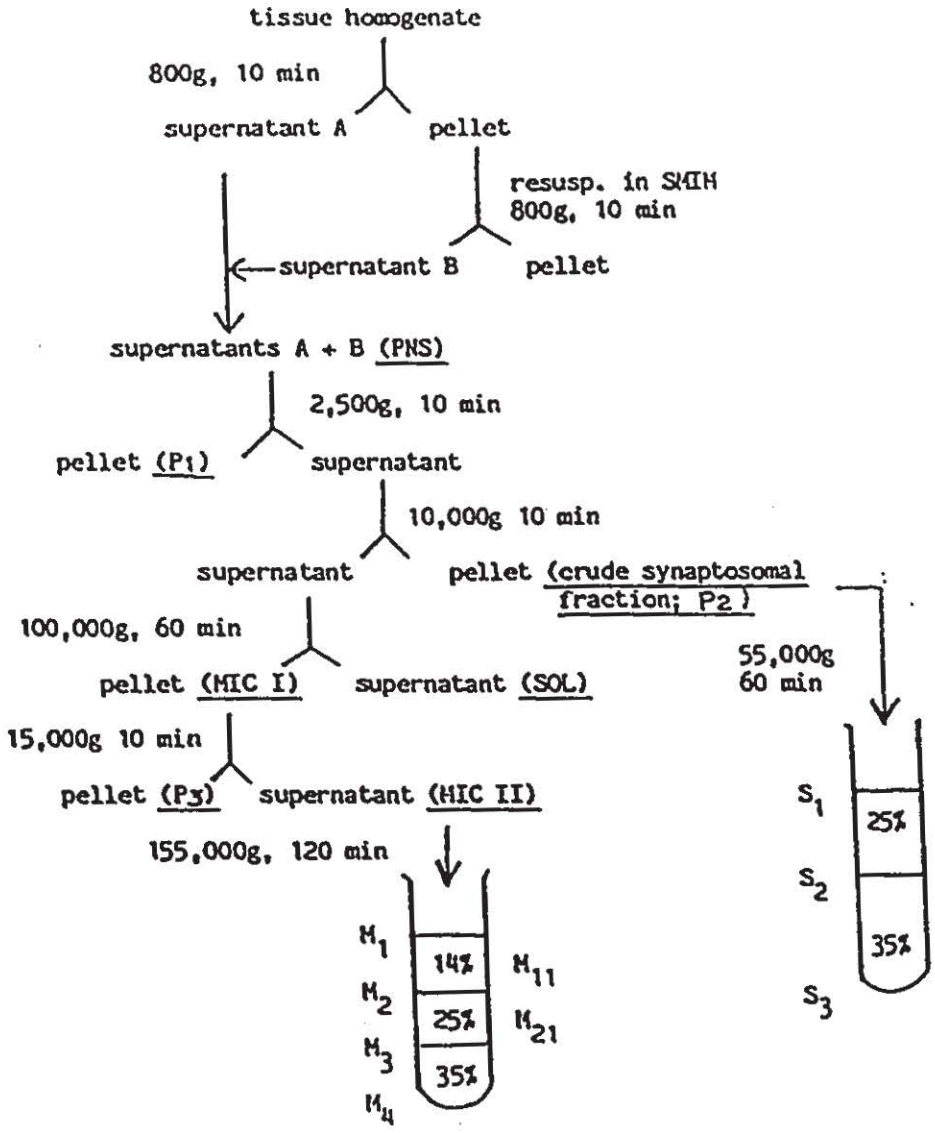
SUCROSE DENSITY GRADIENTS 30,000 rpm, 100 min.



supernatant was centrifuged at 10,000 x g for 10 min. The resultant mitochondrial-synaptosomal pellet is referred to as the crude synaptosomal fraction (P2). The supernatant after a 10,000 x g spin was centrifuged at 100,000 x g for 60 min. to separate the soluble proteins from the crude microsomal pellet (Mic I). The Mic I pellet was resuspended in the buffer and centrifuged at 10,000 x g for 10 min. The pellet thus obtained was designated P3 fraction and the supernatant was Mic II. The fractions P2 and Mic II were subjected to sucrose density gradient centrifugation as follows. Three mL of P2 was loaded on a gradient consisting of 5 mL each of 25% and 35% sucrose and centrifuged at 55,000 x g for 60 min. The protein band at the interface of 8/25% was S1, at 25/35% was S2 and the pellet at the bottom of the tube was S3. Four mL of Mic II fraction was loaded on a gradient consisting of 3 mL each of 14%, 25% and 35% sucrose and centrifuged at 110,000 x g for 120 min. Six fractions were obtained; M1 fraction at 8/14% sucrose interface; M11 - the material suspended in 14% sucrose; M2 at 14/25% sucrose interface; M21 - the material suspended in 25% sucrose; M3 was at the interface of 25/35% sucrose interface and M4 was the pellet at the bottom of 35% sucrose layer.

The flow-chart of the membrane fractionation from the submucous plexus is illustrated in Fig. 4. Briefly, the tissue was finely minced with the scissors and homogenized at 25,000 rpm setting in a Polytron PT-20 homogenizer for 3 x 8 seconds, allowing some time for cooling between the bursts. The

Figure 3. The scheme for the purification of the longitudinal muscle layer to obtain the purified longitudinal smooth muscle plasma membranes and the the synaptosomes from the myenteric plexus of canine small intestine. Reproduced from Kostka, 1987. For abbreviations see legend to figure 2.


tissue homogenate
800g, 10 min
supernatant A
pellet
resusp. in SMIH
800g, 10 min
supernatant B
pellet
supernatants A + B (PNS)
2,500g, 10 min
pellet (P1)
supernatant
10,000g 10 min
supernatant
pellet (crude synaptosomal fraction; P2)
100,000g, 60 min
pellet (MIC I)
supernatant (SOL)
55,000g
60 min
15,000g 10 min
pellet (P3)
supernatant (MIC II)
155,000g, 120 min
M1
M2
M3
M4
14%
25%
35%
M11
M21
S1
S2
S3
25%
35%

homogenate was centrifuged at 750 x g for 10 minutes, the pellet was discarded and the supernatant was designated as the postnuclear supernatant (PNS). The PNS was subjected to a centrifugation at 4,000 x g for 10 minutes, the pellet thus obtained was designated as P1 and the supernatant was S1. The supernatant S1 was again centrifuged at 48,000 x g for 10 minutes, the pellet P2 was collected and the supernatant S2 was subjected to high speed centrifugation at 120,000 x g for 60 minutes. The pellet (Mic I) was resuspended in the buffer using a hand held teflon coated homogenizer, and was subjected to a centrifugation at 17,000 x g for 10 minutes. The pellet thus obtained was designated as P3 and the supernatant was Mic II.

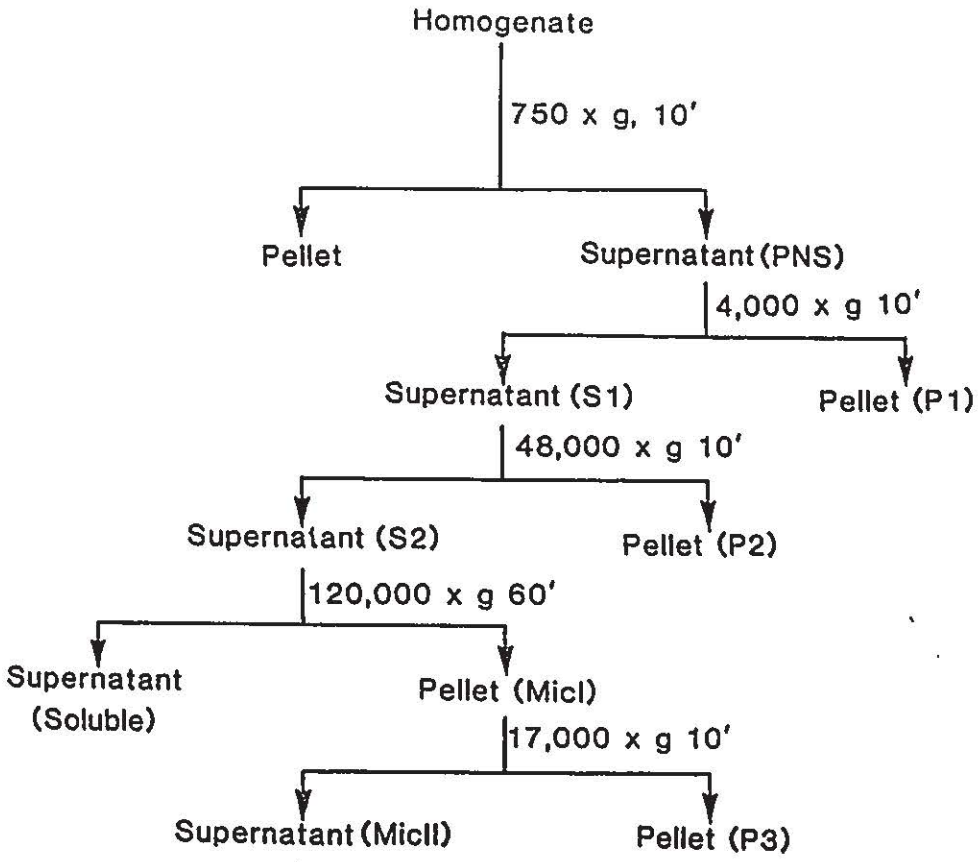
2.3.3. ENZYME, PROTEIN AND VIP RADIOIMMUNO ASSAYS

5'-nucleotidase activity was measured according to the method of Song and Bodansky (1967) using adenosine monophosphate as the substrate and 10 - 25 µg of the membrane proteins. Cytochrome c oxidase and NADPH-cytochrome c reductase activities were measured through continuous assay by the method of Cooperstein and Lazarow (1951) and Sottocasa *et. al.*. (1967) respectively.

Protein was determined by the method of Lowry *et. al.*. (1951). Bovine serum albumin was used as the standard for the protein assay.

VIP-immunoreactivity was measured using the antiserum R-501 (Chijiiwa,1986) by the method described in an earlier study (Manaka,1988).

Figure 4. Purification scheme to obtain the synaptosomes from the submucous plexus from canine small intestine. See legend to figure 2 for comments


Homogenate
750 x g, 10'
Pellet
Supernatant (PNS)
4,000 x g 10'
Supernatant (S1)
Pellet (P1)
48,000 x g 10'
Supernatant (S2)
Pellet (P2)
120,000 x g 60'
Supernatant
(Soluble)
Pellet (MicI)
17,000 x g 10'
Supernatant (MicII)
Pellet (P3)

2.3.4. [^{3}H] SAXITOXIN BINDING

The binding was performed at 20° C in a medium consisting of 50 mM Tris-HCl pH 7.4 containing 0.2% Bovine serum albumin with 0.5 - 0.8 nM radioligand. The non-specific binding was determined simultaneously in the presence of 1 µM tetrodotoxin and the specific binding was the difference between total and the non-specific binding. The specific binding was usually 90 to 95% of the total binding. The reaction was always started by the addition of the membranes (30 - 80 µg) to the incubation tubes. After 15 minutes of incubation, the reaction was terminated by the addition of 3 ml. ice cold buffer and filtration over Whatman GF/F filters using a Millipore filtration apparatus. The tubes and filters were washed twice with the buffer and the radioactivity on the filters was counted on a Beckman model LS6800 beta counter with an efficiency of about 40%.

2.4. RESULTS

2.4.1. PROCESSING OF THE CIRCULAR SMOOTH MUSCLE LAYER

2.3.1.1 MORPHOLOGY

The ultrastructural study of the dissected circular smooth muscle layer showed that this layer was free of the myenteric plexus (Fig. 5 a,b). The myenteric plexus had been dissected out together with the longitudinal smooth muscle layer, along with a thin layer of the circular muscle (Fig. 6). The procedure ensured that all the myenteric plexus went with the longitudinal muscle layer. Various fractions obtained during the purification procedure were also examined under the electron microscope. The mitochondrial fractions contained some intact synaptosomes as well as mitochondria (Fig. 7). As shown in Figs. 8 a and b, the microsomal fraction (Mic II) and the purified plasma membrane fraction from the gradient mainly consisted of intact vesicles and were free of synaptosomes. Any synaptosomes were apparently removed in mitochondrial fractions Mit I and Mit II. The low magnification micrograph of the dissected submucous plexus obtained after the zinc-iodide osmium tetraoxide staining is given in the fig. 9. A dense network of the stained nerve fibres is seen in the micrograph. Blood vessels are also visible in the picture.

2.4.1.2. DISTRIBUTION OF MARKERS

The fractions obtained by differential centrifugation of the tissue homogenate were described in the Methods section. The microsomal fraction Mic I was layered on a continuous sucrose density gradient. After centrifugation, 0.5 mL fractions were withdrawn sequentially from the top of the centrifuge tubes, and were assayed for various marker enzymes and protein. The results are shown in Fig. 10. 5'-nucleotidase activity migrated with a peak at 23.5% sucrose. There were two protein peaks. One minor peak was present at 13% sucrose concentration and a major peak was observed at 38% sucrose concentration. The activities of the ER and mitochondrial membrane marker enzymes, NADPH-cytochrome c reductase and cytochrome c oxidase respectively were also present at around 38% sucrose. When the fraction Mic II was loaded on the gradients instead of the fraction Mic I, similar results were obtained except that the major protein band now was at 10% sucrose concentration and the second protein band was reduced considerably and the activity of the cytochrome c-oxidase was considerably reduced in all the fractions. In subsequent experiments, fraction Mic II was routinely loaded on the gradient. Based on the results of the continuous density gradients, a discontinuous gradient centrifugation scheme was constructed. 14% and 33% sucrose concentrations were taken as the cutoff points for the plasma membranes since between these concentrations, high activity of the plasma membrane marker and

Figure 5. Electron micrograph of the dissected circular muscle of the canine small intestine. Low power electron micrograph showing the outer border of the circular muscles (OCM) near the submucous plexus (a) and the inner border of the circular muscle (ICM) near the myenteric plexus (b) after cleaning. Note that the myenteric plexus has been completely removed after the cleaning. Also, note the absence of submucous plexus from the dissected circular muscles.

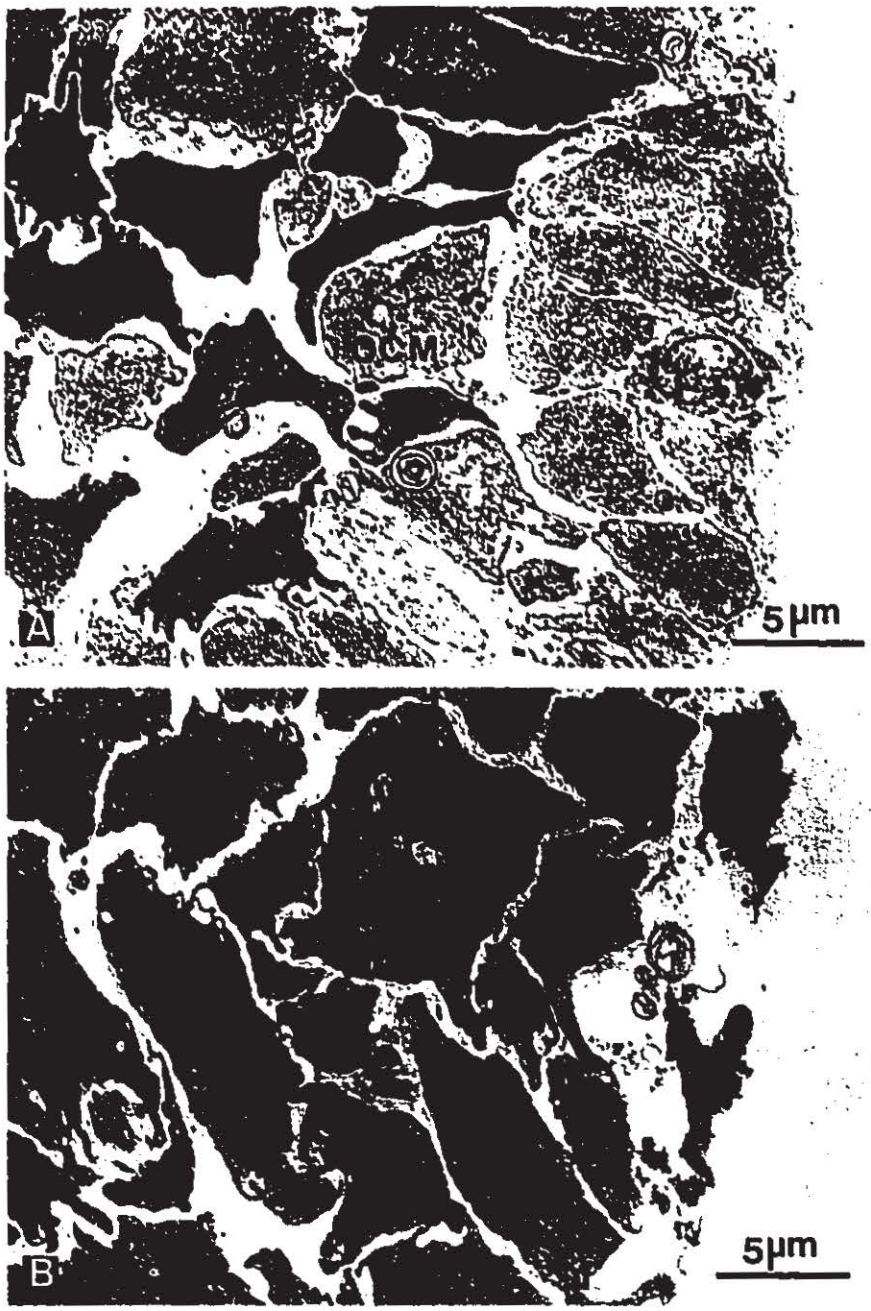
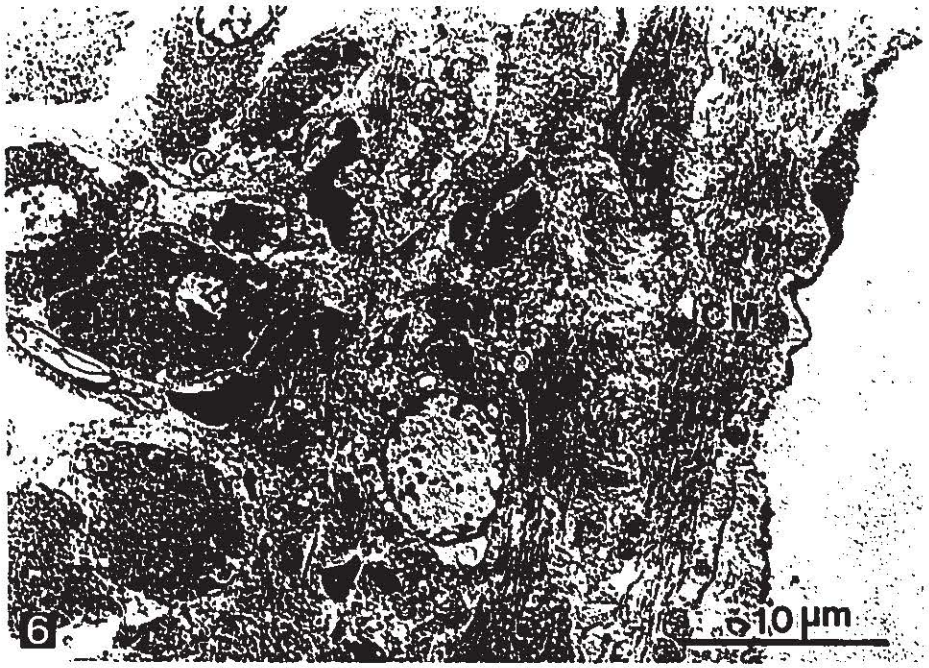

D&M
A
5 µm
B
5µm

Figure 6. Electron micrograph of the separated longitudinal muscle 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 some layers of the circular muscle (CM) have been always left during cleaning.

Figure 7. High magnification electron micrograph of the mitochondrial fraction. Note the nerve varicosities containing large granular vesicles (lgv) and small agranular vesicles (sav) present in this fraction. m, mitochondria.


M
6
10 µm

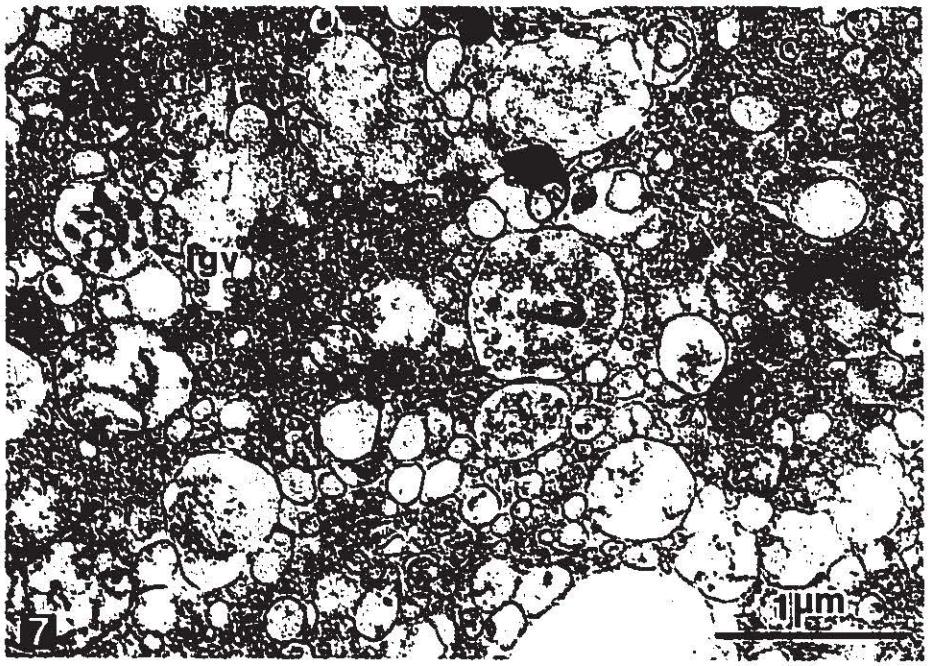
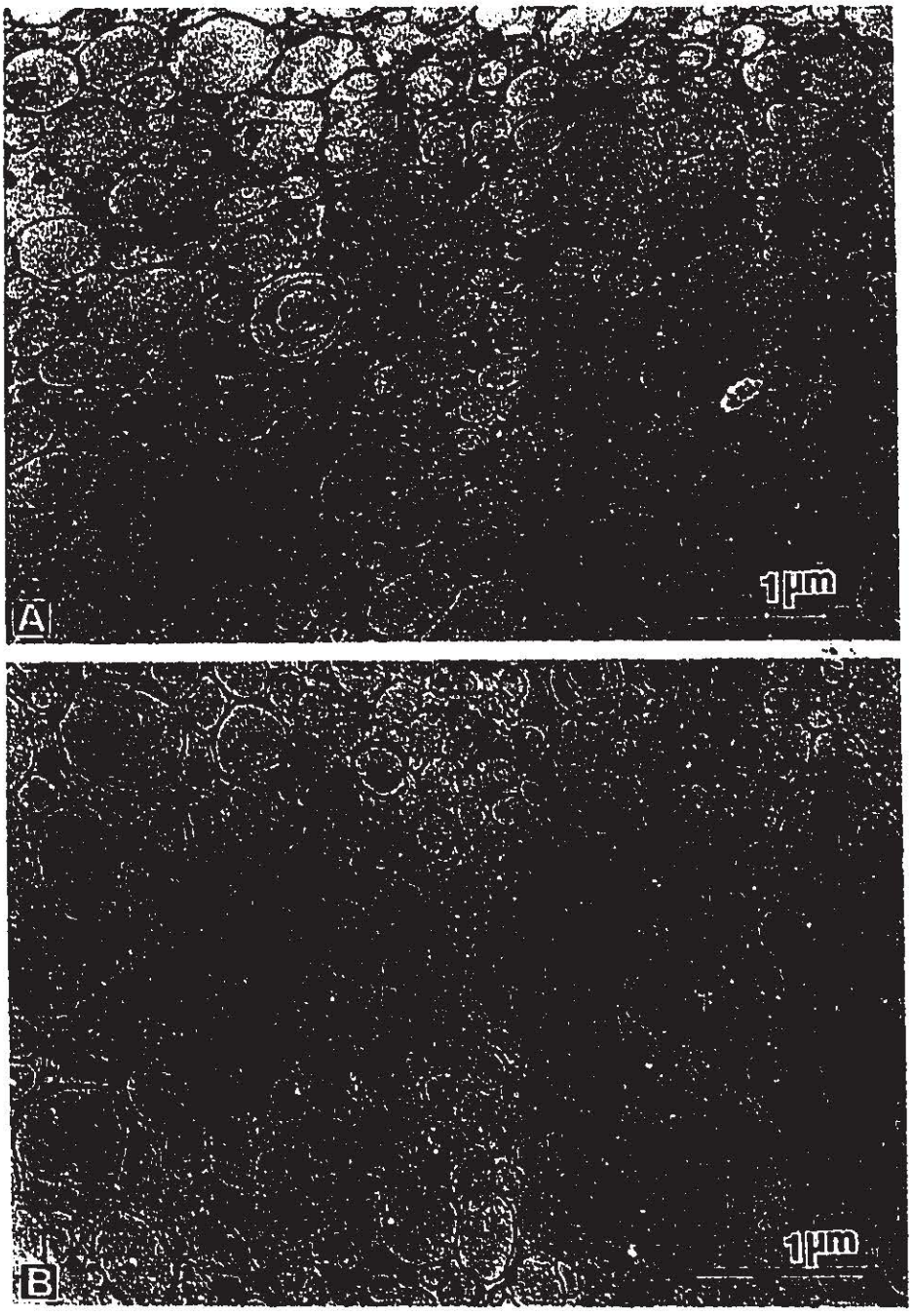

gv
7
1µm

Figure 8. A. High power electron micrograph of the Mic II fraction. Small amounts of ribosomes (R) are present in this fraction.

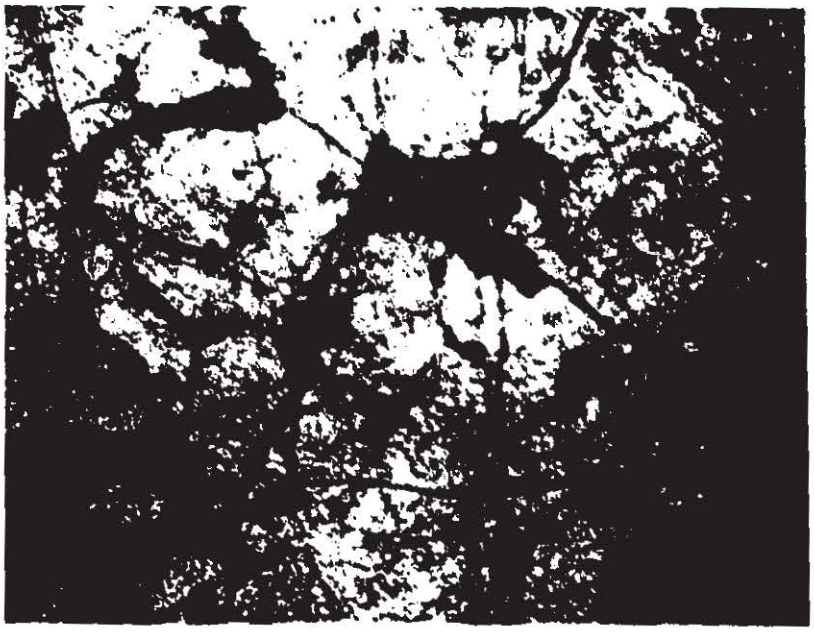
B. High power electron micrograph of the purified plasma membrane fraction from the gradient.


A
1μm
B
1μm

lower activities of the mitochondrial and endoplasmic membrane markers were observed. Therefore, 2.5 mL each of 48%, 40%, 33%, and 14% sucrose concentrations were used for the discontinuous gradient for the preparation of the smooth muscle plasma membranes. The protein bands at each interface were termed as M1 - M4 (from top to bottom). For the preparation of the deep muscular plexus neuronal membranes and the synaptosomes, the stepwise gradient was 2.0 mL each of 48%, 40%, 35%, 25%, and 14% sucrose. The protein bands at each interface and the pellet at the bottom of the tube were termed as P1 - P6 (from top to bottom. The results of the marker enzymes, VIP immunoreactivity, and [^{3}H]saxitoxin binding to the differential centrifugation fractions and the fractions obtained after discontinuous sucrose density gradients are illustrated in the Fig. 11.

It is clear from the Fig. 11 that the fraction M2, obtained between 14 and 33 % sucrose is enriched in smooth muscle plasma membrane marker enzyme, while neuronal membranes and synaptosomes were obtained at a density greater than 35% sucrose. The fraction M2, was enriched more than 20 fold in its smooth muscle plasma membrane marker enzyme activity. The fraction P4 was similarly enriched by about 20 fold in its saxitoxin binding activity and also by about 13 fold in its VIP immunoreactive content. Fractions P2 and M2 contained the specific activity of the 5'-nucleotidase of about 220 μmol Pi/mg.hr, whereas this activity in the fraction P4 and M4 is about 50 μmol Pi/mg.hr. Specific [^{3}H]saxitoxin binding in the fraction P4 amounted to more than 2500 fmol/mg protein.

Figure 9. The zinc-iodide/osmium tetraoxide stained submucous plexus of the canine small intestine.



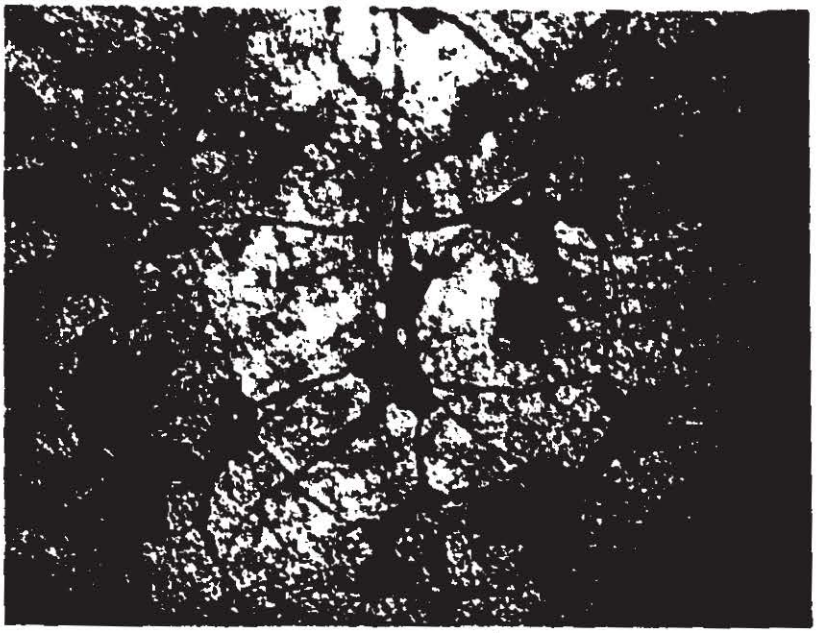
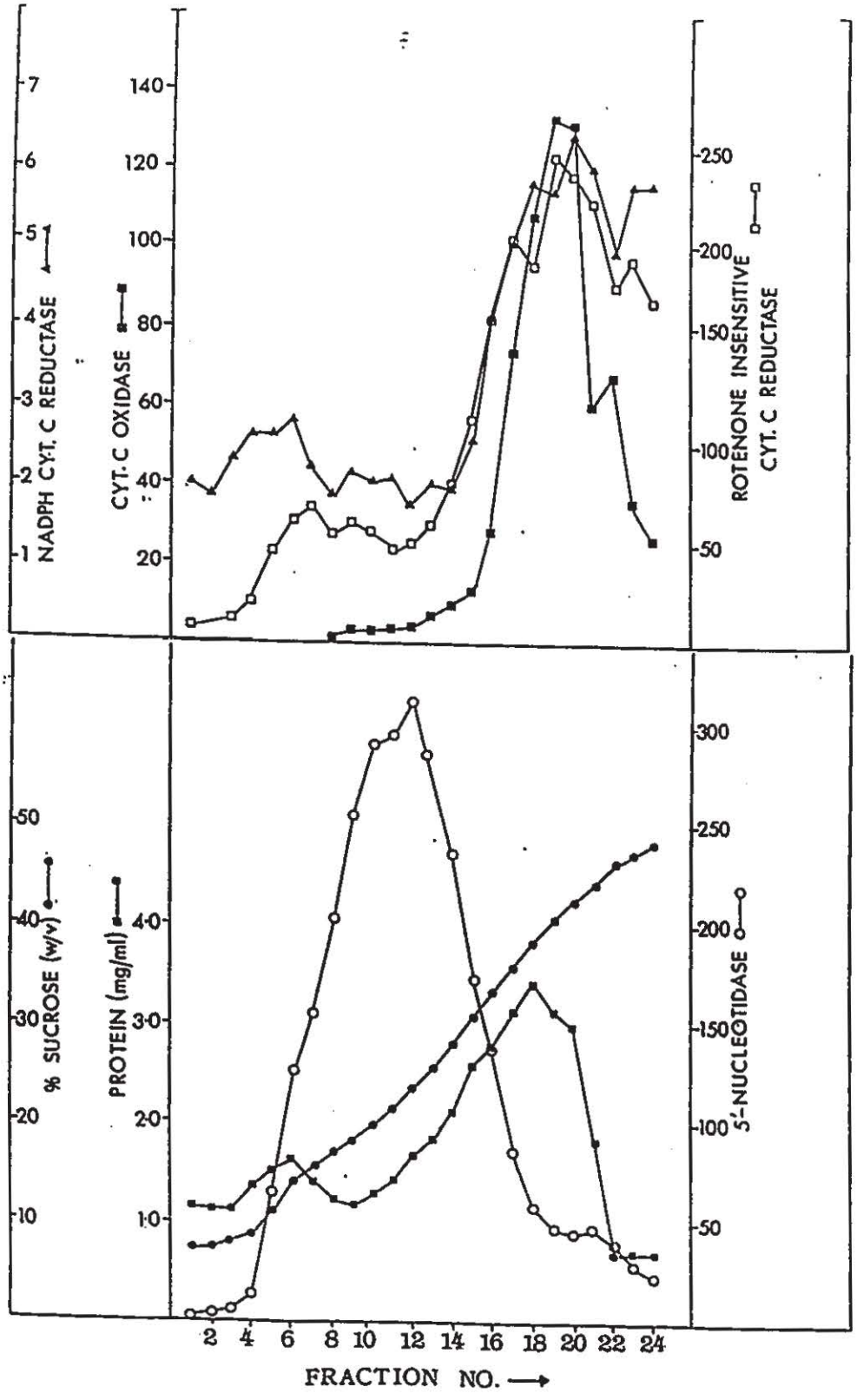


Figure 10. Distribution of the marker enzymes after sucrose density gradient. Microsomes were prepared as given in the Methods and were centrifuged over 8 - 60% sucrose density gradient at 30,000 rpm for 100 min. 0.5 mL fractions were collected from the top and assayed for the enzyme activities. The figure is a representative of five such experiments.


NADPH CYT. C REDUCTASE
CYT. C OXIDASE
ROTENONE INSENSITIVE CYT. C REDUCTASE
% SUCROSE (w/v)
PROTEIN (mg/ml)
5'-NUCLEOTIDASE
FRACTION NO. ⟶

Fractions M2 and P2, both of which were enriched in smooth muscle plasma membranes markers bound relatively less [^{3}H]saxitoxin, especially the fraction M2, which bound less than 200 fmol/mg protein of [^{3}H]saxitoxin. In fact, all the M fractions (M1-M4) bound less than 200 fmol/mg protein of [^{3}H]saxitoxin.

2.4.1.3 RECOVERY OF MARKERS IN VARIOUS FRACTIONS

The recoveries of the protein and two markers 5'-nucleotidase and the specific binding of [^{3}H]saxitoxin in miscellaneous fractions obtained during the processing are listed in table I. As is clear from the table, about 85% of the protein was recovered in the soluble fraction. Soluble fraction also contained a majority of the enzyme 5'-nucleotidase, an observation consistent with the previous findings (see Daniel,1982) of a major soluble fraction of this enzyme in smooth muscles. In the gradient fractions, the highest recovery of 5'-nucleotidase was in the fraction M2, which also had the highest specific activity of this enzyme and therefore, would represent the purified fraction from the circular smooth muscle plasma membranes. The protein recovery in this fraction was 0.36% of that present in the PNS. In the differential centrifugation fractions, about 50% of the [^{3}H]saxitoxin binding sites were recovered in the fraction Mit I. When the fraction Mit I was loaded on the gradient, highest recovery of the binding sites was in the fraction P4, amounting to more than 25% of that present in the PNS. Fraction P4 contained about 0.5% of the total protein and 1% of the 5'nucleotidase.

Figure 11. The distribution of the markers 5'-nucleotidase (5'-ND), [^{3}H]saxitoxin binding (3H-STX) and VIP-immunoreactivity (VIP-IR) in the fractions obtained during the differential and discontinuous sucrose density gradient centrifugations of the homogenate of the circular muscle of the canine small intestine. Note that the N-type calcium channel blocker omega-conotoxin GVIA (125I-wCTX) also labels the nerve membranes specifically, while the L-type calcium channel blocker, nitrendipine (^{3}H-NIT) binds to both nerves and muscles.

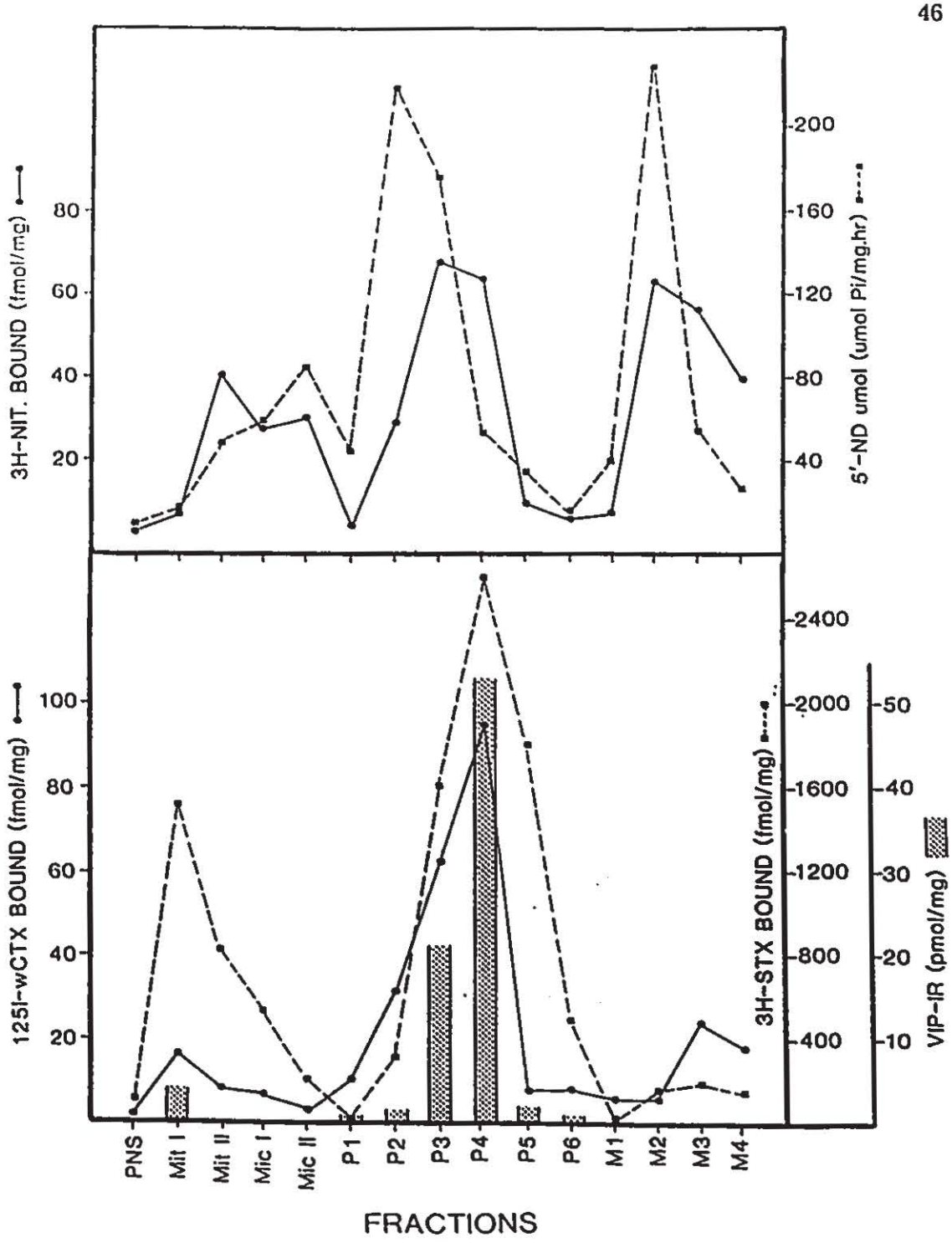

3H-NIT. BOUND (fmol/mg)
5'-ND umol (umol Pi/mg.hr)
125I-wCTX BOUND (fmol/mg)
3H-STX BOUND (fmol/mg)
VIP-IR (pmol/mg)
PNS
Mit I
Mit II
Mic I
Mic II
P1
P2
P3
P4
P5
P6
M1
M2
M3
M4
FRACTIONS

TABLE I

RECOVERY OF PROTEIN, 5'-NUCLEOTIDASE AND [^{3}H]SAXITOXIN BINDING ON DICM FRACTIONATION

FRACTIONS	Protein	5'-ND	[^{3}H]STX
PNS	100	100	100
Mit I	4.45 ± 1.43	8.22 ± 2.73	46.90 ± 15.40
Mit II	1.65 ± 0.31	6.07 ± 2.22	13.70 ± 6.30
Mic I	4.60 ± 0.82	30.48 ± 8.14	20.90 ± 11.30
Mic II	1.58 ± 0.57	17.36 ± 6.38	3.40 ± 2.10
Sol.	85.40 ± 8.40	42.69 ± 18.2	-
P1	0.25 ± 0.15	1.25 ± 0.08	0.10 ± 0.10
P2	0.17 ± 0.05	1.73 ± 0.18	0.28 ± 0.06
P3	0.53 ± 0.31	2.22 ± 0.25	6.35 ± 1.11
P4	0.47 ± 0.06	1.01 ± 0.09	25.73 ± 9.61
P5	0.79 ± 0.33	0.69 ± 0.07	4.48 ± 2.69
P6	1.02 ± 1.09	0.86 ± 0.31	1.45 ± 1.16
M1	0.21 ± 0.02	0.92 ± 0.04	0.02 ± 0.01
M2	0.36 ± 0.01	7.76 ± 0.34	0.44 ± 0.20
M3	0.25 ± 0.01	1.32 ± 0.05	0.29 ± 0.14
M4	0.16 ± 0.01	0.56 ± 0.03	0.14 ± 0.04

The results are mean ± sd of 3 - 6 experiments. The binding was performed with 0.5-0.8 nM [^{3}]saxitoxin in triplicates for both total and nonspecific binding. The results are expressed as percentage with post-nuclear supernatant taken as 100%.

2.4.2. PROCESSING OF THE LONGITUDINAL SMOOTH MUSCLE/MYENTERIC PLEXUS (LM/MP) LAYER

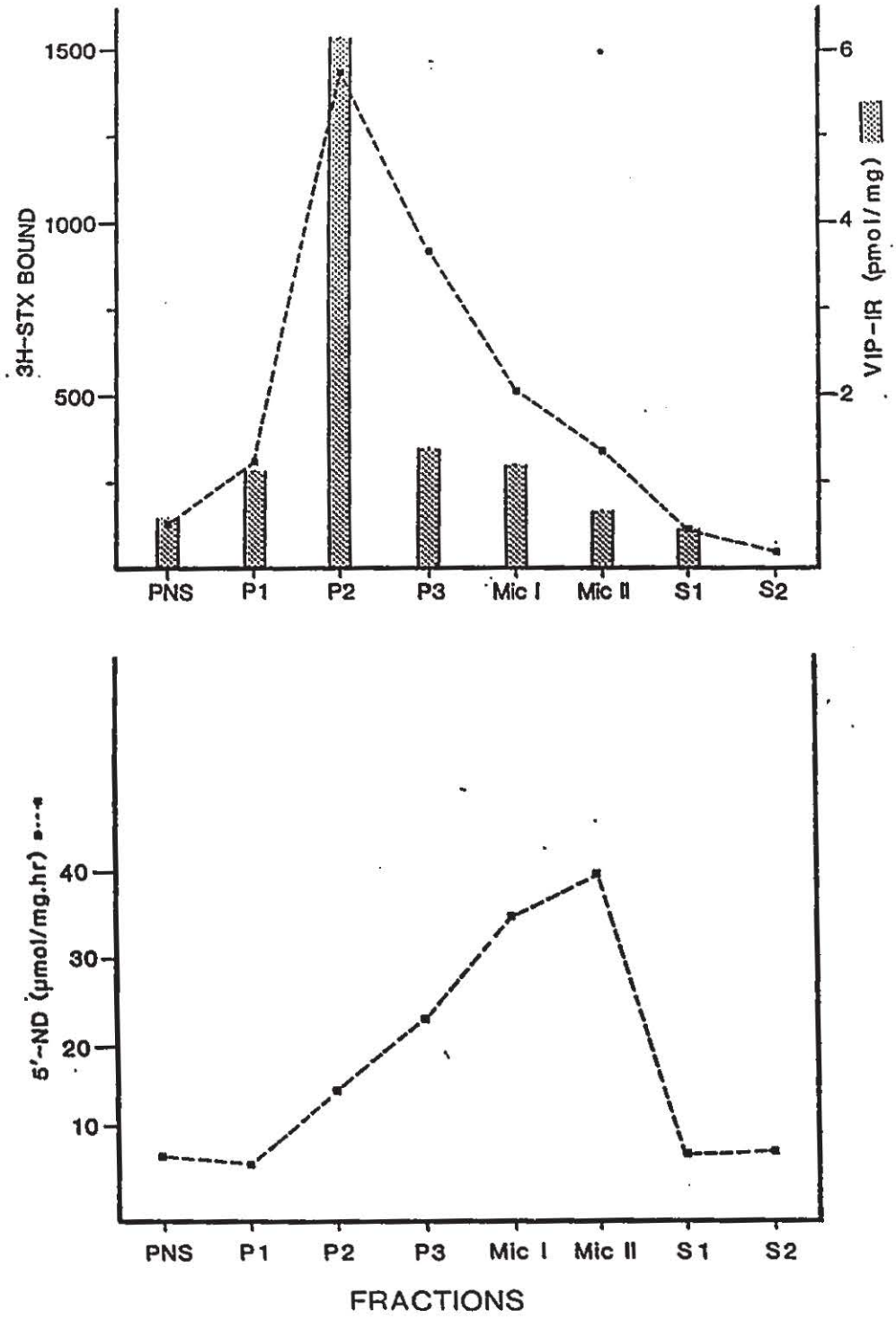
LM/MP layer was processed according to the methods described by Kostka *et. al.* (1987). A scheme for the purification is illustrated in the figure 3. The reader is referred to this article for the pertinent discussion of the results.

2.4.3. PROCESSING OF THE SUBMUCOSA

2.4.3.1 DISTRIBUTION OF MARKERS

The distribution of the marker enzymes, [^{3}H]saxitoxin binding and the VIP-immunoreactivity is illustrated in the Fig. 12. The highest 5'-nucleotidase activity was obtained in the fraction Mic II, which amounted to about 40 µmol Pi/mg.hr. Highest [^{3}H]saxitoxin binding was observed in the fraction P2 (1436 fmol/mg of protein). The activity of 5'-nucleotidase in the fraction P2 was 14.9 µmol Pi/mg.hr. P2 fraction was enriched by about 14 fold in the specific binding of [^{3}H]saxitoxin and by about 10 fold in the content of VIP-immunoreactive material over the post-nuclear supernatant. Specific [^{3}H]saxitoxin binding and the VIP-immunoreactivity paralleled very well in all the fractions, except in the fraction P3, where relatively high binding was observed, but VIP-immunoreactivity in this fraction was quite low.

Figure 12. Distribution of the markers 5'nucleotidase (5'-ND), [^{3}H]saxitoxin binding (3H-STX) and the VIP-immunoreactivity (VIP-IR) in the fractions obtained upon the fractionation of the submucosa.


1500
1000
500
3H-STX BOUND
6
4
2
VIP-IR (pmol/mg)
PNS
P1
P2
P3
Mic I
Mic II
S1
S2
40
30
20
10
5'-ND (μmol/mg.hr)
FRACTIONS

2.4.3.2. RECOVERY OF MARKERS IN MISCELLANEOUS FRACTIONS

The recovery of the protein and the markers 5'-nucleotidase and the specific binding of [^{3}H]saxitoxin is indexed in table II. The fraction Mic II, which was most enriched in the specific activity of 5'-nucleotidase, contained about 16% of the total activity recovered. Specific binding of [^{3}H]saxitoxin in this fraction was low, as was the total number of binding sites recovered. The fraction S1 had the highest share of [^{3}H]saxitoxin binding sites, however, this fraction also contained a preponderance of the recovered protein (>96%). The recovery of 5'-nucleotidase in the fraction S1 was also high (>77%). When the fraction S1 was submitted to a centrifugation step at 48,000 x g for 10 min., the bulk of the protein and 5'-nucleotidase was recovered in the supernatant, while the pellet P2 carried most of the [^{3}H]saxitoxin binding sites. As a result, the pellet P2 manifested high specific binding of [^{3}H]saxitoxin and low specific activity of the enzyme 5'-nucleotidase. The fraction P2, therefore was used for investigation of the peptide receptors on submucous plexus synaptosomes.

2.4.4. PROPERTIES OF [3H]SAXITOXIN BINDING SITES

Table III lists the binding parameters obtained for the [^{3}H]saxitoxin binding sites in deep muscular and submucous plexus. [^{3}H]saxitoxin bound with high affinity to a single class of non-interacting sites with a Kd value in subnanomolar range and a very high Bmax value (~ 4-6 pmol/mg of protein). Unlabelled

TABLE II

RECOVERY OF PROTEIN, 5'-NUCLEOTIDASE AND [^{3}H]SAXITOXIN BINDING ON SMP FRACTIONATION

	PROTEIN	5'-ND	[^{3}H]STX
PNS	100	100	100
P1	3.20 ± 0.48	2.04 ± 0.95	10.36 ± 1.29
P2	3.14 ± 0.77	5.46 ± 2.28	49.89 ± 5.68
P3	1.42 ± 0.34	4.00 ± 3.05	14.77 ± 7.26
Mic I	6.41 ± 2.16	21.23 ± 4.70	31.55 ± 3.23
Mic II	4.69 ± 1.22	15.89 ± 3.77	15.37 ± 2.37
S1	96.46 ± 8.97	77.64 ± 31.14	81.85 ± 11.29
S2	83.26 ± 2.27	63.32 ± 25.03	27.12 ± 8.29

The results are mean ± sd of 3 - 6 experiments. The binding was performed with 0.5-0.8 nM [^{3}]saxitoxin in triplicates for both total and nonspecific binding. The results are expressed as percentage with post-nuclear supernatant taken as 100%.

TABLE III

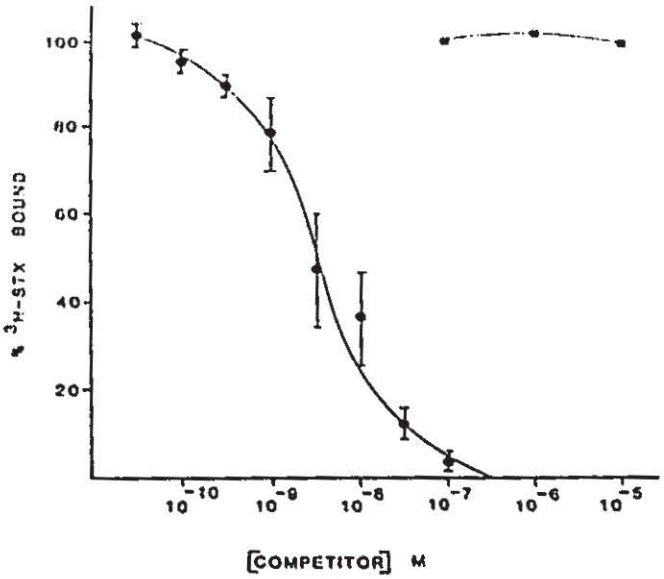
PROPERTIES OF [^{3}H]SAXITOXIN BINDING TO THE DEEP MUSCULAR PLEXUS AND SUBMUCOUS PLEXUS MEMBRANES

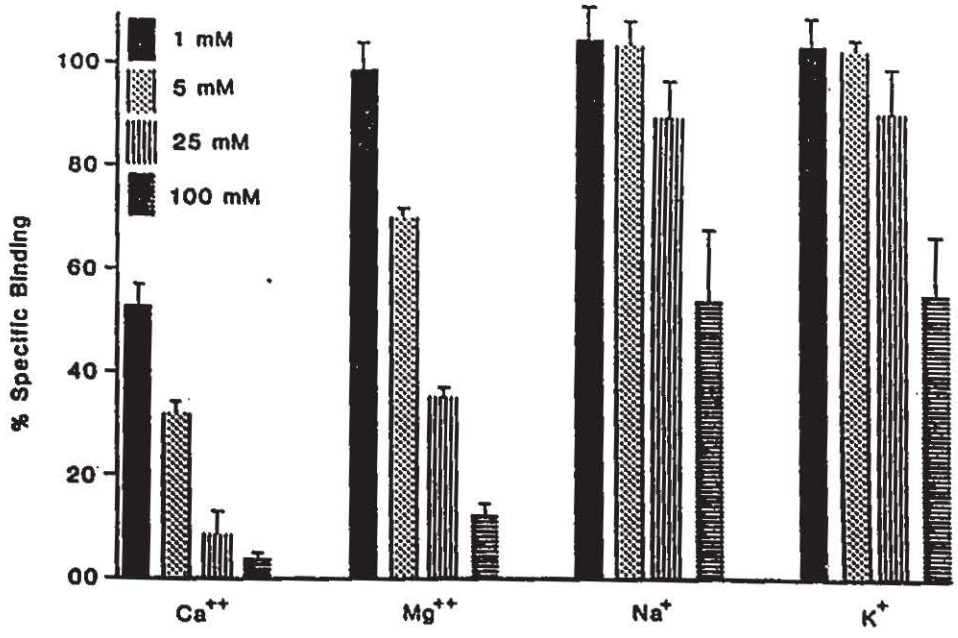
	DMP	SMP
Kd (nM)	0.69 ± 0.29	0.21 ± 0.09
Bmax (fmol/mg)	5705 ± 1029	4525 ± 813
Hill Coefficient	0.93 ± 0.05	0.89 ± 0.04

The results are the mean ± sd from 3 separate experiments performed in triplicate simultaneously for both total and nonspecific binding.

Figure 13. Competition of the [^{3}H]saxitoxin (0.5-0.8 nM) binding to the deep muscular synaptosomes by tetrodotoxin (closed circles) and by veratridine (closed squares). Note veratridine failed to inhibit the binding up to a concentration of 10 μM.

Figure 14. Effect of mono- and divalent ions on the specific binding of [^{3}H]saxitoxin (0.5-0.8 nM) to the deep muscular plexus synaptosomes.


100
80
60
40
20
% ^{3}H-STX BOUND
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[COMPETITOR] M


1 mM
5 mM
25 mM
100 mM
% Specific Binding
100
80
60
40
20
00
Ca^{++}
Mg^{++}
Na^{+}
K^{+}

tetrodotoxin could completely inhibit the specific [^{3}H]saxitoxin binding to the deep muscular plexus membranes in a concentration dependent manner, supporting the specificity of the binding to Na-channels (Fig. 13). At 0.54 nM radioligand concentration, the IC_{50} for TTX displacement was 5×10^{-9} M corresponding to the K_i value of 2.1×10^{-9} M. Veratridine and guanidinium hydrochloride had no effect on the binding up to a concentration of 10^{-5} M.

Fig. 14 illustrates the effect of ions on the specific binding of [^{3}H]saxitoxin. Divalent ions were more potent inhibitors of the binding. Ca^{++}ions even at 1 mM concentration inhibited the specific binding by 47%, while other ions at this concentration were ineffective. At higher concentrations, all the ions tested inhibited the specific binding.

2.5. DISCUSSION

The technique of the membrane fractionation relies heavily on the use of markers to distinguish various membranes from a complex homogenate. Therefore, the validity of the procedure must obviously be judged by the validity of the markers utilized for the purpose, and by the degree of purification accomplished. Some excellent reviews have appeared in the recent past (Daniel,1982; Kwan,1987), which have critically evaluated the procedures involved in the membrane fractionation technique and the use of various markers. The membranes of mitochondrial and endoplasmic reticulum origin do not interfere severely with the interpretation on the locus of receptors in radioreceptor binding assays since these organelles are not the target of neurotransmitters. However, in the experiments involving the Ca^{++}-uptake, for example, it becomes extremely important to assess the purity of these membranes as well since both mitochondria and the endoplasmic reticulum are sites of Ca^{++}-uptake and handling (Daniel,1982). Moreover, the presence of the membranes other than the desired ones decreases the "signal to noise" ratio. On the other hand, various cell types present in a tissue may be the target of the action of various mediators. Therefore, for radioreceptor binding assays, reasonably pure membranes from a single cell type are highly desirable if one is to make any conclusion regarding the locus of action of the mediator in question and the properties thereof. To obtain "pure" membranes from

desirable cell type, a "good" marker for that particular membrane is required. The properties that make a marker a good one include the sensitivity, specificity of that marker for the given membrane, and the ease with which an assay can be performed. The enzyme 5'-nucleotidase, which has been used repeatedly as a marker for the smooth muscle plasma membrane, turned out to be a good marker in the present studies as well, mainly because of the high activity of this enzyme present in this tissue. However, about 50% of this enzyme is present in the soluble fraction, as was also observed in previous studies (see Daniel,1982). Furthermore, 5'-nucleotidase activity is not restricted to the plasma membranes of the smooth muscle cells. Immunohistochemical localization of 5'-nucleotidase has been studied previously and observations on its presence (although low levels) in cell types other than the smooth muscle have been made. In rat and mouse stomach, most of the activity of the 5'-nucleotidase activity was observed to be present in the circular layer (Hardonk, 1968), or in a narrow rim at the innermost surface of the circular layer (Klaushofer,1975). In dog intestine, Freiman *et. al.* (1961) observed a strong activity in both the longitudinal and circular smooth muscle. Forsman (1985) observed that the strongest activity of 5'-nucleotidase was present in the membranes of smooth muscle cells in guinea pig ileum, iris and vas deferens. The cellular elements in the myenteric ganglia showed lower activity; most of the activity in ganglia was present at the non-specialized neuron-to-glia boundaries and at some synaptic specializations (Forsman,1985). Stefanescu-Gavat (1980) reported

that in monkey and rabbit intestine, the activity of 5'-nucleotidase was weak in nerve cells but higher in satellite neuroglia, nerve fibres and capillaries. In rat cerebellum, 5'-nucleotidase immunoreactivity was found on the plasma membranes of glial elements; in this case however, neuronal cells or processes were devoid of immunoreactivity (Schoen,1987). The gradient purified fractions in the present study, enriched in the [^{3}H]saxitoxin binding and VIP-immunoreactivity (enriched synaptosomal fractions) also contained low levels (approximately 15-20% of that in the purified smooth muscle plasma membrane fractions) of the activity of 5'-nucleotidase. Some of this activity may come from the contaminating smooth muscle membranes in these fractions. A part of this activity also may represent that present in the neuronal membranes, or contaminating glial membranes in these fractions.

Use of [^{3}H]saxitoxin as a marker for the neuronal membranes also deserves critical assessment. Voltage sensitive sodium channels are present in many types of excitable tissues (Agnew,1984; Catterall,1984; Lombet,1982; Rogart,1981). The molecular events underlying the nerve action potential are correlated with sequential time and voltage dependent opening and closing of this transmembrane protein (Hodgkin,1952; Catterall,1984). Several classes of neurotoxins exert their effects by binding to these channels and altering their properties (Catterall,1984; Lazdunski,1986). Saxitoxin and tetrodotoxin, the water soluble heterocyclic guanidines, are proposed to bind to a common receptor site

that is thought to be located near the extracelluar opening of the sodium ion conducting pore of the transmembrane protein. Saxitoxin and tetrodotoxin bind to these channels on neuronal membranes with high affinity and inactivate them (Ritchie,1977). Smooth muscles, on the other hand, are believed to be lacking in sodium channels since tetrodotoxin does not affect the action potential which is carried mainly by Ca^{2+} (Bulbring,1963; Mirroneau,1973). These toxins have been proven valuable tools for the study of VSSCs. Much of the progress in understanding the structure and molecular events underlying the function and the of these channels has come about by the use of these toxins. VSSCs are perhaps the most thoroughly studied membrane channels. Recent years have seen these channels solubilized, purified, reconstituted in vesicles and cloned (see Numa,1986; Lazdunski,1986), and STX and TTX have been indispensable tools for all of these studies.

In the present study, another potential of these toxins was explored. We attempted to use radioactively labelled saxitoxin to label the neuronal membranes, in order to assess the contamination of the smooth muscle plasma membranes with neuronal membranes and to purify neuronal membranes from the plexuses of gastrointestinal tract. [^{3}H]saxitoxin binding turned out to be highly sensitive assay due to the low background, high affinity and the presence of a very high density of binding sites. The affinity (Kd) of [^{3}H]saxitoxin for all the fractions enriched in this binding (from DMP, SMP and MP) was in the nanomolar range (0.2 - 0.7 nM)

and the maximum number of binding sites (Bmax) was very high (4 - 6 pmol/mg of protein). These parameters make [^{3}H]saxitoxin binding a highly sensitive assay.

The specificity of an assay is another requirement for it to be used as a marker. As mentioned earlier, smooth muscles have been believed to lack the sodium channels, since the action potential is TTX insensitive and is carried by Ca^{++} ions (Bulbring,1963; Mirroneau,1973). Some recent studies (Amidee,1986) suggest however, the presence of apparently silent or non-functional sodium channels and low affinity STX/TTX binding sites in myometrial smooth muscle plasma membranes. Amedee *et. al.* (1986) have described the presence of low affinity tetrodotoxin binding sites in the myometrial smooth muscle cell homogenate and also the induction of functional sodium channels by veratridine and sea anemone toxin. These channels as opposed to the present study and the previous studies on neuronal membranes, were of low affinity for tetrodotoxin (K_d $2x10^{-6}$ M). There was no evidence of such low affinity binding sites in the present study. We found negligible binding of [^{3}H]-saxitoxin to all the smooth muscle plasma membrane enriched fractions. These data justify the conclusion that VSSCs are present in negligible quantity, or, are absent from the plasma membrane of intestinal smooth muscles.

To further validate the results of the [^{3}H]saxitoxin binding, the content of the VIP-immunoreactive material in these fractions was measured. VIP-IR and [^{3}H]saxitoxin binding parallelled one another well in terms of enrichment in all the

fractions. The fractions containing the highest binding also contained the highest VIP-immunoreactivity. This supports the validity of [^{3}H]saxitoxin binding as a marker for neuronal membranes. Further support is also provided by our other sets of experiments demonstrating that 1) in rat myometrium, the specific binding of [^{3}H]saxitoxin is decreased by more than 85% by day 22 of pregnancy (Kyozuka,1988), when natural denervation of the tissue is known to take place, 2) dog aorta, a vessel known to be minimally innervated also had minimal [^{3}H]saxitoxin binding (~ 10% compared to dog mesenteric arteries, Shi,in press), 3) rat mesenteric nerves contained about 400% higher [^{3}H]saxitoxin binding compared to rat mesenteric arteries (Shi,in press) and 4) by dissecting the smooth muscle carefully from the dog mesenteric arteries, it was possible to reduce the [^{3}H]saxitoxin binding to about 1/3 the value of that obtained with crude dissection (Shi,in press). All these observations support the view that [^{3}H]saxitoxin can label the neuronal membranes not only from the gastrointestinal tract, but also from other smooth muscle containing tissues: It can therefore serve as a more universal marker for the neuronal membranes in smooth muscle tissues.

In the preceding paragraph, we have discussed some of the properties of the [^{3}H]saxitoxin binding emphasizing its sensitivity and universality as a marker for the neuronal membranes. The validity for any parameter as a marker also implies its specificity for a particular cell or membrane type. The presence of the voltage sensitive sodium channels is very well established in the neurons, skeletal

muscle and cardiac muscle. Studies done largely within the last decade, have tried to identify VSSCs in other cell types classically considered as non-excitable, one such cells are the glial cells. Kuffler (1966) and later others (reviewed in Orkand 1977) demonstrated that the resting membrane potential in leech glial cells is very close to the potassium equilibrium potential, and it was commonly accepted that the ionic channels of the glial cell membranes were possibly exclusively potassium channels. However, several recent studies have demonstrated the presence of tetrodotoxin-sensitive sodium channels in cultured glial cells (Bowman,1984; Raiser,1983; Munson,1979; Lombet,1982; Pouyssegur,1980; Shrager,1985). Veratridine and scorpion toxin, sodium channel activators, cause depolarization of the cultured glial cells (Bowman,1984) and also increased the sodium permeability (Munson,1979). In some cases (Nowak,1987), TTX-sensitive, voltage dependent activation of the cultured Schwann cells or the astrocytes has also been demonstrated. These studies indicate that the glial cells *in situ* may also be electrically active and may contain the voltage-sensitive sodium channels and the TTX/STX binding sites. Ritchie (1983) observed that in the guinea pig and rats, the sectioning of sciatic nerves lead to the disappearance of the STX binding, while maintaining high level of STX binding in the rabbit sciatic nerves. It was suggested that the high levels of binding in the severed, degenerated nerves in rabbit was to the Schwann cell membranes. In the present studies, we can not rule out the possibility of [^{3}H]saxitoxin binding to the glial membranes and therefore the co-

purification of these membranes with the neuronal membranes. Appropriate markers for the glial membranes need to developed for the *in vitro* assay of the glial membranes. Such a membrane marker has not been validated at present. The best available marker for the glial cells is antibody to the glial fibrillary acidic protein (GFAP). Since GFAP is an intracellular protein, GFAP marker may not be useful for the membrane purification procedure.

However, in the present studies, the enrichment and recovery of the VIP-immunoreactivity in various fractions parallelled that of the [^{3}H]saxitoxin binding (allowing for the presence of some broken or leaky synaptosomes and extrasynaptic membranes). Since, in the gastrointestinal tract, VIP immunoreactivity is present exclusively in the neurons and their axonal varicosities, and absent from the glial cells, a majority of the membranes present in the fractions containing highest [^{3}H]saxitoxin binding must represent the neuronal membranes and synaptosomes.

Arguments similar to those in the preceding paragraph can also be made regarding the possible contamination of the neuronal membranes by the membranes from the interstitial cells of Cajal. However, again, the lack of any markers for these cells precludes any definitive statement on this aspect, barring that the combined data on the [^{3}H]saxitoxin binding and the VIP-immunoreactivity strongly suggest that the majority (if not all) of the membranes in the fractions enriched in these two markers would be of the neuronal origin.

In summary, we have developed the use of [^{3}H]saxitoxin as a marker for the neuronal membranes in complex homogenates of gastrointestinal and other tissues. In complement with the VIP-immunoreactivity and 5'-nucleotidase, we have used the specific binding of [^{3}H]saxitoxin to purify the membranes from the deep muscular, myenteric and submucous plexus, and from the circular and the longitudinal smooth muscle of canine small intestine, appropriate for the use in radioreceptor binding assays. The following two chapters will deal with the binding assays carried out using these membranes.

CHAPTER 3

NEUROTENSIN RECEPTORS

3.1.0. INTRODUCTION

Neurotensin is a tridecapeptide, which was first isolated by Carraway and Leeman (1973) from bovine hypothalamus. In subsequent years, its amino acid composition was established (Carraway,1975,1976) as the following:

pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ileu-Leu

Neurotensin has since then been isolated from several sources. Bovine intestinal neurotensin was isolated (Kitabgi,1976) and was sequenced (Carraway,1978) and its identity was established as that of the hypothalamic neurotensin. Hammer *et. al.* (1980) isolated neurotensin from the human small intestine which was found identical to the bovine hypothalamic and intestinal neurotensin.

More recently, Dobner *et. al.* (1987), using cDNA libraries from primary cultures of canine enteric mucosal cells, have demonstrated that one neurotensin related peptide, neuromedin-N is encoded for by the same gene. The precursor protein, preproneurotensin/neuromedin-N, a 170 amino acid peptide contains both neurotensin and neuromedin-N sequences. Similar genes were also isolated from the rat genomic and bovine hypothalamic cDNA libraries (Kislauskis,1988). The general features of the predicted precursor from rat and bovine were similar to those of the canine precursor. Furthermore, the comparison of the complete precursor protein from three species revealed 76% homology in the

amino acids (Kislauskis,1988). Neurotensin has been demonstrated to be present in several species including human, pig, guinea pig, chicken, frog and pig. It is now clear that several variants of neurotensin, with extensive sequence homologies especially at the carboxy-terminus, are present in various systems. A neurotensin variant, [Ser^{7}]neurotensin has been isolated from the guinea pig small intestine (Shaw, 1986). A peptide from the chicken small intestine LANT-6 ([Lys^{8}, Asn^{9}]neurotensin), and xenopsin from the skin of *xenopus laevus* have extensive sequence homology with the biologically active c-terminus of neurotensin (Araki,1973; Carraway,1983). Similarly, Neuromedin-N from the porcine spinal cord also has extensive sequence homology to the C-terminal end of the neurotensin

3.1.1. DISTRIBUTION

3.1.1.1. NEUROTENSIN IN GASTROINTESTINAL TRACT

Although, neurotensin was first isolated and characterized from the bovine hypothalamus, this tissue contains only a minor fraction of the total neurotensin. A major portion of the neurotensin is present in the periphery, in fact, more than 85% of the total body neurotensin is located in the gut (Carraway,1976). Neurotensin-like immunoreactivity has been observed in the gastrointestinal tract of many mammalian and non-mammalian species. The distribution of immunoreactive neurotensin along the digestive tract of many species is very similar (Holzer,1982). The highest concentration of neurotensin was

observed in the distal part of the small intestine. Stomach, oesophagus and large intestine contained only a fraction of the total gut neurotensin. In many species (cat, guinea pig, pig, rabbit, rat, man), proximal jejunum and terminal ileum contained more than 90% of the total gut neurotensin (Holzer,1982). High neurotensin-like immunoreactivity (NT-LI) has been observed in the gastrointestinal tract of the cat, dog and man while relatively low levels of this peptide were observed in rat, guinea pig, rabbit and the pig Holzer,1982; Sundler,1977).

Most of the peptide in many mammalian species is stored in specific endocrine type cells in mucosa, called N-cells which are located mainly in the ileum, and to a lesser extent, in other parts of the gut (Polack,1977). Ferri *et. al.* (1982) studied separate layers of the human gut for the contents of peptides and observed that of the total content of immunoreactive neurotensin in the whole wall of the gut, more than 99% was recovered from the mucosal layers. Polak *et. al.* (1977) observed restricted localization of NT-LI to the human mucosa, again the majority of immunoreactive material was present in the ileum. Sundler *et. al.* (1977) also observed NT-LI restricted to the endocrine-like cells of jejunum and ileum of many mammalian species. No NT-containing cells could be demonstrated in the stomach, colon and pancreas, and they were rare in the duodenum. In chicken, however, NT-containing cells were found throughout the intestine, where NT-containing cells were more numerous than in mammalian species (Sundler 1977). Sundler *et. al.* (1977) could find no NT immunoreactive nerves in any mammalian,

or the chicken gut. Some other studies also failed to detect NT immunoreactive nerves in the mammalian gut (Orci,1976; Daniel,1985).

There are, however, some studies describing the presence of NT-immunoreactive nerves in the gastrointestinal tract of some species. Neurotensin containing nerves were observed in the gastrointestinal tract of some teleost species (Langer,1979), where the immunoreactivity was confined to the nerve fibres of the myenteric plexus. Schulzberg *et. al.* (1980) studied the distribution of the NT-immunoreactivity in the gastrointestinal tract of rat and guinea pig. Nerves immunoreactive to the antisera against neurotensin were observed in the gastrointestinal tract of rat, where the immunoreactivity was confined to the nerve fibres (no cell bodies) of 1) the myenteric plexus of oesophagus and duodenum, 2) some parts of stomach, and 3)the circular muscle of caecum. No NT-immunoreactive nerves were identified in the guinea pig gastrointestinal tract. However, Reinecke *et. al.* (1983) observed NT-immunoreactive nerve fibres in the myenteric plexus of the upper duodenum of guinea pig. Doyle *et. al.* (1985), described the presence of NT, as measured by radio-immunoassay, in the canine gastrointestinal tract. Jejunum and ileum contained 73 ng/gm and 187 ng/gm neurotensin in seromuscular layer as compared to 422 ng/gm and 3025 ng/gm in mucosal extracts. The immunoreactivity in the seromuscular layer was attributed to its presence in the "neurotensinergic fibres". However, since NT immunoreactivity in the seromucular layer of ileum represents only about 6% of

that in mucosal extract, the possibility of the contamination of the seromuscular layer with mucosal neurotensin can not be completely ruled out. Buchan *et. al.* (1987) described the extensive presence of neurotensin-immunoreactive nerves in the submucous plexus, and to some extent in the myenteric plexus of canine small intestine. The NT-containing neurons in the submucous plexus were calculated to represent 55% of the total submucous plexus neurons. Tange (1983) also described the occasional presence of neurotensin-immunoreactive fibres in canine ileum.

As is clear from the above discussion, the presence of neurotensin in the enteric neurons, and therefore its role as the enteric neurotransmitter, is controversial. Some studies have reported the presence of NT-containing neurons (cell bodies or fibres), while other reporters failed to detect any NT-containing fibres in the gastrointestinal tract. The reason for this discrepancy is unclear. It is possible that some antisera recognize some peptide/s in some nerves that has some degree of homology with neurotensin sequence, but other antisera do not recognize such peptide/s. Such peptide may in fact be neurotensin-like peptide such as neuromedin-N or LANT-6. Extensive studies with antisera to the family of neurotensin-like peptides needs to be performed in order to resolve the issue of the neurotensinergic nerves. It is however clear that endocrine cells do contain a majority, if not all, of the neurotensin-like immunoreactivity in the gastrointestinal tract. Barber *et. al.* (1986a,b) have developed a technique to maintain NT-containing canine ileal mucosal cells for the studies of the regulation of the release

of the peptide from this model system. The pheochromocytoma PC12 cell cultures, were demonstrated to contain neurotensin-like immunoreactivity which was increased by about 100 folds by supplementing the cells with nerve growth factor and dexamethasone (Tischler,1982). Medullary thyroid carcinoma cell line were also shown to contain releasable (by K^+-depolarization and norepinephrine) neurotensin-like immunoreactivity (Zeytinoglu,1983).

3.1.1.2. NEUROTENSIN OUTSIDE THE GASTROINTESTINAL TRACT

As mentioned in the preceding section, neurotensin was first isolated from the hypothalamus. Neurotensin has been detected in other areas of the central nervous system of many species. Neurotensin-like immunoreactivity is widely distributed in different brain areas. Both in rats and human, strong neurotensin-like immunoreactivity was localized in hypothalamus, the bed nucleus of stria terminalis, septal area and the central grey area (Carraway,1973; Carraway,1976; Cooper,1981; Goedert,1984a; Kataoka,1979). In rats, certain cells of anterior pituitary also contained neurotensin-like immunoreactivity (Goedert,1982). Other areas of the brain, eg. hippocampus, cerebral cortex or cerebellum did not contain any detectable neurotensin-like immunoreactivity (Emson,1982,1985; Govoni,1980; Jennes,1982; Kobayashi,1977; Uhl,1976; Uhl,1977). Alterations in the levels of the NT-LI in specific brain areas has been observed in some disease states. In Huntington's disease, increased NT-LI was observed

specifically in nucleus caudatus and globus pallidus (Emson,1985; Nemeroff,1983). In schizophrenia, elevated levels were observed in frontal cortex (Nemeroff,1983). Alzheimer's and Parkinson's brains did not reveal any change in NT-LI, however, in Parkinson's patients, high NT-LI was measured in the cerebrospinal fluid (Emson,1985; Agid,1985).

In the periphery beside the gastrointestinal tract, NT-LI has been localized in cardiovascular system (Reinecke,1982; Weihe,1981), adrenal medulla (Goedert,1983), and retina (Osborne,1983).

Neurotensin-like immunoreactivity is also present in plasma of man and other animals. The levels of plasma NT-LI is altered by several stimuli including food; lipid perfusion of the small intestine is very potent stimulator of the neurotensin release (Hammer,1982; Pederson,1986,1988; Ferris,1985a,b). Other stimulators include some peptides (bombesin and gastrin releasing peptide) and duodenal acidification (Rokaeus,1982; Mogard,1986). Isocaloric and isovolumetric glucose and amino acids had either no effect, or were considerably less potent than lipid (Rosell,1979; Go,1981; Ferris,1981,1985b). In plasma, a great majority of the neurotensin-like immunoreactivity is due to the N-terminal fragments of neurotensin (Hammer,1982). The prevalence of N-terminal neurotensin fragments in plasma has been observed in the basal state as well as after the ingestion of meals (Hammer,1982). These N-terminal fragments are biologically inactive, and are probably the consequence of the rapid proteolytic degradation of the native

neurotensin. Such a rapid proteolytic inactivation of the peptide raises some questions relating to its physiological role as a hormone. This will be discussed later.

3.1.2. ACTIONS OF NEUROTENSIN

Neurotensin has a wide variety of actions both in the central nervous system as well as in the periphery. Intracisternal (i.c.) or intracerebrovascular (i.c.v.) administration but not the intravenous injections caused hypothermia in rats and mice (Bissette,1976; Jolicouer,1981; Martin,1981; Nemeroff,1977). In rats and mice i.c. and i.c.v. neurotensin induced antinociception (Clineschmidt,1977,1979; Martin,1981). Intravenous as well as intracisternal injections of neurotensin caused hypotension in rats; both centrally and peripherally mediated effects were discussed (Rioux,1981; Carraway,1973). Intracerebrovascular or intracisternal administration of neurotensin produced a dose-related decrease in food intake in deprived rats (Hawkins,1986; Luttinger,1982).

Centrally administered neurotensin inhibited gastric acid secretion in rats (Osumi,1978), it also provided cytoprotection and significantly reduced incidence of gastric ulcers in cold-plus-restraint-stressed rats (Nemeroff,1985; Hernandez,1983). Neurotensin also decreased gastric acid secretion in dogs and human (Holst,1986; Andersson,1976). Contraction of guinea pig gall bladder also occurred through a direct action on smooth muscle and an indirect action through

cholinergic neurons (Yamasato,1988).

Neurotensin produced positive chronotropic and inotropic effects in anaesthetized guinea pig, in isolated hearts of guinea pig and in rat and guinea pig isolated atria (Bachelard,1985; Kerouac,1981; Quirion,1980a,b).

Neurotensin, in the presence of low glucose concentrations, stimulated the insulin release, while in the presence of high glucose concentrations, it inhibited insulin release from isolated pancreatic islets (Dolais-Kitabgi,1979). *in vitro*, neurotensin increased prolactin release from rat anterior pituitary (Memo,1986; Vijayan,1979). In rats, neurotensin i.v. elevated and i.c. decreased prolactin release (Maeda,1978).

Neurotensin released histamine from rat peritoneal and human intestinal mast cells (Kurose,1981; Selbekk,1984).

3.1.2.1. ACTION OF NEUROTENSIN ON GASTROINTESTINAL TRACT

Several studies have demonstrated the secretomotor action of neurotensin on the gastrointestinal tract of several species. The response to exogenous neurotensin depends upon the mode of study (in vivo or *in vitro*), the tissue and the species studied.

Intravenous neurotensin delays gastric emptying in humans (Blackburn,1980), dogs (Keineke,1986) and rats (Hellstrom,1986). Infusion of neurotensin i.v. in human, stimulated the duodenum and inhibited the antrum

(Thor,1983). Furthermore, there was a decrease in the number and velocity of contractions migrating from antrum to duodenum (Rosell,1984). Hellstrom (1986) reported that vagal innervation was required for the slowing of the gastrointestinal transit by i.v. administration of neurotensin. Similar observations were made by Bueno *et. al.*. (1985) regarding the role of vagus on the action of i.c.v. neurotensin on the motility of the canine gastrointestinal tract. However, the action of the systemic administration of neurotensin on the gastrointestinal motility was not vagally mediated and appeared to be due to a peripheral action of the peptide.

In dogs, Andersson *et. al.*. (1977) observed that vagally innervated antral pouches were more sensitive to the action of Gln^4-neurotensin than vagally denervated fundic pouches, suggesting both central as well as peripheral actions of the peptide. *In vitro*, in dogs, neurotensin produced a biphasic response in gastric corpus muscles (McLean,1983). The inhibitory response was insensitive to TTX and scorpion venom, therefore, direct inhibition of smooth muscle was suggested. The excitatory response appeared to be due to the activation of the mast cells since it was diminished after mast cell degranulation.

Neurotensin contracted guinea pig ileal longitudinal muscle/myenteric plexus (MP/LM) preparation (Kitabgi,1978,1979a,b; Rökaeus,1977; Huidobro-Toro,1984); however in the precontracted preparation, it produced a biphasic response, i.e. relaxation followed by contraction (Kitabgi,1978; Huidobro-Toro,1984). The contractile phase was abolished by TTX and was reduced by atropine or

substance P tachyphylaxis (Kitabgi,1978; Monier,1980). The inhibitory phase of the response was TTX-resistant. Thus both direct (relaxation) and indirect (contraction) effects of neurotensin were proposed. Guinea pig ileal circular smooth muscle also relaxed to neurotensin *in vitro*, this response was also TTX-resistant and a direct action on smooth muscle was proposed (Goedert,1984b). The nerve-mediated action of neurotensin in guinea pig MP/LM preparation was due to the release of acetylcholine since atropine inhibited the contractile action and neostigmine increased the effectiveness of neurotensin doses (Huidobro-Toro,1984; Kitabgi,1979b). More direct evidence for neurotensin action via cholinergic pathway was provided by Nakamoto *et. al.*. (1987) who observed a neurotensin-evoked release of acetylcholine and gamma-amino butyric acid from guinea pig ileal strips. In guinea pig MP/LM preparation, in another study, (Yamanaka,1987), the contraction was TTX-resistant, indicative of a non-neuronal action of the peptide, and may result from regional differences in the TTX-sensitivity of the response. The contraction in this tissue was not accompanied by the depolarization of the membrane, while the circular muscle hyperpolarized in the presence of neurotensin. NT also relaxed the precontracted (with 17.8 mM but not with 39.6 mM KCl) circular muscle strips. This might imply a role for the K^+-gradient and K^+-channels in the response.

Rat fundus strips contracted to neurotensin (Donoso,1986; Huidobro-Toro,1985; Quirion,1980a,b; Rökaeus,1977). A broad spectrum of antagonists

including TTX did not influence the action of neurotensin, suggesting a direct action on smooth muscle cells (Huidobro-Toro,1985; Quirion,1980a). The action of neurotensin on rat fundus depended on external calcium, was potentiated by the calcium channel agonist Bay-K 8644 and was inhibited by dihydropyridine calcium channel antagonist, indicating that an influx of calcium was involved in neurotensin-mediated contractile response (Donoso,1986). Rat ileum and duodenum relaxed in response to neurotensin (Carraway,1973; Rökaeus,1977; Kitabgi,1978).

Rabbit colon circular muscles also contracted in response to added neurotensin *in vitro* (Snape,1987a,b). The action was TTX resistant, and was not affected by adrenergic, cholinergic or opioid antagonism (Snape, 1987a). Rabbit ileum responded to neurotensin with inhibition of spontaneous phasic contractions (Huidobro-Toro,1983).

Intraarterial injections of neurotensin to the canine small intestine inhibited field-stimulated, cholinergic-mediated contractions (Fox,1987; Sakai,1984). The inhibition was atropine-insensitive. TTX and α_2-selective adrenergic antagonists decreased the sensitivity of neurotensin response and it was proposed that neurotensin *in vivo* was acting on neural receptors to release norepinephrine which acted at the presynaptic α_2-adrenoceptors to inhibit the acetylcholine release, thereby inhibiting the ongoing contractile activity (Sakai,1984). There may have also been a direct smooth muscle component of neurotensin relaxing action since this response was still obtained after TTX, adrenergic antagonists or

reserpinization. In the study of Fox *et. al.*. (1987), i.a. neurotensin produced, after a delay of several minutes, an enhancement of contraction produced by submaximal concentration of acetylcholine administered intraarterially.

In vitro, the circular smooth muscle strips from canine small intestine responded in a biphasic manner. At lower concentrations of neurotensin (10^{-12}-10^{-9}M), an increase in frequency and amplitude of the phasic contractions occurred. Higher concentration (10^{-8}-10^{-6}M) caused an initial inhibition of the contractions followed by a tonic contraction (Fox,1987). TTX did not affect any of these responses, therefore, a direct action on the smooth muscle was proposed. At the highest concentration (10^{-6}M) an action on the mast cells was also suggested (Fox,1987).

3.1.3. NEUROTENSIN RECEPTORS

Autoradiographic studies with ^{3}H-NT have demonstrated the presence of NT-receptors on circular smooth muscle and to a lesser extent on longitudinal smooth muscle of guinea-pig ileum (Goedert,1984b). Using ^{3}H-NT and a crude membrane preparation (Goedert,1984b) or the homogenate of isolated longitudinal smooth muscle cells (Kitabgi,1979a), the equilibrium constant (Kd) of NT was measured to be 3.4 and 4.0 nM respectively. The obvious experimental difficulty in these studies was the use of tritium-labelled neurotensin with low specific activity (56 - 77 Ci/mmol). Such a low specific activity combined with the low capacity (Bmax) of binding sites may have precluded the detection of high affinity binding sites in these preparations.

Mazella *et. al.* (1983) reported iodination of a neurotensin analogue, Trp^{11}-NT. Iodination at Tyr^{11} causes a drastic reduction in the biological potency of the peptide, but selective iodination of Trp^{11}-NT at Tyr^{3} yielded a radiolabelled peptide which retained high biological activity at the receptors in some tissues. Using this high specific activity iodinated derivative of Trp^{11}-NT, Kitabgi *et. al.*. (Kitabgi,1984) characterized the NT-receptors on the purified membrane preparation from rat gastric fundus. A high affinity as well as a low affinity site were clearly recognized.

Iodinated Trp^{11}-NT served as a good label for NT-receptors in murine

species, however in guinea-pig (Checler,1982) and human brain (Sadoul,1984a) it was ineffective. Sadoul *et. al.* (1984b) later reported the iodination and chromatographic separation of native NT selectively iodinated at Tyr^3 residue.

At the beginning of the present studies, no binding studies on the canine gastrointestinal tract had been performed to study the neurotensin receptors. Extensive studies have been performed on this model *in vivo* and *in vitro* (discussed earlier), which suggest the presence of peripheral neurotensin receptors, both on the smooth muscle and on the neuronal structures of the canine small intestine. The suggestion for the presence of receptors on smooth muscle cells comes from the studies with the neurotoxin tetrodotoxin. However, TTX-insensitive neural responses are known to occur (Daniel,1987). Furthermore, in previously described functional studies, the identification of the nerves (myenteric plexus, deep muscular plexus, or the submucous plexus) responsible for the TTX-sensitive responses was not possible. Therefore, the present studies had as one objective, to elucidate the locus of receptors for neurotensin action within the gastrointestinal tract and the nature of these receptor sites.

3.2 MATERIALS

^{125}I-Na (2000 Ci/mmol) was from Amersham (Arlington Heights, IL). Neurotensin, its analogues and partial fragments were from Sigma Chemical Company (St. Louis, MO). dl-dithiothreitol (DTT), 1,10-phenanthroline, cysteine and cystine were also from Sigma. Other salts and chemicals were either from Sigma or BDH Chemicals. Racemates of levocabastine were kindly provided by Dr. Pierre Laduron.

3.3. METHODS

3.3.1 IODINATION OF NEUROTENSIN

The iodination of neurotensin selectively at tyr^3 was performed by the hydrogen peroxide-lactoperoxidase method as described by Sadoul *et. al.*. (1984b). Briefly, 2 equivalents of neurotensin were incubated at room temperature with 1 equivalent of ^{125}I-Na and 0.1 mg lactoperoxidase in 5 mM sodium phosphate buffer at pH 7.4. Four aliquot (0.25 equiv) of H_2O_2 were then added at 30-s intervals after which the reaction was terminated by the addition of 1 equivalent of sodium metabisufite and 1 mg bovine serum albumin. The iodinated product was then separated over a SP-Sephadex C-25 column. The elution was performed by a stepwise gradient of 1 mM, 25 mM and 50 mM phosphate buffer (pH 8.6). The radioactive peak corresponding to the monoiodinated product (Sadoul,1984b) was screened for its binding activity and the fraction having the highest binding activity was aliquoted and quick frozen in liquid nitrogen. The specific activity of the monoiodinated peptide was taken as that of iodine. When required (i.e. in the case of saturation experiments), these fractions were diluted with uniodinated neurotensin to quench the specific activity, in such cases, new specific activities were defined.

3.3.2. BINDING OF (TYR^3 ^{125}I)-NEUROTENSIN

The binding of radioactively labelled neurotensin to the membranes was performed by slight modification of the method of Sadoul *et. al.*. (1984b). The incubation buffer consisted of 50 mM Tris-HCl (pH 7.4) containing 0.2% bovine serum albumin and 1 mM 1,10-phenanthroline. All the reactions were carried out simultaneously for the total and the nonspecific binding each in triplicate. To the incubation tubes, 25 µL of radioactively labelled neurotensin and 25 µL of the buffer (for the total binding) or 25 µL of unlabelled neurotensin (10^{-5} M) were added. The reaction was started by the addition 200 µL (20-80 µg) of the appropriate membranes diluted in the incubation buffer. The reaction was carried out at 37°C for 20 min except for the experiments on the time course, where the incubation was performed for the indicated times. The separation of the bound from the free ligand was effected by filtration under vacuum using a Millipore filtration apparatus and Gelman GA-8 (0.2 µm pore size) or the Gelman GN-6 filters. The filters were soaked in the incubation buffer for at least 4 hours before use. The reaction, unless otherwise indicated, was always terminated by dilution of the incubation sample with 2 mL ice cold incubation buffer and immediate filtration under reduced pressure (20 micron). The tube and filters were washed twice with 2mL incubation buffer. Total time taken for the filtration and washing was between 5-10 s. The radioactivity on the filters was measured in a gamma-counter (Beckman 5500 or Searle model 1285). Non-specific binding was defined

as that not displaced in the presence of excess (1μM) unlabelled neurotensin. The specific binding was defined as the difference between the total binding and the non-specific 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.

3.3.2.1. PROTEIN BINDING PROFILE

Varying amounts of appropriate membrane protein (0-180 μg per assay tube) were incubated with 70-80 pM labelled neurotensin under the reaction conditions described above. At 25 min. the incubation mixture was diluted with cold buffer and filtered immediately as described earlier.

3.3.2.2. ASSOCIATION AND DISSOCIATION KINETICS

Membranes (0.1 - 0.3 mg/mL) without (for total binding) or with (for nonspecific binding) excess (10^{-6} M) of unlabelled neurotensin, diluted in the incubation buffer were incubated at 37°C. The association was started by the addition of the labelled neurotensin. At various time intervals, 0.25 mL aliquot were withdrawn, diluted in cold incubation buffer, filtered and the tubes and filters were washed twice with the buffer.

After 25 min. of association, the dissociation was initiated by a) addition of the excess (10^{-6} M) unlabelled neurotensin, b) dilution of the incubation medium

by 40 fold, or c) dilution of the incubation medium in the presence of an excess (10^{-6} M) 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 aliquot were taken and filtered as usual.

3.3.2.3. EQUILIBRIUM BINDING EXPERIMENTS

The membranes (20-80 µg protein per assay tube) were incubated with increasing concentrations of radioactively labelled neurotensin (specific activity ~2000 Ci/mmol, or serially reduced specific activities by dilution with unlabelled neurotensin), in a total volume of 0.25 mL. The reaction was carried out as described earlier.

3.3.2.4. COMPETITION EXPERIMENTS, EFFECT OF Gpp(NH)P, LEVOCABASTINE, AND THE EFFECT OF VARIOUS IONS

The membranes were incubated in the absence (for control i.e. 100% binding), or in the presence of competing peptides and fragments or ions. In the case of competition experiments, the incubation was performed in quadruplicate, non-specific binding was simultaneously measured only in the control tubes. In the experiments on the study of ions, non-specific binding was measured at each concentration of the ion. The effect of levocabastine and GTP analogue Gpp(NH)p was studied similar to the experiments on the effect of ions. The data for the

competition as well as for the effect of ions are always presented as the specific binding at each concentration.

3.3.2.5. COMPUTATION OF THE DATA

Ligand binding data were analyzed using an IBM-PC/XT microprocessor and the programmes EBDA and LIGAND prepared by Munson and Rodbard (1980) and adapted by G.A. McPherson (1983).

3.3. CONTRACTILITY EXPERIMENTS

Parallel strips of the circular smooth muscle were cut in the circular orientation. These were suspended in 3.0 mL jacketed organ baths filled with the Krebs at 37° C and gassed with 95% O_2 , 5% CO_2. One end of the tissue strip was tied to the electrode and the other end was tied to a Grass FT03C force transducer with the help of silk suture. Changes in the tension were recorded on a Beckman R611 dynograph. Drugs were serially diluted and added to the bath in 30 µL aliquots.

3.4. RADIATION INACTIVATION EXPERIMENTS

For the radiation inactivation experiments, the membranes were prepared as described before and were quick-frozen in aluminium trays in liquid nitrogen and stored at -70° C. These samples were radiated at -45° C using a Van

de Graaf generator and stored again at -70° C until the binding was performed. The radiation dose was simultaneously determined using blue cellophane at the radiation temperature. Pyruvate kinase, yeast alcohol dehydrogenase and galactose oxidase were used as the molecular weight standards. These standards were also frozen and radiated in the same manner.

3.4. RESULTS

3.4.1. DISTRIBUTION OF THE BINDING SITES

The distribution of the neurotensin binding sites in the fractions obtained upon homogenization and separation of the fractions from the circular smooth muscle layer is listed in the table IV. These results are plotted in fig. 15. In fig. 15 are also plotted the results of the distribution of the markers 5'-nucleotidase (for the smooth muscle plasma membrane), specific binding of the [^{3}H]saxitoxin (for nerve membrane) and the content of the VIP-immunoreactivity (for intact synaptosomes) for comparison. As is clear from the table and the figure, the binding and the enrichment of the binding does not follow any simple unimodal distribution pattern. The plasma membrane enriched fraction M2 is enriched about 20 fold over the post nuclear supernatant (PNS). In the fractions obtained from the gradient fractionation of Mit I, high neurotensin binding activity is present in the P2 fraction enriched in the smooth muscle plasma membrane marker 5'-nucleotidase. However, high neurotensin binding is also observed in the fractions P3-P5. The fraction P4 is a synaptosome-enriched fraction and the fraction P5 is also rich in saxitoxin binding: however, the specific activity of the 5'-nucleotidase is relatively low in these fraction, therefore, the neurotensin binding in these fractions must represent the binding to the neural structures. The high level of the

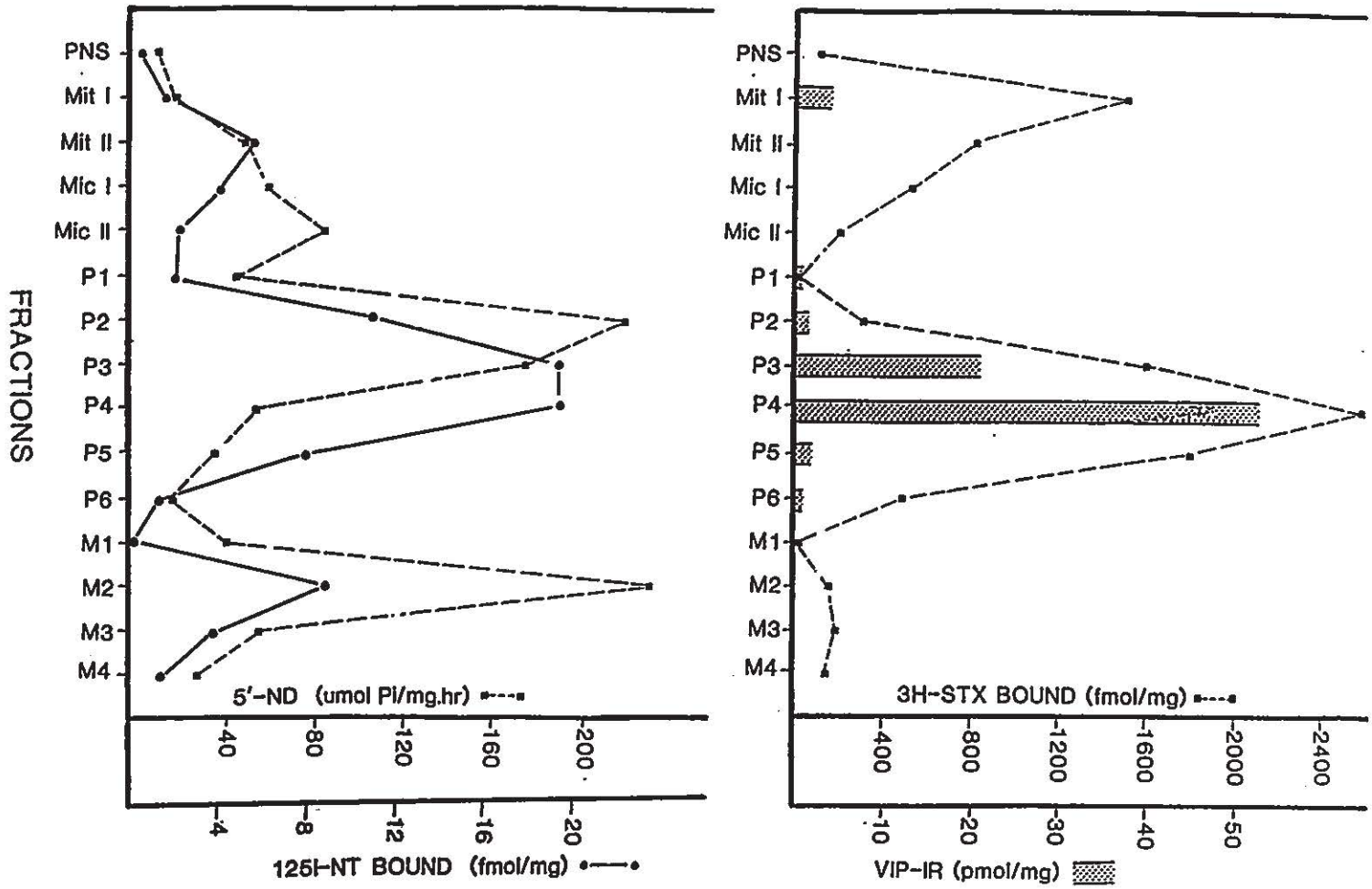
TABLE IV

DISTRIBUTION OF NEUROTENSIN BINDING
IN CANINE SMALL INTESTINE CIRCULAR MUSCLE LAYER

FRACTION	BOUND (fmol/mg)
PNS	0.45 ± 0.17
Mit I	2.38 ± 1.10
Mit II	6.93 ± 1.71
Mic I	4.07 ± 1.06
Mic II	2.85 ± 0.91
P1	2.52 ± 1.69
P2	11.3 ± 4.95
P3	19.3 ± 7.93
P4	19.4 ± 9.74
P5	8.12 ± 4.58
P6	1.57 ± 1.49
M1	0.18 ± 0.12
M2	8.74 ± 2.90
M3	3.29 ± 1.54
M4	1.61 ± 0.85

Results are the means ± sd from 3-7 experiments performed in triplicate simultaneously for the total and nonspecific binding. Approximately 0.1 nM [^{125}I]tyr^{3}-neurotensin was incubated with appropriate membranes under the incubation conditions described in the "methods".

Figure 15. Distribution of the [^{125}I]tyr^{3}-neurotensin binding (125I-NT, 70-80 pM)) to the fractions obtained upon the fraction of the homogenate of the circular muscle of canine small intestine. For comparison, the distribution of the markers (from fig. 11) is also plotted here.


FRACTIONS
PNS
Mit I
Mit II
Mic I
Mic II
P1
P2
P3
P4
P5
P6
M1
M2
M3
M4
5'-ND (umol Pi/mg.hr)
40
80
120
160
200
4
8
12
16
20
125I-NT BOUND (fmol/mg)
3H-STX BOUND (fmol/mg)
400
800
1200
1600
2000
2400
10
20
30
40
50
VIP-IR (pmol/mg)

binding in the fraction P3 may reflect the binding of neurotensin to a mixture of smooth muscle plasma membranes and to the neural structures.

The distribution of the binding in the longitudinal muscle/myenteric plexus fractions is listed in the table V. The upper three lines list the binding observed in the fractions P1, P2 and Mic II. In all these three fractions, the binding was very low. Since, during the initial dissecting procedure, the longitudinal smooth muscle layer was dissected out with a thin layer of the circular muscle attached to it, the binding in these fractions may be to the membranes from remnants of the circular muscle in these fractions. The middle four lines of the table V list the results of another set of experiments, where, for comparison, the binding was performed simultaneously with the DMP fraction. Since, the LM/MP fractions were prepared in the imidazole buffer, the effect of the imidazole buffer on the binding was also studied. In this experiment, more purified fractions from the gradient were taken for the binding. As is clear from these data, binding to the DMP fraction was quite high and the presence of the imidazole buffer in the incubation medium had no effect on the binding. However, the binding to the purified longitudinal smooth muscle plasma membranes (M2) and the purified synaptosomal fraction from the myenteric plexus (S2) was extremely low, near the background level. The bottom three lines in the table V, again compare the binding between the DMP fraction and the purified synaptosomal fraction from the myenteric plexus. Again high level of binding was observed in the DMP fraction

TABLE V

NEUROTENSIN BINDING TO THE LONGITUDINAL MUSCLE LAYER OF CANINE SMALL INTESTINE

FRACTION	BOUND (fmol/mg)
LM P1	0.83 ± 0.37
LM P2	1.09 ± 0.20
Mic II	0.33 ± 0.06
CM P4	20.86
CM P4 + imidazole	21.31
LM M2	0.86
LM S2	0.31
CM P4	16.23
LM S2	0.17
CM P4 + LM S2	18.29

See table IV for the legend. CM = circular smooth muscle; LM = longitudinal smooth muscle. The results in the upper three lines are the mean ± sd from two separate experiments performed in triplicate. The rest are the results from individual experiments.

and MP fraction had very low binding. In order to examine if the lower level of binding in the MP fraction could be explained due to a rapid degradation of the peptide in this particular fraction, the fraction DMP was contaminated with the MP fraction and the binding was studied. This procedure did not impair the binding in the DMP fraction. Therefore, it is concluded that under our reaction conditions, we could not detect any binding in the longitudinal smooth muscle/myenteric plexus preparation. This lack of binding could not be explained due to the use of a different buffer or the rapid degradation of the peptide by these fractions.

The distribution of the neurotensin binding in the submucous plexus preparation is given in the table VI and the fig. 16. In this case, the binding demonstrated a unimodal pattern of distribution. Highest binding was observed in the fraction P2, which was also highly enriched in the neuronal markers. The binding in the Mic II fraction was very low and could be explained by neuronal contamination in this fraction.

3.4.2. CHARACTERIZATION OF THE BINDING

3.4.2.1. PROTEIN BINDING PROFILE

To determine the working range for the membrane protein, binding with varying concentrations of protein (0 - 180 µg protein) was performed. The result for the circular smooth muscle plasma membrane is depicted in the fig 17. The amount of the specifically bound label increased linearly with the increase in the

TABLE VI

DISTRIBUTION OF NEUROTENSIN BINDING SITES
IN CANINE SMALL INTESTINE SUBMUCOUS FRACTIONS

FRACTIONS	BOUND (fmol/mg)
PNS	0.53 ± 0.32
P1	1.00 ± 1.23
P2	7.83 ± 2.23
P3	4.41 ± 0.02
S1	0.50 ± 0.33
S2	1.23 ± 0.78
Mic I	2.39 ± 0.48
Mic II	0.43 ± 0.31

See legend for the table table IV. The results are the mean ± sd from three experiments.

Figure 16. Distribution of the [^{125}I]tyr^{3}-neurotensin binding (125I-NT, 70-80 pM)) to the subcellular fractions from the homogenate of the the submucosa. For comparison, the distribution of the markers (from fig. 12) is also plotted here.

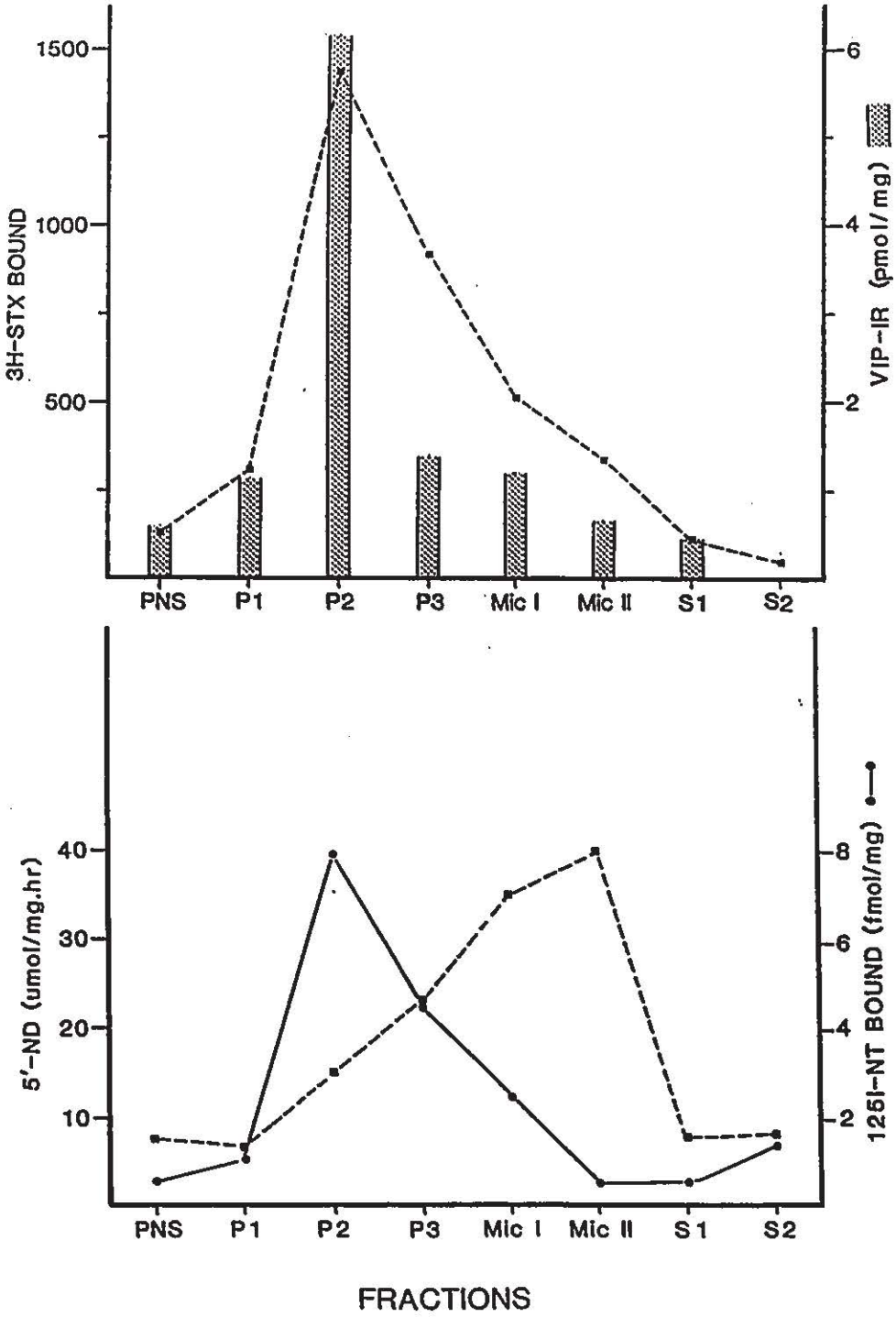
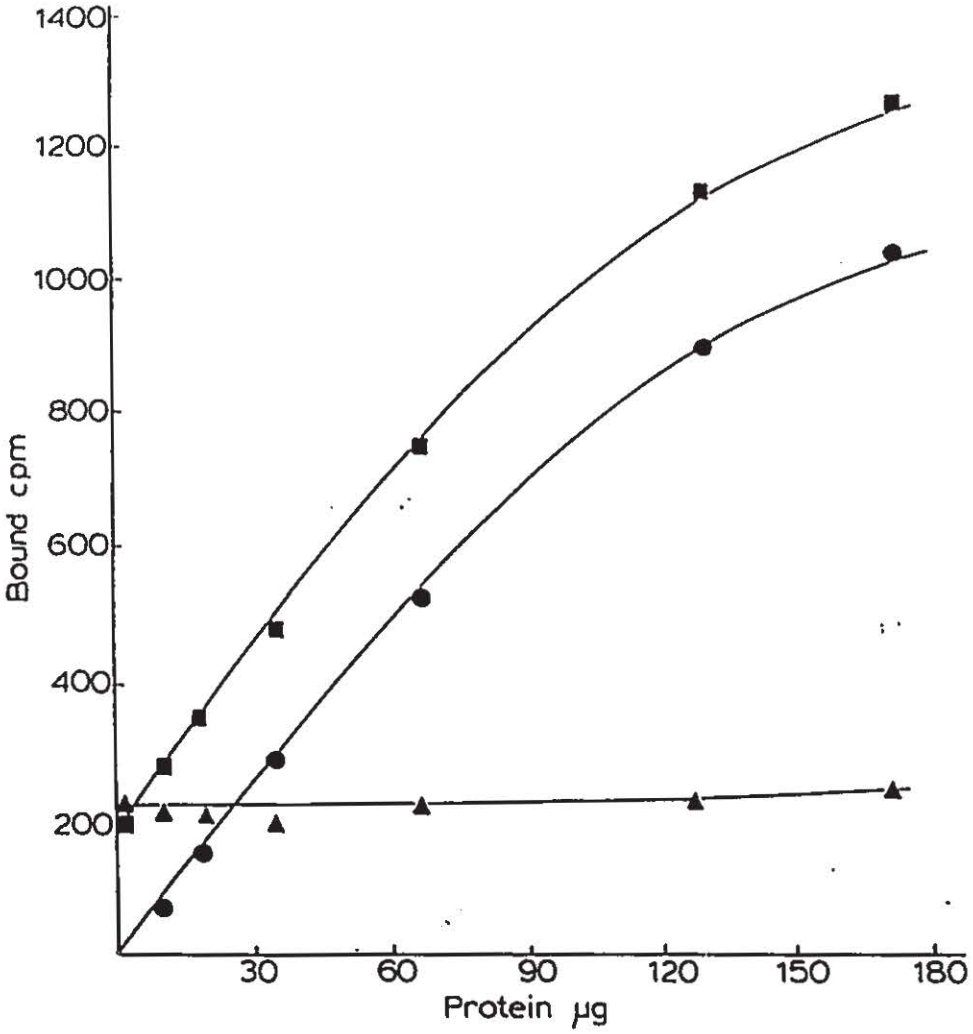

1500
1000
500
3H-STX BOUND
6
4
2
VIP-IR (pmol/mg)
PNS
P1
P2
P3
Mic I
Mic II
S1
S2
40
30
20
10
5'-ND (umol/mg.hr)
8
6
4
2
125I-NT BOUND (fmol/mg)
PNS
P1
P2
P3
Mic I
Mic II
S1
S2
FRACTIONS

Figure 17. Protein binding profile. Labelled [^{125}I]tyr^{3}-neurotensin (200 Ci/mmol; 70-80 pM) was incubated at 37°C in the presence of increasing concentration of the circular smooth muscle plasma membranes (0-180 μg) under the conditions described in the methods. Filled squares, total binding; filled triangles, non-specific binding; filled circles, specific binding.


1400
1200
1000
800
600
400
200
Bound cpm
30
60
90
120
150
180
Protein µg

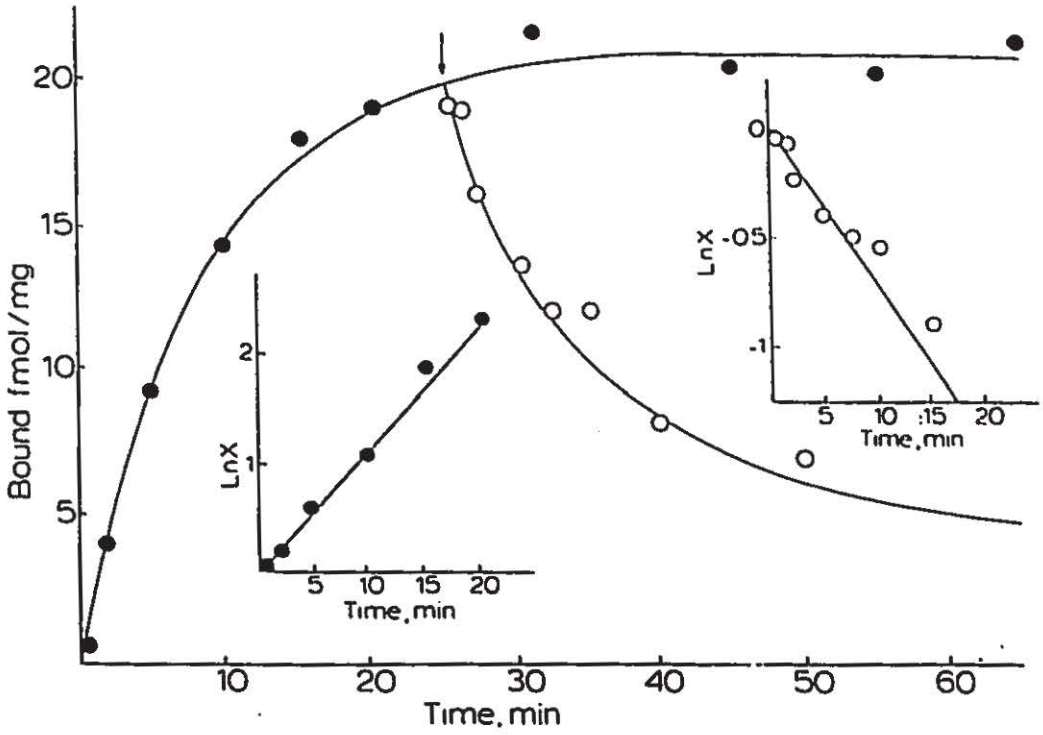
content of the membrane protein in the incubation medium up to about 100 μg per assay tube. Non-specific binding to the membranes was found to be negligible this concentration (80 pM) of the label; all the non-specific binding observed was on the filters. The non-specific binding did not increase when the concentration of the protein in the incubation medium was increased. Furthermore, no specific binding to the filters (i.e. in the absence of membranes) was observed. In all other experiments, no more than 100 μg of the protein per assay tube was used.

3.4.2.2. TIME COURSE OF ASSOCIATION AND DISSOCIATION

The specific binding of radioactively labelled neurotensin to the circular smooth muscle plasma membranes as a function of time is depicted in fig. 18. The binding increased with time and reached equilibrium by 20 min. The half time of association ($T_{1/2}$) was 3.5 min, about 90% of the total binding at equilibrium (B_{eq}) was achieved by 15 min. The levels of specific binding did not change for up to 80 min of incubation. At equilibrium, at the working range of the concentration of radioactively labelled neurotensin (0.06 - 0.1 nM), bound neurotensin represented only about 2 - 3% of the total neurotensin label added in the incubation medium, hence at equilibrium, free ligand can be considered equal to the total ligand added. For this reason, the association kinetics could be analyzed as pseudo-first order reaction according to the equation

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

Figure 18. Time course of [^{125}I]tyr^{3}-neurotensin to the circular smooth muscle plasma membranes. Labelled peptide (2000 Ci/mmol; 70 pM) was incubated with the membranes. At indicated times 0.25 mL aliquot 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 simultaneously in a different set of samples which contained 1 μM unlabelled neurotensin from the beginning of the incubation. The data are plotted as the specific binding determined at various times. The insets show the same data plotted according to the pseudo first order reaction for the 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 $[B_0]$ is the concentration of the bound ligand at time zero of dissociation.


Bound fmol/mg
20
15
10
5
10
20
30
40
50
60
Time, min
Ln X
2
1
5
10
15
20
Time, min
Ln X
0
-.05
-.1
5
10
15
20
Time, min

where $[B_{eq}]$ is the concentration of the bound ligand at equilibrium, $[B]_t$ is the concentration of the bound ligand at 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 of $\ln([B_{eq}]/[B_{eq}]-[B]_t)$ vs time gave a straight line with a slope value $k = k_1[L] + k_{-1} = 0.118\ min^{-1}$, where [L] = 69 pM.

Addition of excess (1 μM) of unlabelled neurotensin induced a rapid dissociation to the levels approaching the nonspecific binding. The dissociation proceeded quickly for about 10 min and slowly thereafter. Such a biphasic dissociation might be attributed to the presence of two classes of binding sites (see 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 $\ln([B]_t/[B]_0)$ vs time gave a straight line. A logarithmic representation of the data are represented in fig. 18 inset, the slope of this line represents the rate constant of dissociation, which was $0.072\ min^{-1}$.

Introduction of k_{-1} into the expression for k (see above) gave the value of 5.8×10^8 for k_1. The dissociation constant calculated from these values ($K_d = k_{-1}/k_1$) was 0.11 nM.

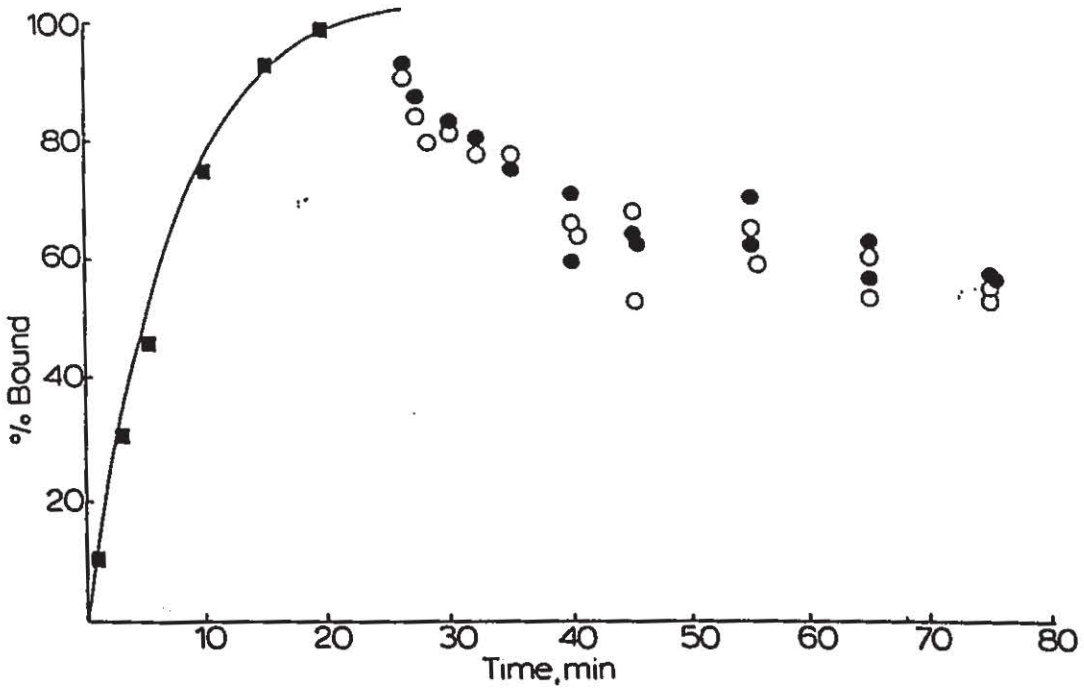
The dissociation rate was also assessed by diluting the incubation medium (40 fold) with excess of the incubation buffer at 37°C and diluting the incubation medium in the presence of excess unlabelled (10^{-6} M) neurotensin to explore the possibility of the presence/absence of cooperativity among the receptor

sites. The results are given in fig. 19. As is evident from the figure, the rate as well as the extent of dissociation was same in both the cases thus favouring the multiple site model over the negative cooperativity. However, upon dilution, the dissociation was incomplete as compared to the experiment where only excess of unlabelled ligand was added to initiate the dissociation (compare figs. 18 and 19). The reason for this discrepancy is unclear.

3.4.3.3. EQUILIBRIUM BINDING CHARACTERISTICS

The binding as a function of increasing concentration of the radiolabeled ligand for the smooth muscle plasma membranes is illustrated in the fig. 20. A summary of the data for the binding parameters for the smooth muscle plasma membranes as well as for the deep muscular plexus membranes is given in table VII. When analyzed with the EBDA and LIGAND programmes, the binding to both membrane types revealed heterogenous population of binding sites. The data were fitted better by a two site model as compared to a one site model. At least three sets (or affinity states) of the binding sites were observed. The high affinity sites on the circular smooth muscle plasma membranes and on the deep muscular plexus membranes were of the similar Kd value (0.1 - 0.2 nM). The low affinity sites on the smooth muscle plasma membranes had the Kd value of about 3 nM and those on the deep muscular plexus membranes were of much lower affinity i.e. in 30 - 40 nM range kd.

Figure 19. Dissociation of the bound labelled neurotensin from the circular smooth muscle plasma membranes. The association was performed as described in the Methods with 70-80 pM radiolabel. At 25 min, dissociation was initiated by diluting the incubation mixture 40-fold by the incubation buffer (filled circles), or by diluting 40-fold in the presence of 1 μM unlabelled neurotensin. The data are plotted as percent of specifically bound labelled neurotensin at equilibrium.


100
80
60
40
20
% Bound
10
20
30
40
50
60
70
80
Time, min

3.4.3.4. COMPETITION STUDIES WITH NEUROTENSIN-RELATED PEPTIDES

The structural requirements for the neurotensin binding was studied by performing the competition studies with neurotensin analogues and fragments. The competitive inhibition of the binding by unlabelled neurotensin, partial sequences and analogues on the circular smooth muscle plasma membranes is illustrated in fig. 21. The IC_{50} values for the binding to the circular smooth muscle plasma membranes as well as to the deep muscular plexus membranes is summarized in the table VIII. The rank order of potency and the IC_{50} value of the peptides tested for the competition was similar for both types of membranes.

3.4.3.6. EFFECT OF IONS, LEVOCABASTINE AND GTP ANALOGUE

The results for the effects of ions on the binding of the labelled neurotensin on the circular smooth muscle plasma membrane receptors is listed in the table IX. The specific binding of the peptide was modulated by all the ions tested, the maximum inhibition of the binding was by $CaCl_2$.

The guanine triphosphate analogue, Gpp[NH]p modulated the specific binding of the peptide (at the concentration near the high affinity Kd value) to the circular smooth muscle plasma membranes. At 10^{-8} M of Gpp[NH]p, no significant effect on the specific binding was noticed (98 ± 3% binding remained, mean ± sd, n=3). The binding decreased with increasing concentration of the nucleotide; at

Figure 20. Binding of [^{125}I]tyr^{3}-neurotensin to the circular smooth muscle plasma membranes as a function of increasing concentration of the ligand. Plasma membranes were incubated at 37°C with the increasing concentrations of the ligand. After 25 min the reaction was stopped and the bound ligand was separated from the free by filtration. Closed squares represent the non-specific binding and closed circles and the open circles represent the specific binding with the specific radioactivity of 2000 Co/mmol and with serially decreasing radioactivities respectively. The inset is the representation of the same data according to the Scatchard analysis.

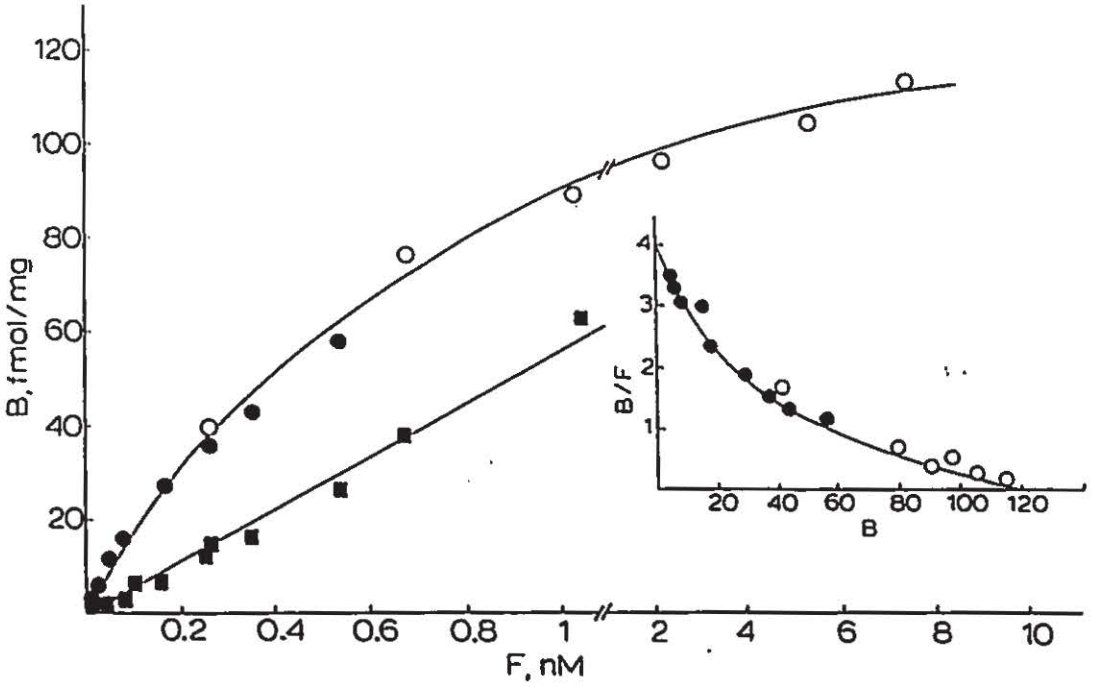

B, fmol/mg
120
100
80
60
40
20
0.2
0.4
0.6
0.8
1
2
4
6
8
10
F, nM
B/F
4
3
2
1
20
40
60
80
100
120
B

Figure 21. Competitive inhibition of [^{125}I]tyr^{3}-neurotensin binding by neurotensin, neurotensin analogues and fragments. The membranes were incubated with 70 - 80 pM labelled peptide with increasing concentration of of the competitors under the incubation conditions described in the Methods. neurotensin, ●——●; neurotensin[8-13], ■——■; neurotensin[1-11], □——□; [phe^{11}]neurotensin, ▽——▽; neurotensin[1-8], △——△ ; [L-Trp^{11}]neurotensin, ▲——▲; [Trp^{11}]-neurotensin. ○——○

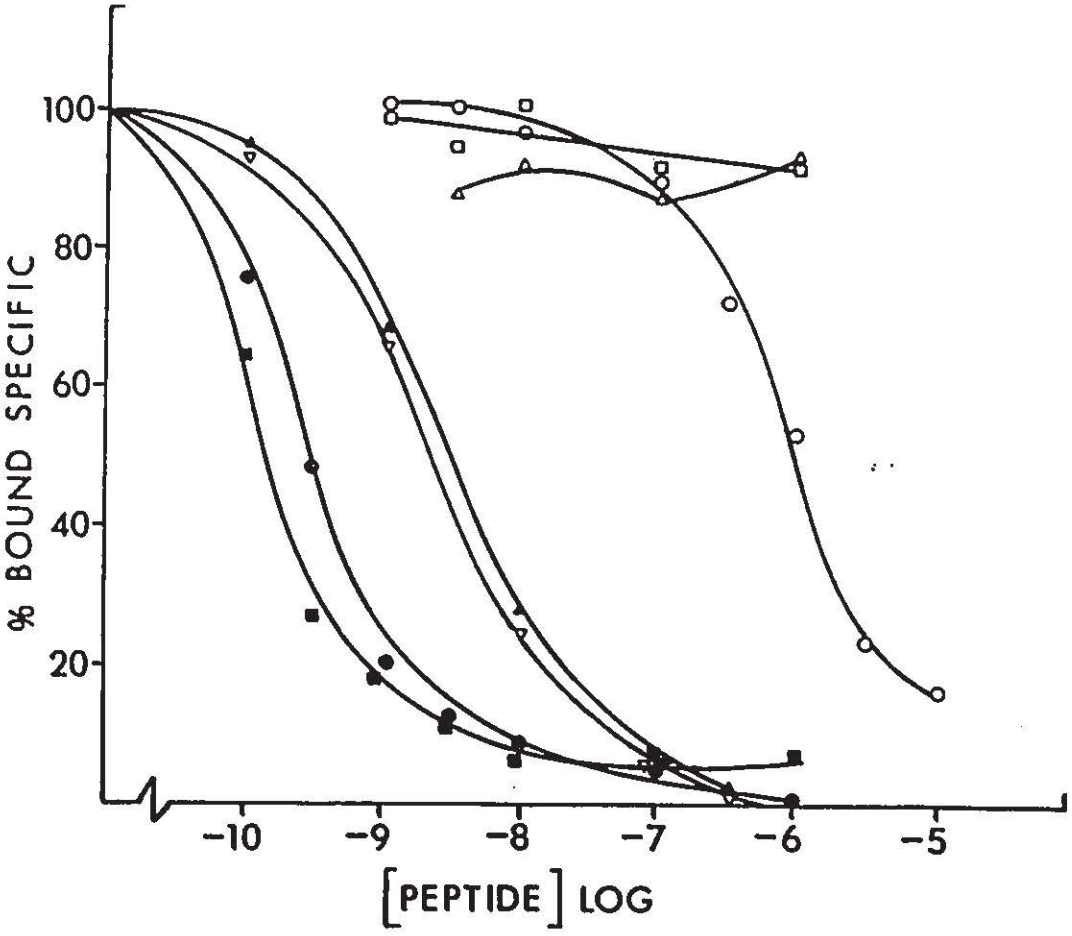

100
80
60
40
20
% BOUND SPECIFIC
-10
-9
-8
-7
-6
-5
[PEPTIDE] LOG

TABLE VII

NEUROTENSIN SATURATION DATA

	PM	DMP
Kd_1 (nM)	0.12 ± 0.04	0.23 ± 0.06
Kd_2 (nM)	3.18 ± 1.37	39.71 ± 10.32
$Bmax_1$ (fmol/mg)	9.73 ± 2.20	34.05 ± 4.45
$Bmax_2$ (fmol/mg)	129.80 ± 48.30	772.00 ± 118.88

The results are the mean ± sd from 3 experiments for DMP and 6 experiments for PM. DMP = deep muscular plexus synaptosomes; PM = circular smooth muscular plasma membranes.

TABLE VIII

NEUROTENSIN COMPETITION DATA

	PM		DMP	
	IC_{50}	POTENCY	IC_{50}	POTENCY
Neurotensin	0.50 ± 0.15	100	0.50 ± 0.20	100
Neurotensin(8-13)	0.15 ± 0.08	333	0.21 ± 0.01	238
Trp^{11}-Neurotensin	4.50 ± 0.64	11.1	5.00 ± 1.27	10
Phe^{11}-Neurotensin	4.20 ± 0.52	11.9	3.50 ± 1.12	14.3
D-Trp^{11}-Neurotensin	250 ± 266	0.04	750 ± 146	0.06
Neurotensin(1-11)	>20,000	<0.002	>20,000	<0.002
Neurotensin(1-8)	>20,000	<0.002	>20,000	<0.002

The binding was performed at approximately 0.1 nM concentration of the radiolabel. The results are the mean ± sd from 3-10 experiments performed in quadruplicate. The potency is expressed as compared to neurotensin taken as 100.

10^{-7} M, 88 ± 2% binding was observed; at 10^{-6} M 83 ± 3%; 10^{-5} M, 74 ± 5% and at 10^{-4} M, only 58 ± 4% specific binding remained.

The antihistamine, levocabastine has been shown to inhibit the specific neurotensin binding in several systems. The effect of this compound was also tested in the present study: the results are furnished in the table X. Levocabastine and its racemic mixture, at a concentration of 10^{-6} M, had no effect on the specific neurotensin binding to either the circular smooth muscle plasma membrane receptors, or to the deep muscular plexus receptors.

3.4.3.5. TARGET SIZE OF THE NEUROTENSIN RECEPTORS

The target size was determined by the rate of radiation inactivation of the neurotensin receptors from the circular smooth muscle plasma membranes as well as from the deep muscular plexus membranes. The inactivation profile of the receptors is given in the fig. 22. External standards were used for the calibration of the inactivation profiles. The standards were galactose oxidase, alcohol dehydrogenase and pyruvate kinase. Using these standards, curves for the slope of inactivation vs radiation dose were constructed. As is apparent from the figure, the inactivation profile for the neurotensin receptors from the two sources differed considerably. The calculated slope of the line for the deep muscular plexus synaptosomal neurotensin receptors was 0.067 and that for the circular smooth muscle plasma membranes was 0.10, yielding the molecular size of about 119,333

TABLE IX

EFFECT OF IONS ON THE SPECIFIC NEUROTENSIN BINDING IN CANINE SMALL INTESTINE CIRCULAR SMOOTH MUSCLE PLASMA MEMBRANES

	Percent binding at			
	10 mM	25 mM	50 mM	75 mM
NaCl	64.8 ± 3.6	32.9 ± 9.2	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 ± 2.2	6.1 ± 2.7

The binding was performed at approximately 0.1 nM ligand concentration. The data are the mean and sd from at least 3 different experiments performed in triplicate for both total and nonspecific binding.

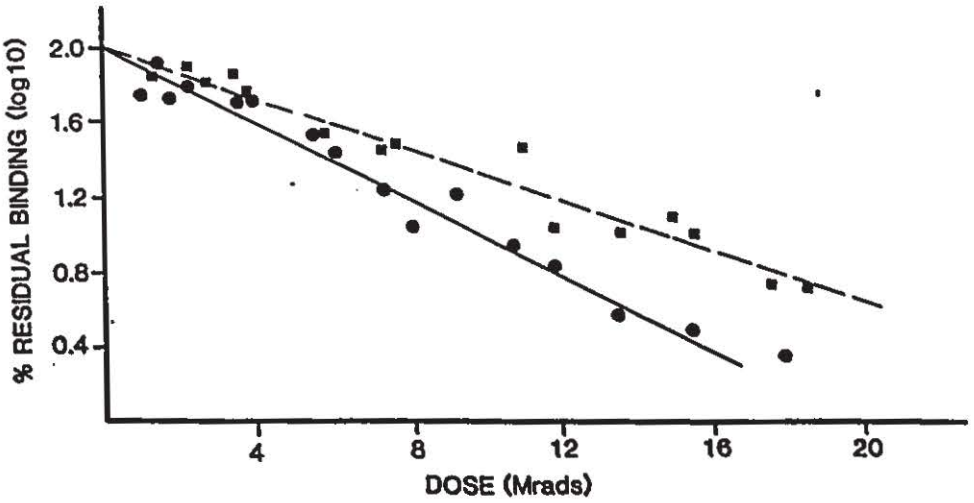
TABLE X

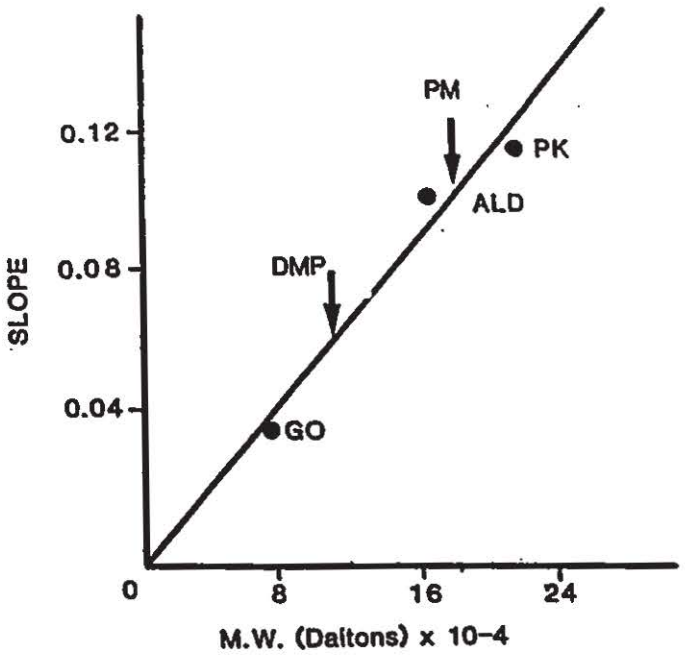
EFFECT OF LEVOCABASTINE RECEMERS ON THE SPECIFIC NEUROTENSIN BINDING

	PM	DMP
	% SPECIFIC BINDING	
R-64034	122.4 ± 15.5	103.5 ± 4.8
R-61816	114.5 ± 16.6	98.55 ± 16.7

The effect of two racemates of the antihistamine levocabastine was studied. The binding was performed in the presence of approximately 0.1 nM radiolabel. Results are the mean ± sd from three experiments performed in triplicate.

Figure 22. Target size analysis of neurotensin receptors on the deep muscular plexus synaptosomes and the circular smooth muscle plasma membranes. A, The $\log_{10}$ of the inactivation of the binding plotted against the dose of radiation. B, The slopes of the known molecular weight standards, galactose oxidase, GO, alcohol dehydrogenase, ALD and the pyruvate kinase, PK plotted against the slope of inactivation. The molecular wight of the neurotensin receptors were then extrapolated on x-axis by their slope of inactivation on the y-axis. Arrow with DMP indicates the slope for deep muscular plexus neurotensin receptors, and the arrow with PM indicated the slope of inactivation of the circular smooth muscle plasma membrane receptors.


% RESIDUAL BINDING (log10)
2.0
1.6
1.2
0.8
0.4
4
8
12
16
20
DOSE (Mrads)


SLOPE
0.12
0.08
0.04
0
PM
PK
ALD
DMP
GO
8
16
24
M.W. (Daltons) x 10−4

± 13,316 da. for the deep muscular plexus neuronal membranes and 187,308 ± 14,179 da for the circular smooth muscle plasma membranes.

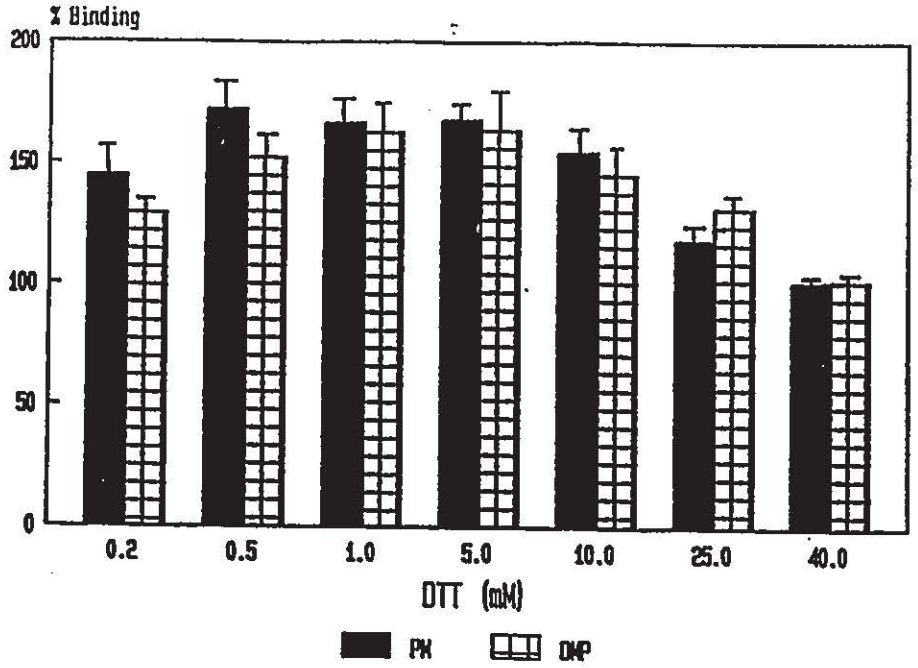
3.4.3.6. EFFECT OF SUFHYDRYL AGENTS ON THE BINDING

In order to account for the difference in the target size of the neurotensin receptors from the two sources, the possibility of the disulfide-linked subunit structures was explored. Dithiothreitol (DTT) the disulfide reducing agent augmented the binding in the case of the circular smooth muscle plasma membrane as well as to the deep muscular plexus receptors (fig. 23). The maximum effect of DTT was seen at about 1 mM DTT. The maximum enhancement of the binding was about 80% in the smooth muscle plasma membranes and 70% in deep muscular plexus neurons. Apparently, no preincubation of the membranes with DTT was required for its effect on the binding. As shown in the fig. 24, the effect of DTT was maximal within 2 min of preincubation, which was the minimum preincubation time studied.

Effects of two other sulfhydryl agents having opposing actions were also studied on the binding. Cysteine (a sulfhydryl reducing agent) and cystine (an oxidant) influenced the binding in opposite direction (fig. 25a,b). In a concentration-dependent manner cysteine enhanced the binding, while cystine decreased the binding to both the circular smooth muscle plasma membranes and the deep muscular plexus synaptosomes.

Figure 23. The effect of dithiothreitol (DTT) on the specific binding of [^{125}I]tyr^{3}-neurotensin (70-80 pM) to the circular smooth muscle plasma membranes (PM) and the deep muscular plexus synaptosomes (DMP). The results are plotted as percentage with control (in the absence of DTT) taken as 100%.

Figure 24. Time course of the stimulation of the binding of [^{125}I]tyr^{3}-neurotensin (70-80 pM) to the circular smooth muscle plasma membranes by DTT. The membranes were preincubated for indicated times with 1.0 mM DTT. At indicated times, the binding was started by the addition of 70-80 pM [^{125}I]tyr^{3}-neurotensin. Nonspecific binding was also simultaneously measured. After 25 min the reaction was terminated as usual. 0 indicates the control i.e. in the absence of any added DTT.


% Binding
200
150
100
50
0
0.2
0.5
1.0
5.0
10.0
25.0
40.0
DTT (mM)
PM
DMP

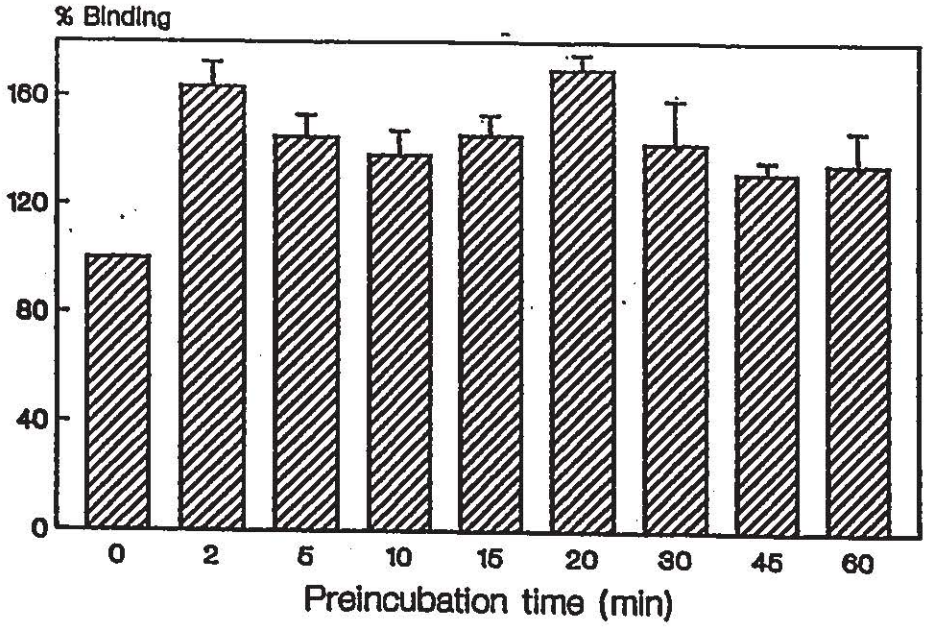
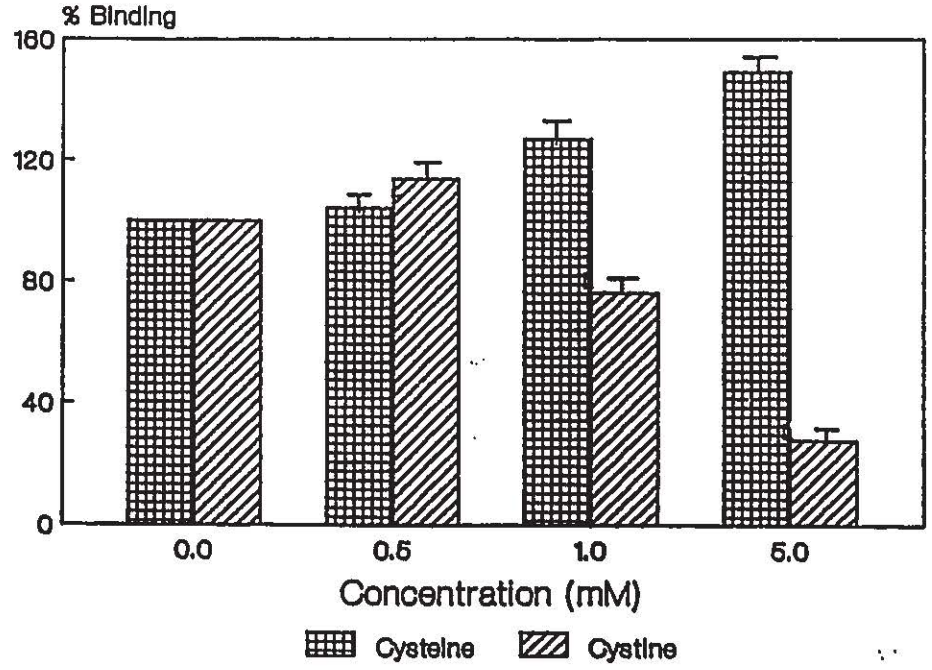
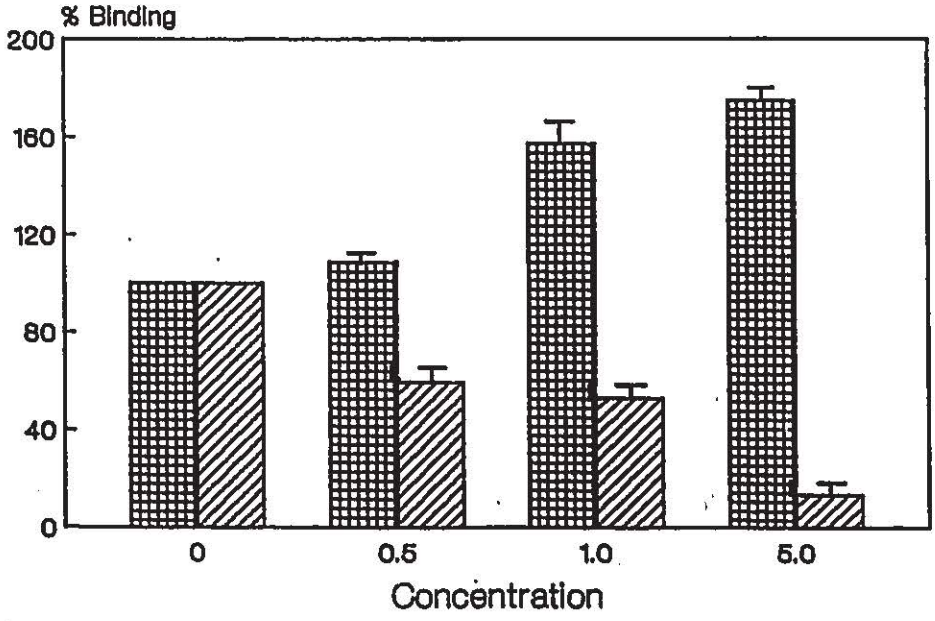

% Binding
160
120
80
40
0
0
2
5
10
15
20
30
45
60
Preincubation time (min)

Figure 25. Effects of cysteine and cystine of the specific binding of [^{125}I]tyr^{3}-neurotensin (70-80 pM) to the circular smooth muscle plasma membranes (A) and the deep muscular plexus synaptosomes (B). The reaction was carried out in the absence (control, 100%) or in the presence of indicated concentrations of the sulfhydryl agents and 70-80 pM [^{125}I]tyr^{3}-neurotensin. The reaction was carried out as usual as described in the Methods.

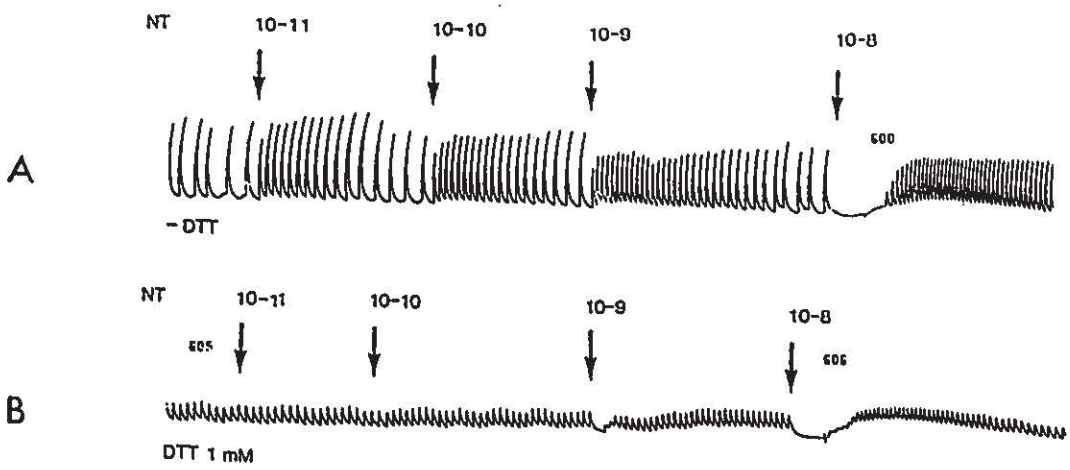

% Binding
160
120
80
40
0
0.0
0.5
1.0
5.0
Concentration (mM)
Cysteine
Cystine

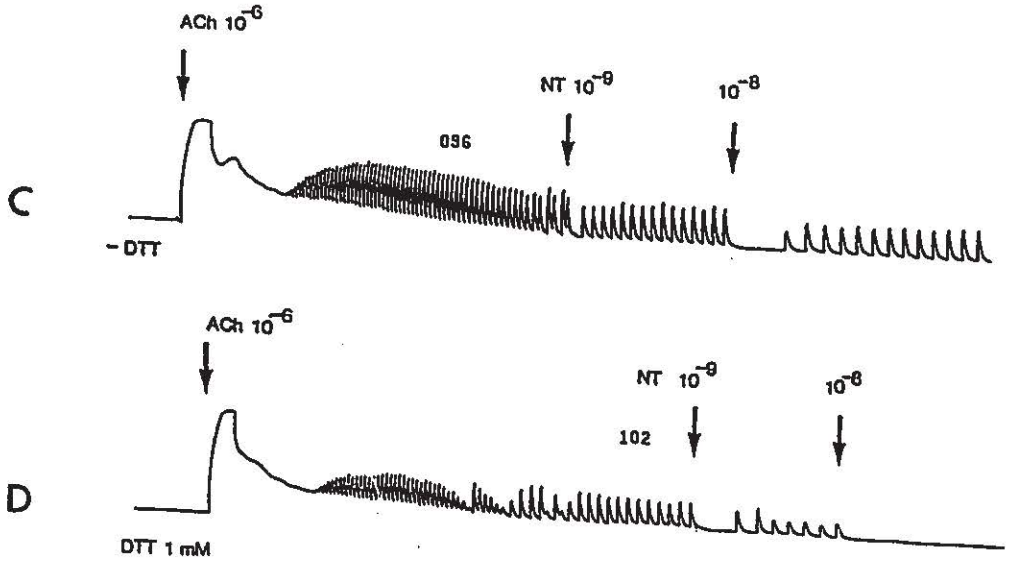

% Binding
200
160
120
80
40
0
0
0.5
1.0
5.0
Concentration

3.4.3.7. EFFECT OF DTT ON THE *in vitro* RESPONSES TO NEUROTENSIN

Neurotensin *in vitro* stimulated the circular muscle of canine small intestine at lower doses (10^{-12} - 10^{-8} M). At doses higher than 10^{-8} M, it first inhibited and then caused phasic and tonic contractions (fig. 26A; and Fox,1987). DTT (1 mM) prompted phasic and tonic contraction of the muscle which diminished with time. When neurotensin was added after the contractions due to DTT subsided, the contractions could not be produced by any dose of neurotensin. The inhibitory response to neurotensin was unaffected (fig. 26B). DTT inhibited only marginally, the response to acetylcholine, indicating that the drug did not cause non-specific interference with the contractile machinery of the tissue (fig. 26C & D). Neurotensin inhibited the contractile response of acetylcholine in the absence as well as in the presence of DTT.

Figure 26. *in vitro* response of the circular muscle from canine small intestine to the added neurotensin. A; the control response. B; the response to added neurotensin in the presence of 1 mM DTT. C; the control response to acetylcholine and neurotensin. D; the response to acetylcholine and neurotensin in the presence of 1 mM DTT.3.5.


A
NT
10-11
10-10
10-9
10-8
500
- DTT
B
NT
10-11
10-10
10-9
10-8
605
606
DTT 1 mM


C
ACh 10-6
NT 10-9
10-8
036
- DTT
D
ACh 10-6
NT 10-9
10-8
102
DTT 1 mM

DISCUSSION

3.5.1. LOCUS OF NEUROTENSIN RECEPTORS IN CANINE SMALL INTESTINE

Dissection of the tissue and the extensive separation of the membranes from the canine small intestine and the study of the distribution of the binding sites in the present work revealed that neurotensin receptors are primarily located on two plexuses i.e. the deep muscular and the submucous plexus and on the circular smooth muscle plasma membrane. The membranes from the myenteric plexus and the longitudinal smooth muscle did not demonstrate any significant amount of binding.

The highest neurotensin binding activity was observed in the gradient fractions of Mit I. The fraction P4, which is highest in the saxitoxin binding and the VIP-immunoreactivity bound more than 19 fmol/mg of neurotensin at approximately 0.1 nM radioligand concentration. The fraction P2, which was low in saxitoxin binding and the VIP-immunoreactivity bound about 11 fmol/mg of protein. The fraction P3 also had very high levels of the binding probably due to the presence of both neuronal and smooth muscle membranes which is suggested by the relatively high 5'-nucleotidase activity and high saxitoxin binding as well as high VIP-immunoreactivity.

In the gradient fractions of the purified microsomal fraction Mic II, highest binding of neurotensin was obtained at the sucrose interface of 14-33%

(fraction M2). The fraction M2 was highly purified plasma membrane fraction from the circular smooth muscle with minimal saxitoxin binding and negligible VIP-immunoreactivity but more than 20 fold enriched in the smooth muscle marker enzyme 5'-nucleotidase. Therefore it was concluded that the binding observed in the fraction M2 was to the circular smooth muscle plasma membranes.

In the fractions obtained upon the differential centrifugation of the submucous plexus, the peak of the neurotensin binding was obtained in the fraction P2. Neurotensin binding in the fraction P2 was enriched by about 15 fold; a similar purification was obtained for the saxitoxin binding, therefore, it was concluded that submucous plexus also has neurotensin receptors. The purified microsomal fraction Mic II had minimal neurotensin binding although saxitoxin binding in this fraction was approximately 3 fold higher compared to the PNS. The reason for this lack of correspondence is unclear. It is possible however, that in the submucosa, in addition to the neuronal membranes, some other membranes (glial cells) also have sodium channels, which are being copurified with the smooth muscle membranes. These smooth muscle membranes may arise from the remnants of the muscularis mucosa or the blood vessels. We did not try to further characterize these membranes.

We consistently failed to detect any neurotensin binding to either the longitudinal smooth muscle plasma membranes or the membranes from myenteric plexus. Guinea pig ileal longitudinal muscle/myenteric plexus strips have been used

in a number of studies and neurotensin was shown to contract these strips by acting on cholinergic nerves (Kitabgi,1979b). Therefore, in guinea pig ileum at least, a portion of the response appeared to be mediated through the action of the peptide on the myenteric neurons. Electrophysiological studies with single myenteric neurons from the guinea pig ileum have shown that neurotensin, in nanomolar range of concentration, stimulated 50% of these neurons (William,1979). In rat small intestine, neurotensin caused relaxation rather than the contraction, and this response was apparently through the action of neurotensin at the post junctional (smooth muscle) level (Kitabgi,1982). In precontracted longitudinal muscle strips from guinea pig ileum, neurotensin manifested a biphasic response i.e. a relaxation followed by contraction (Kitabgi,1978). Due to the selective inhibition of contractile response by TTX, the relaxatory phase of the response was suggested to be due to the post junctional effect of the peptide (Kitabgi,1978). Rat stomach longitudinal muscle strips, however, contracted to the added neurotensin with an EC_{50} of 3 to 11 nM; an action on the smooth muscle was proposed (Kitabgi, 1982). The lack of any detectable binding to the membranes either from the longitudinal smooth muscle or the myenteric plexus in the present studies was a cause of concern. Since the longitudinal muscle/myenteric plexus preparation was processed according to the method of Kostka *et. al.*. (1987) in the imidazole buffer instead of the sucrose MOPS buffer, the possibility of imidazole buffer inhibiting the neurotensin binding was investigated. Imidazole buffer added to the incubation

media for the binding to the circular smooth muscle fraction did not inhibit the binding to this fraction. The next possibility was that, perhaps, there was a rapid degradation of the added neurotensin by the longitudinal muscle/myenteric plexus fractions. To investigate the possibility of degradation of added neurotensin the fraction from the deep muscular plexus was contaminated by the synaptosomal fraction from the myenteric plexus. If the myenteric plexus synaptosomal fraction did degrade added neurotensin fast enough to account for the lack of detectable binding there, one would have expected a decrease in the binding to the deep muscular plexus fraction due to the presence of the contaminating membranes from the myenteric plexus. Since no such decrease in the binding was observed, the possibility of rapid degradation of neurotensin by the longitudinal smooth muscle or the myenteric plexus membranes can be ruled out. We have not exhausted all the possibilities explaining the lack of the detectable binding in the longitudinal muscle/myenteric plexus fraction. One explanation can be that the receptors of these membranes are extremely labile such that they get inactivated upon the homogenization and fractionation procedure. If this is the case, then the neurotensin receptors on these membranes will have to be very different from the ones studied in any other system. The other explanation can be the species differences in the locus of neurotensin receptors, which would not be very surprising since interspecies differences in responses to neurotensin have been observed (see introduction).

3.5.2. PROPERTIES OF NEUROTENSIN RECEPTORS IN CANINE SMALL INTESTINE

3.5.2.1. BINDING PARAMETERS

A summary of the binding parameters obtained in miscellaneous studies on neurotensin receptors in a number of tissues is summarized in the table XI. As is explicit from the table, a whole range of the value for the affinity constant and the number of binding sites has been obtained. This may be an indication of the tissue and species difference, the difference in the reaction conditions employed and whether the neurotensin used for the binding was tritium-labelled, iodine-labelled and the position of the iodination in the neurotensin molecule (see the Introduction section).

In the present studies, at least three classes or the states of neurotensin receptors were identified. The high affinity receptors on the circular smooth muscle and the deep muscular plexus were of the similar affinity with Kd value in the subnanomolar range (0.1 - 0.2 nM). A class of site with an intermediate affinity was recognized in the circular smooth muscle plasma membranes (Kd 3 nM). A low affinity class (Kd 40 nM) of sites was also recognized in the deep muscular plexus membranes. Such a class of low affinity neurotensin binding sites has never been identified in any tissue except in the rat mast cells where only one class of site with a Kd value of 154 nM were identified (Lazarus,1977). However,

TABLE XI

A SUMMARY OF NEUROTENSIN RECEPTORS

Tissue	Kd	Bmax	Reference
Rat cerebral cortex	3 nM	3.1 pmol/g tissue	Uhl,1977
Rat uterus	0.5;9 nM	41;100 fmol/mg	Pettibone,1987
Pr.cultured neurons	0.3 nM	178 fmol/mg	Schote,1988
N1E-115	9-11 nM	200 fmol/10^6cells	Gilbert,1986
N1E-115	0.15 nM	9 fmol/mg	Poustis,1984
N1E-115	0.75 nM	45 fmol/10^6cells	Amar,1985
Rat liver	0.4;8 nM	13;122 fmol/mg	Muraki,1987
Rat brain	0.1;5 nM	17;126 fmol/mg	Mazella,1983
Rat mast cells	154 nM	1.27 pmol/mg	Lazarus,1977
Bovine brain, purified	5.5 nM	4.7 x 10^6fmol/mg	Mills,1988
Mouse brain, solubilized	0.36 nM	63 fmol/mg	Mazella,1988
Human Brain	0.3;4 nM	26;89 fmol/mg	Sadoul,1984b
Rat ant. pituitary	1.4 nM	121 fmol/mg	Memo,1986
Rat fundus	0.06; 1.96 nM	6.6; fmol/mg 11.4 fmol/mg	Kitabgi,1984
Guinea pig ileum,CM	3.4 nM	14 fmol/mg	Goedert,1984b
Guinea pig ileum, LM	4 nM	10 fmol/mg	Kitabgi,1979a

The binding parameters reported for the neurotensin receptors in various systems.

the studies in the rat mast cells were performed with chloramine T labelled neurotensin, therefore, a direct comparison of the results is not possible.

The number of both low and high affinity sites in the present studies were 4 to 6 times higher in the deep muscular plexus membranes compared to the circular smooth muscle plasma membranes. The number of the binding sites observed especially in the deep muscular plexus membranes is higher than that observed in most of the other studies (see table XI). Again an exception was the mast cell receptors (Lazarus,1977). In terms of the affinity and the density of the binding sites, the low affinity class of receptors on the deep muscular plexus resemble those on the mast cells, however, differences in the molecular organization are apparent in terms of the role of disulfide bonds in the binding activity (discussed below).

3.5.2.2. STRUCTURE-ACTIVITY RELATIONSHIP

In terms of the structurural requirements for the binding, the receptors in the canine small intestine are similar to those in other species and tissues, however, some differences are apparent. The carboxy terminal amino acid residues 8-13 were essential for the binding activity. In fact, neurotensin[8-13] was about 3 times more potent than the native neurotensin while neurotensin[1-8] and neurotensin[1-11] were inactive up to 10^{-6} M. In murine species, the recognition properties of the neurotensin receptors were slightly different than those in the

dog. In rat brain (Mazella,1983) and rat fundus (Kitabgi,1984), neurotensin and [Trp^{11}]neurotensin were of the same potency and [D-Trp^{11}]neurotensin was only slightly less potent. In human brain, [Trp^{11}]neurotensin was about 10 times less potent compared to the native neurotensin (Sadoul,1984). The canine intestinal receptors, in the present study, also like the human brain, discriminate between neurotensin, [Trp^{11}]neurotensin and [D-Trp^{11}]neurotensin. The activity of [Trp^{11}]neurotensin was about 1/10 that of the native neurotensin and that of [D-Trp^{11}]neurotensin was about 200 fold less in the canine small intestine. Functional studies of these receptors in canine small intestine show a corresponding lack of potency of [Trp^{11}]neurotensin, which was about 10 times less active than the native neurotensin (Fox, J.E.T. and Daniel, E.E., personal communication).

3.5.2.3. LACK OF EFFECT OF LEVOCABASTINE

The antihistamine, levocabastine has been reported to inhibit neurotensin binding to rat (Schote,1986; Kitabgi,1987) and mouse brain membranes (Mazella,1988). Levocabastine selectively inhibited neurotensin binding to low affinity sites without affecting the high affinity sites (Schote,1986; Kitabgi,1987; Mazella,1988). In the present studies, no effect of levocabastine was found on the neurotensin binding to either the smooth muscle plasma membrane receptors, or, to the deep muscular plexus neuronal receptors. Several other non-murine tissues are also known to be insensitive to the inhibitory effects of levocabastine

(Schote,1986; Kitabgi,1987).

3.5.2.4. TARGET SIZE OF THE NEUROTENSIN RECEPTORS

Determination of the target size by the method of the radiation inactivation revealed a difference in the neurotensin receptors in the circular smooth muscle plasma membranes and in the deep muscular plexus synaptosomal membranes. The size of the deep muscular plexus receptors obtained with this technique was similar to that obtained for the rat fundus and the rat brain neurotensin receptors using the same technique (Ahmad,1987). However, on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the neurotensin receptors from rat brain were found to be composed of two subunits of 49,000 and 51,000 dalton whereas the rat fundus neurotensin receptors migrated as a single entity of 110,000 dalton (Mazella,1985a,b). Solubilized neurotensin receptors from mouse brain also migrated with a molecular size of 100,000 dalton (Mazella,1988).

When the neurotensin receptors from the bovine brain were purified to homogeneity, a single polypeptide band at 72,000 dalton was detected under the reducing conditions (Mills,1988). Under non-reducing conditions, the apparent molecular weight was 50,000 suggesting the presence of the intramolecular disulfide bonds. These results suggested the difference in the molecular organization of the neurotensin receptors from difference sources and the presence of the disulfide

linkage at least in bovine brain neurotensin receptors was apparent. For these reasons and to investigate the possibility of the disulfide-linked dimers in the case of the circular smooth muscle plasma membrane receptors, the effect of some thiol modifying agents on the binding was studied.

3.5.2.5 ROLE OF DISULFIDE BRIDGES IN THE BINDING

The disulfide reducing agents dithiothreitol and cysteine enhanced neurotensin binding to the circular smooth muscle plasma membranes and to the membranes from the deep muscular plexus. The effect was concentration dependent and did not require any preincubation. Cystine, on the other hand decreased the binding in a concentration-dependent manner. The fact that this effect was seen to a similar extent on both the circular smooth muscle plasma membranes and the deep muscular plexus membranes precludes the possibility that disulfide-linked subunits may account for the difference in the target size of these receptors from the two sources. However, the receptors in the canine small intestine appear to be different from those on the mast cells where DTT and N-ethylmalyimide failed to influence the binding (Lazarus,1977).

The effect of DTT and other thiol modifying agents has been studied in other receptor systems such as nicotinic cholinergic receptors in mouse brain (Stitzel,1988), opioid receptors in bovine adrenal medulla (Kamikubo,1988), ß-adrenergic receptors (Moxham,1985) and histamine receptors in guinea pig ileum

(Donaldson,1987). In many of the previous studies, the thiol reducing agents decreased the binding and a preincubation with the reducing agents was required; the maximal effect was seen after 60 minutes of preincubation (Kamikubo,1988).

In the present studies, no preincubation was required for the effect of thiol reducing agents and these agents increased rather than decreased the binding. These observations suggest that the sulfhydryl groups rather than the disulfide bonds in the receptors are required for the binding activity. In the many previous studies where DTT attenuated the binding, antagonists were used as the ligands (Kamikubo,1988; Moxham,1985). In the case of the histamine receptors, where both agonist and antagonist were used, it was observed that DTT did not affect the binding of the antagonist but revealed a high affinity state of the agonist (Donaldson,1987). The lack of a proper neurotensin antagonist precludes further studies along this line. However, it is clear that the receptors in the canine small intestine are different from those on the rat mast cells where DTT failed to influence the binding, even though the binding parameters of the deep muscular plexus receptors were similar to those of the mast cell receptors.

3.5.2.6. DISULFIDE BRIDGES AND THE *in vitro* RESPONSES TO NEUROTENSIN

Pretreatment of the tissue with DTT abolished the stimulatory effect of neurotensin at the lower doses while the inhibitory response was unaffected. At

higher concentration of the neurotensin, the stimulatory response, that was suggested to be due to the action of neurotensin on mast cells (Fox,1987) was not affected. This may in fact be an action of the mast cells since the binding to the rat mast cells was not affected by DTT and other sulfhydryl agents. Reduction of the disulfide bridges has been envisaged as a mechanism of receptor activation by ligands (Moxham,1985; Sweet,1986; Wilden,1986). Sulfhydryl reagents have been reported to inhibit the response to insulin and reducing agents acted as insulinomimetic agents (Sweet,1986). Furthermore, insulin receptor/kinase activity was shown to be inhibited by N-ethylmaleimide, a sulfhydryl alkylating agent, and activated by DTT (Sweet,1986). ß-adrenergic receptor activation in the absence of agonist and even in the presence of antagonist was observed upon exposure to thiol agents capable of cleaving disulfide bridges (see Moxham,1985 and references therein). DTT caused massive stimulation of the tissue in the present study which subsided with time before neurotensin was added. Therefore, in the canine intestinal circular muscle as well, DTT may be activating diverse receptors causing contraction of the tissue. However, if DTT was activating neurotensin receptors as well, one would have expected a decrease in the affinity of these receptors for the ligand, resulting in a decrease in binding, not the increase as observed. Therefore, these receptors do not fit in the hypothesis of the disulfide exchange mechanism of the receptor activation (Moxham,1985). Further studies are required to clarify this issue.

3.5.2.7. RELATIONSHIP OF THE PRESENT STUDIES TO THE FUNCTIONAL STUDIES

The demonstration of the presence of neurotensin binding sites on the circular smooth muscle and the deep muscular plexus neurons provide an explanation for the direct (on smooth muscle) and indirect (through the action on adrenergic nerves) action of neurotensin observed *in vivo* in dogs (Sakai,1984). However, complications arise due to the difference in *in vivo* and *in vitro* responses in dogs. *In vivo*, neurotensin, given intra-arterially, at low doses inhibited field-stimulated contractile responses in the ileum (mean ED_{50} = 2.1 x 10^{-11} mol). This action was sensitive to α_2-adrenergic blockers and to reserpine and was perhaps due to an action on the adrenergic nerves. At higher doses (10^{-10} - 10^{-9} mol) other neurotensin receptors, likely on the smooth muscle (TTX-insensitive) appeared to be activated, again causing inhibition of the contraction (Sakai,1984, Fox,1987). Excitation of the contraction of ileum, either in quiescence or field-stimulated, could not be demonstrated *in vivo*. However, neurotensin produced a delayed potentiation of the response to exogenous acetylcholine (Fox,1987). *In vivo*, neurotensin may act to increase VIP release (J.E.T. Fox and E.E. Daniel, personal communication) and this may contribute to the inhibition in that circumstance. Also after TTX, VIP release is abolished so any residual effect is direct. This might reveal an excitatory effect never seen *in vivo* or seen only after a delay.

In vitro, in lower doses (10^{-12} - 10^{-10} M), neurotensin caused an increase the frequency and amplitude of the phasic contraction. At higher doses (10^{-9} - 10^{-7} M), an initial inhibition, followed by a tonic and phasic contractions was observed (Fox,1987). The absence of the inhibition of the contractions at lower concentrations may be because the neuronal circuitry involved for the release of VIP is lost under *in vitro* conditions. All the *in vitro* responses were unaffected by tetrodotoxin in concentrations sufficient to block field-stimulated nerve responses. If TTX-insensitive responses are to be taken as the direct response on smooth muscle, *in vitro* demonstration of high affinity neurotensin receptors was possible. In electrophysiological studies in the sucrose gap, neurotensin (10^{-11} - 10^{-7} M) caused hyperpolarization of the circular smooth muscle from canine ileum (Christinck,1989) by activating apamin-sensitive K^+-channels, apparently by releasing intracellular calcium. Contractions, which required calcium entry through dihydropyridine-sensitive calcium channels, occurred during the recovery phase of the hyperpolarization. These responses were TTX-insensitive and may reflect a direct action on the smooth muscle (Christinck,1989). In the present studies, at least three sets (or states) of neurotensin receptors were observed. High affinity receptors on the smooth muscle as well as on the deep muscular plexus membranes with affinity in subnanomolar range (Kd = 0.1 - 0.2 nM). A low affinity class of the receptors on the smooth muscle (Kd = ~3 nM), and a much lower affinity type on the deep muscular plexus neuronal membranes with the affinity of ~40 nM. *In vivo*,

the high affinity neuronal receptors (mediating release of noradrenalin and VIP etc.) may be dominant. *In vitro*, since the neuronal circuitries are lost, the high affinity receptors on the smooth muscle are able to produce contractile response; at high concentrations, the low affinity receptors on the smooth muscles also appear to be activated increasing the calcium activated potassium conductance, hyperpolarization of the tissue, and the resulting inhibition of the response. However, we can not describe the functional significance of the low affinity receptors (Kd ~40 nM) on the deep muscular plexus.

3.5.2.8. NEUROTENSIN; A NEUROTRANSMITTER, HORMONE, OR A PARACRINE AGENT

The cellular localization of neurotensin-like immunoreactivity (NT-LI) has already been discussed in the Introduction section. Majority of NT-LI is present in the mucosa of the gut. Some studies, however, described its presence in enteric nerves (Langer,1979; Schulzberg,1980; Reinecke,1983; Doyle,1985; Tange,1983), while others (Sundler,1977; Orci,1976; Daniel,1985) failed to register any immunoreactivity in neurons. The reason for this discrepancy is unclear and may reflect the use of different antibodies against different segment of the neurotensin molecule such that some antibodies would also recognize some neurotensin-related peptides (i.e. neuromedin-N), while others will not. The presence of neurotensin in the enteric nerves, therefore, is still questionable. Therefore, it will be premature

to comment on the role of neurotensin as a neurotransmitter in the gastrointestinal tract.

High levels of NT-LI have also been measured in the plasma (Hammer,1982; Pederson,1986,1988; Ferris,1985). The plasma levels of NT-LI increase in response to food (especially lipids), duodenal acidification and other peptides such as bombesin and gastrin releasing peptide (Rökaeus,1982; Mogard,1986). Experiments with eviscerated rats have demonstrated that a majority of plasma NT-LI is of the gut origin (Ferris,1981). Furthermore, indirect evidence, by measurement of NT-LI in mesenteric and femoral veins suggest that the intestine is the primary source of elevated levels of NT-LI in the plasma (Ferris,1981). Therefore, it would seem a plausible explanation that the luminal presence of food, lipid etc. stimulate the mucosal N-cells to release neurotensin on the abluminal side. Barber *et. al.*. (1986) have demonstrated the regulation of NT-LI release from the enteric primary cell cultures by ß-adrenergic agents and forskolin (causing stimulation) and by carbachol and somatostatin (causing inhibition of the release). The action of the lipids on the release on NT-LI, however, was not studied.

The stimulus-released NT-LI, can then either enter the blood stream and be circulated to the target organs and therefore act as an hormone, or it can diffuse to nearby target cells (eg. circular smooth muscle cells, deep muscular plexus or the submucous plexus neurons) and act in a paracrine fashion. The local

spread of a mediator and, therefore, the paracrine function is extremely difficult to assess as such, therefore, it will be discussed in the light of the possible hormonal action of neurotensin.

Bulk of the NT-LI in the plasma is due to the inactive metabolites of neurotensin i.e. NT[1-8] and NT[1-11] (Hammer,1982) and probably reflects the proteolytic degradation of the peptide. Hammer *et. al.*. (1982) calculated the percentage of the NT metabolites in peripheral plasma of human volunteers and concluded that 72 to 84% of the total identified NT was represented by these metabolites. The identification of the NT metabolites, rather than NT as the major component of the total NT-LI in the plasma has been the major criticism against the possibility of NT acting against as an hormone (Ferris,1981). However, The metabolites in the plasma may have resulted from the degradation of NT after it had functioned as an hormone. The elevated concentration of neurotensin or its active fragments measured (by C-terminal directed antibodies) in the blood of dog (Mogard,1986), human (Hammer,1982) or rats (Ferris,1981) was in the range of 0.01 - 0.1 nM. An assumption that the plasma levels are the close approximation of the concentration at the target tissue, would lead to the conclusion that hormonal neurotensin acts at the high affinity receptor sites at the circular smooth muscle, deep muscular plexus and the submucous plexus to inhibit the intestinal transit and acid secretion, in addition to other central and peripheral actions.

Alternatively, the presence of neurotensin and its metabolic products in

the plasma may represent the removal of excess peptide from the local site of action, suggesting the paracrine mode of action of the peptide. If such is the case, then the local concentrations of the peptide are more likely to reach to higher levels, making it possible for the low affinity receptors to be activated as well. Physiologically, it is also possible that only the low affinity binding sites are functional, since at the physiological ionic concentrations the high affinity binding may be completely suppressed.

However, such high levels of NT-LI is plasma do not favour the suggestion that NT acts exclusively in a paracrine fashion. It is highly likely, therefore, that the neurotensin acts as an hormone and a paracrine agent, and possibly as a neurotransmitter. If the role of neurotensin as an enteric neurotransmitter has to be advocated, its presence in the enteric neurons must be established. Isolated synaptosomes from all three major plexuses now provide a unique opportunity for such a study.

In summary, this study has demonstrated the binding sites for neurotensin in the canine small intestine. The binding sites were localized to the circular smooth muscle, the deep muscular plexus and the submucous plexus. No binding to either the longitudinal smooth muscle or the myenteric plexus could be detected. The binding sites on the circular smooth muscle and the deep muscular plexus demonstrated some unique properties in terms of the binding parameters. Reduced sulfhydryl groups were required for the binding to the receptors on both

the circular smooth muscle as well as to the deep muscular plexus.

CHAPTER 4

OPIOID RECEPTORS

4.1. INTRODUCTION

Several ancient cultures have been aware that extracts of opium poppies have analgesic or euphoric effects. Even in the modern age, these compounds are being both used and abused for these purposes. The pharmacological actions include addiction liability and respiratory depression and have long been a matter of concern. The actions of these compounds on the function of the gastrointestinal tract, to inhibit transit of the gut contents, have also been known for long and much research has had the goal to understand the mechanism of action of these compounds known as opioids. A related therapeutic goal has been to obtain opioid related compounds which have antidiarrheal and no addicting action.

There is a wide array of opioid compounds e.g. the plant alkaloids morphine, codeine and heroin etc. The term opioid will be used in the present chapter to encompass the alkaloid opioids, endogenous opioid peptides and other exogenous opioid compounds such as morphiceptin and ß-casomorphin etc.

4.1.1. ENDOGENOUS OPIOID PEPTIDES

The endogenous opioid peptides primarily originate from three major precursor proteins, *viz.* pro-opiomelanocortin, pro-enkephalin and pro-dynorphin. These three precursor proteins are of similar molecular size and are composed of approximately 260 amino acids (Hender,1987). These precursors are biologically

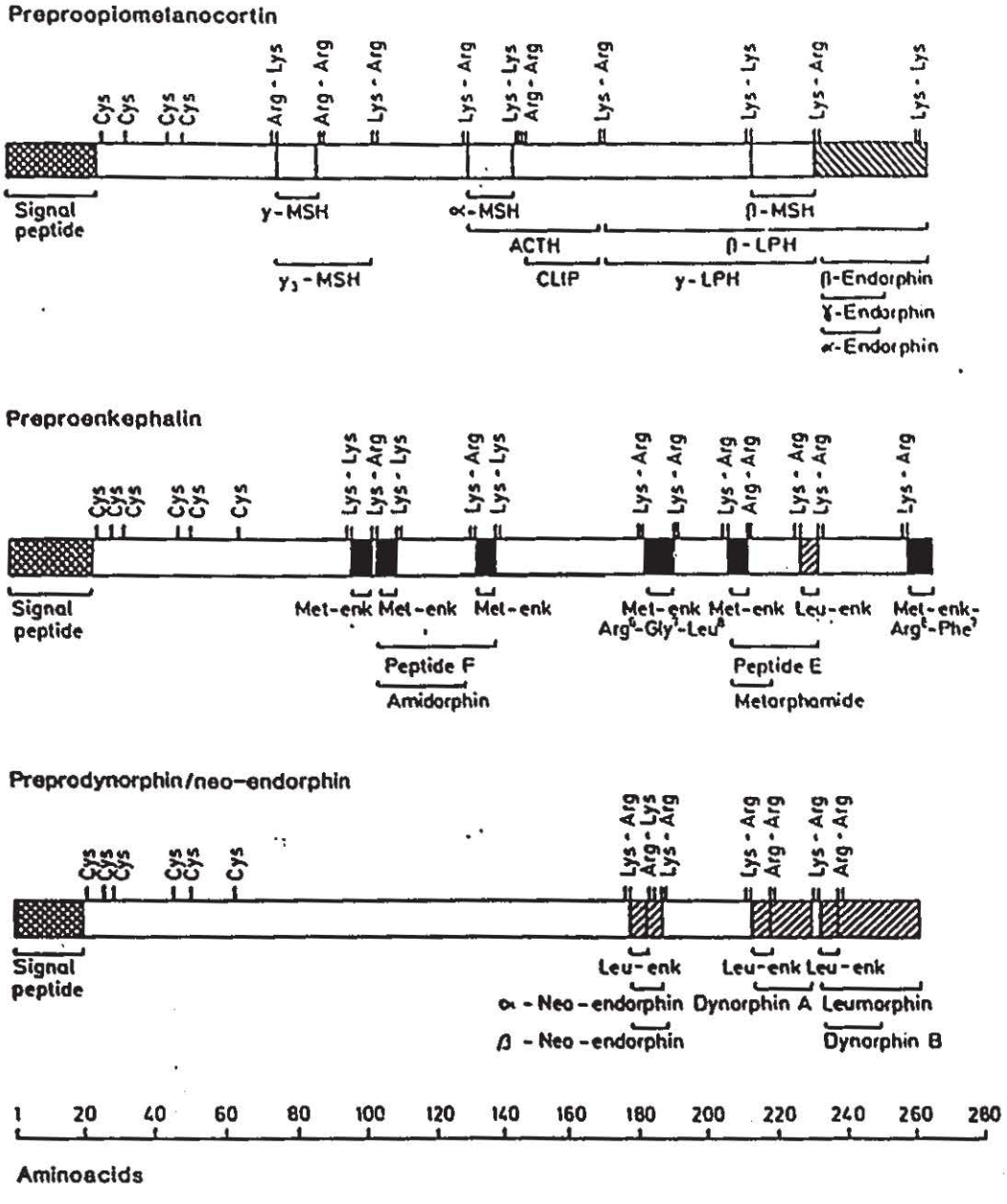
inactive and are subject to proteolytic cleavage at the dibasic amino acid sites (Hender,1987). However, single arginine cleavage also appears to be involved in the generation of some active peptides such as metorphamide from pro-enkephalin, dynorphin 1-8 from pro-dynorphin and dynorphin B 1-13 from dynorphin B-29 (Rapaka,1986).

The precursor pro-opiomelanocortin is a multifunctional protein which contains sequences for ß-endorphin and non-opioid proteins adreno-corticotrophic hormone (ACTH) and melanocyte-stimulating hormone (MSH). Proenkephalin contains met- and leu-enkephalins in a 4:1 ratio, while prodynorphin contains dynorphin A and dynorphin B, which within their structures contain leu-enkephalin sequences. The structure of the precursor proteins is depicted in fig. 27.

4.1.2. DISTRIBUTION OF OPIOID PEPTIDES IN THE GASTROINTESTINAL TRACT

Within the gastrointestinal tract of several species including human, opioid peptides have been localized by immunohistochemical and radioimmunological techniques (Furness,1987). Shultzberg at al. (1980) studied the detailed distribution of opioid-containing nerves throughout the gastrointestinal tract of rat and guinea pig. Enkephalin-like immunoreactivity was observed in all parts of the gastrointestinal tract examined, both in the cell bodies and in the fibres (Schultzberg,1980). Enkephalin-immunoreactive cells were abundant in the myenteric plexus and within the circular muscle while submucous plexus (especially

Figure 27. Opioid precursor proteins with indicated structures present within. Reproduced from Rapaka,1986.


Preproopiomelanocortin
Cys Cys Cys Cys
Arg - Lys
Arg - Arg
Lys - Arg
Lys - Arg
Lys - Lys
Arg - Arg
Lys - Arg
Lys - Lys
Lys - Arg
Lys - Lys
Signal peptide
γ-MSH
α-MSH
β-MSH
ACTH
β-LPH
γ3-MSH
CLIP
γ-LPH
β-Endorphin
γ-Endorphin
α-Endorphin
Preproenkephalin
Cys Cys Cys Cys Cys Cys
Lys - Lys
Lys - Arg
Lys - Lys
Lys - Arg
Lys - Lys
Lys - Arg
Lys - Arg
Lys - Arg
Arg - Arg
Lys - Arg
Lys - Arg
Lys - Arg
Signal peptide
Met-enk
Met-enk
Met-enk
Met-enk
Arg6-Gly7-Leu8
Met-enk
Leu-enk
Met-enk-
Arg6-Phe7
Peptide F
Amidorphin
Peptide E
Metorphamide
Preprodynorphin/neo-endorphin
Cys Cys Cys Cys Cys
Lys - Arg
Arg - Lys
Lys - Arg
Lys - Arg
Arg - Arg
Lys - Arg
Arg - Arg
Signal peptide
Leu-enk
Leu-enk
Leu-enk
α-Neo-endorphin
Dynorphin A
Leumorphin
β-Neo-endorphin
Dynorphin B
1 20 40 60 80 100 120 140 160 180 200 220 240 260 280
Aminoacids

in rat), muscularis mucosa and mucosa contained fewer fibres (Schultzberg,1980). Linnoila and DiAugustine (1978) examined the contents of met^5- and leu^5-enkephalin in the gastrointestinal tract of guinea pigs and rats. The highest amount of met^5-immunoreactive material was obtained from the extracts of duodenum of both rats and guinea pig. Caecum had the lowest content of both met^5- and leu^5-enkephalin immunoreactive material. Generally, the amount of leu^5-enkephalin was 5-10 times less than that of met^5-enkephalin immunoreactivity. In the guinea pig ileum and duodenum, most of the enkephalin-immunoreactive nerve terminals were observed in the submucosal plexus and in the circular muscle layer, while myenteric plexus stained much less (Linnoila,1978). However, no cell bodies could be demonstrated using antisera to ACTH, α-MSH or [met^5]- or [leu^5]-enkephalin in any part of the guinea pig gastrointestinal tract in this particular study (Linnoila,1978).

Furness *et. al.*. (1980,1983) observed enkephalin-immunoreactive cell bodies in myenteric ganglia of guinea pig ileum. Processes were observed in the circular muscle (including deep muscular plexus) and in submucosa in addition to the myenteric plexus. No fibres were observed in the mucosa.

Bu'lock *et. al.*. (1983) also observed a dense population of nerve cell bodies immunoreactive to met^5-enkephalin and its c-terminally extended variant met^5-enkephalin Arg^{6}Phe^7 in the myenteric plexus of guinea pig, rat and mouse. Occasional cells immunoreactive to the antisera were seen in the submucous plexus.

In addition, in rat and mouse, antisera to met^5-enkephalin Arg^{6}Phe^7, but not to met^5-enkephalin or met^5-enk Arg^6 also stained the gastrin containing cells of antral mucosa. Immunoreactive fibres and endocrine cells were sparse in the mucosal layer of other parts.

In tissue sections, Jessen *et. al.*. (1980) also observed enkephalin-immunoreactive nerves in the myenteric plexus and the circular muscle (including the deep muscular plexus) of the guinea pig ileum. In the guinea pig caecum, fibres in the longitudinal muscle layer also were immunoreactive. Enkephalin-immunoreactive neurons were absent from the submucous plexus.

Sosa *et. al.* (1977) observed incorporation of labelled amino acids into met^5- and leu^5-enkephalin, demonstrating the biosynthesis of opioid peptides within the enteric nerves. Met^5-enkephalin-immunoreactive fibres were also observed in the myenteric plexus and in circular muscle layer of rat intestinal tissue transplants (Schultzberg,1980) and fetal mouse intestinal tissue cultures (Schultzberg,1978), both devoid of the extrinsic neuronal connections.

In the canine intestine, Tange *et. al.*. (1983) and Daniel *et. al.*. (1985) studied the distribution of opioid containing nerves, using antisera that did not distinguish between enkephalins and dynorphin. Some major differences in the distribution of the opioid-immunoreactive nerves were observed in canine intestine from those of guinea pig or rats. Most of the studies on guinea pig and rat gastrointestinal tract have observed highest density of enkephalinergic nerves in the

myenteric plexus and in the circular muscle layer. Very low, or no enkephalin-immunoreactive fibres have been identified in the submucous plexus with the exception of one study, where nerve terminals but no nerve cell bodies to immunoreactive to enkephalin were identified (Linnoila,1978). In canine small intestine, enkephalin-immunoreactive fibres appear to be abundant in the submucous plexus, and extensive enkephalinergic innervation of muscularis mucosa and occasionally glands was also apparent (Daniel,1985). These differences were observed using the same antibody and immunohistochemical procedures that had been used previously in the guinea pig (Daniel,1985).

The presence of enkephalin-like material has also been demonstrated in the endocrine cells of the gastrointestinal tract. Polak *et. al.*. (1977) observed human antral G-cells stained with antibodies to met^5-enkephalin. Similar observations were also made by other workers (Ito,1979; Larsson,1981; Tanaka,1982) in several species.

Not much effort has been devoted to study the distribution of endorphin in the gastrointestinal tract. The available studies report conflicting results. Opmeer *et. al.* (1980) extracted endorphin from the guinea pig intestine, while Hughes (1977) failed to detect it in the same tissue. Leander *et. al.* (1984) and Wolter (1984) localised ß-endorphin immunoreactivity in the myenteric and submucous plexus neurons of guinea pig ileum and rat duodenum, but Furness *et. al.* (1987) could not detect ß-endorphin immunoreactivity in the guinea pig ileal

enteric nerves (Furness,1987). In the mucosa and the external muscle layer of the human antrum and pylorus, Tari *et. al.*. (1985) detected ß-endorphin by radioimmunoassay.

In summary, several studies have demonstrated the presence of enkephalin-like immunoreactivities in the nerve cell bodies and fibres of the gastrointestinal tract of several species, and also in the endocrine cells of antral mucosa. Some major species differences have been observed suggesting some possible differences in the action of these enkephalinergic nerves.

4.1.3. OPIOID RECEPTORS IN GASTROINTESTINAL TRACT

The presence of opioid receptors in the gastrointestinal tract using the ligand binding approach was first suggested by Terenius (1972) who reported the "specific uptake" of [^{3}H] labelled dihydromorphine by a crude membrane fraction from the guinea-pig ileum longitudinal muscle (perhaps also containing myenteric plexus membranes) preparation. With a refinement of binding techniques, better characterization followed. Pert and Snyder (1973), using crude membrane preparation, reported the presence of opioid receptors in guinea-pig ileum myenteric plexus but no binding to the longitudinal muscle was detected. In 1975, Terenius (1975) and Creese and Snyder (1975) separately reported the detailed characterization of opioid receptors in guinea-pig ileum longitudinal muscle/myenteric plexus. Good correlation was found between receptor binding and

pharmacological potency of both agonists and antagonists (Creese,1975). Since then, specific opioid binding has been reported in several gastrointestinal systems including parietal cells from guinea-pig ileum (Kromer,1983) and rat small intestine (Monferini,1981). No binding, however, was found on rat intestinal epithelial cells (Gaginella,1983). Opioid receptors have even been demonstrated in the midgut of an insect *Leucophaea moderae* (Stefano,1982).

In autoradiographic studies, binding was detected only in the mucosa, in the fibres within the circular muscles and submucous plexus of rat stomach (Nishimura,1984) or in villi and crypts of rat small intestine (Dashwood,1985). Nishimura (1986) in his autoradiographic studies on rats and guinea-pig reported the presence of both μ and δ receptors in the circular muscle and the muscularis mucosa of gastric fundus. In rat ileum and duodenum, no binding sites could be detected in the muscle layers while in guinea-pigs only μ-receptors were diffusely present over muscular layers (1986). However, owing to the limited resolution of light microscopic studies, the binding sites detected in muscular layers could be present on the axons/varicosities of myenteric, deep muscular or submucous plexus. Autoradiographic studies with high resolution at the electron microscopic level need to be performed to resolve this issue. So far ligand binding studies have presented no convincing evidence suggesting the presence of opioid receptors on any muscle layer of the gastrointestinal tract. Most of the opioid receptors found were primarily present on neuronal membranes.

4.1.4. OPIOID ACTION ON THE GASTROINTESTINAL TRACT

4.1.4.1. ACTIONS ON MOTILITY

The inhibition of the electrically induced contractions of the myenteric plexus longitudinal muscle preparation of the guinea pig ileum *in vitro* has been a valuable tool to study opioid pharmacology and opioid receptors (see Daniel,1989). Exogenous application of morphine (Paton,1957) and enkephalins (Vizi,1985) inhibited the release of acetylcholine from the guinea pig ileal myenteric plexus neurons. Release of many other neurotransmitters has also been shown to be inhibited by opioids, these include substance P (Gintzer,1982), serotonin (Gintzer,1979) and VIP (Manaka,1988).

In the guinea pig ileum preparation, opioids caused inhibition of the field-stimulated release of acetylcholine (Paton,1957) and at higher concentration they also inhibited the release caused by the prostacyclin PGI_2 (Gaion,1984), substance P and neurotensin (Yau,1986). k-selective ligands are more potent in inhibiting the electrically-induced contractions than the μ- or δ-selective opioids (Yoshimura,1982; Takemori,1986; also see Daniel,1989). In the guinea pig ileum, dynorphin was 600-800 times more potent than either met^5- or leu^5-enkephalin (Yoshimura,1982). Although, less degraded δ-opioids (DADLE, DSLET or DAME) had similar potency as μ-agonist such as morphine and buprenorphine (Takemori,1986), naloxone was found to be equipotent (Ki 3-5 nM) for μ and δ

agonist (Yoshimura,1982), suggesting that δ-selective opioids were acting on the μ-receptors. Further evidence for this came from the cross-tolerance studies in which when the ileum of the guinea pig was made tolerant to either μ- or δ-opioids, no tolerance to k-opioids developed (Schultz,1981). Conversely, little cross-tolerance to μ- and δ-agonist developed upon chronic administration of k-agonist (Schultz,1981). Furthermore, there appeared to be complete cross-tolerance between μ- and δ-agonists. Microelectrode studies also demonstrated a similar pattern of cross-tolerance and have further demonstrated that the μ- and k-subtypes of opioid receptors can exist on a single neuron (Karras,1981).

The inhibition of the acetylcholine release by μ-opioid and k-opioid receptors involves different mechanisms. μ-opioid receptors inhibit the neurotransmitter release by increasing the Ca^{2+}-dependent K^{+}-conductance by releasing intracellular calcium (Cherubini,1985). In submucous plexus, the δ-agonist Tyr-D-Pen-Gly-Phe-D-Pen also caused a strong hyperpolarization by increasing the conductance of inwardly rectifying potassium conductance (North,1987). The k-opioid receptor agonist appeared to involve a different mechanism for the depression of the neurotransmitter release. In guinea pig myenteric plexus neurons, the k-receptor agonists (dynorphin, tifluadom, U50488H) depressed the calcium conductance directly; no effect on the potassium conductance was observed (Cherubini,1985). Similar mechanism of k-opioid agonists in the mouse dorsal root ganglion neurons was observed (Macdonald,1986).

Generally, the neurotransmitter release is insensitive to the L-type of calcium channel blockers, while the blockade of N-channels very effectively blocks the neurotransmitter release in both central as well as peripheral systems (see Miller,1987). Gross *et. al.* (1987) observed that dynorphin-A selectively reduced N-type calcium channel currents in the mouse dorsal root ganglion neurons in culture.

Intra-venous or intra-arterial application of opioids caused the excitation of the canine (Plant,1926; Daniel,1959,1982; Burks,1982; Fox,1987) and human (Daniel,1959; Camilleri,1986) small intestine. Intra-arterially, μ- and δ-selective but not k-selective opioid agonists were effective in initiating the phasic and tonic contractions in canine small intestine (Daniel,1982; Fox,1987). These contractions were reduced but not abolished by atropine and were apparently partially sensitive to tetrodotoxin (TTX), suggesting that the stimulation of the opioid receptors (μ and δ), present on the cholinergic nerves, cause the excitation of the tissue by releasing acetylcholine to the postsynaptic site (Daniel,1982; Fox,1987). Since the response was apparently partially insensitive to the nerve action potential blockade by TTX, the direct excitatory action of opioids on smooth muscle was also considered possible.

When the segment of the ileum was quiescent, no excitation to the k-agonists ***in vivo*** could be elicited. However, in the phasically active tissue (by field-stimulation of cholinergic nerves, or by motilin), all opioids inhibited the tissue ***in vivo***, with k-agonists being most potent (Fox,1987). The inhibitory action

of these opioid agonists did not appear to involve the direct action on the smooth muscle since TTX abolished the inhibitory responses. Consistent with this proposition is the finding that dynorphin, in doses effective in inhibiting field-stimulated contractions, failed to inhibit the responses to submaximal intra-arterial doses of acetylcholine (Fox,1987). These findings suggest that kappa receptors inhibiting cholinergic nerve effects are present on such nerves and that μ and δ receptors may be present there as well. Alternatively, high concentrations of μ- or δ-agonists may activate k-receptors.

In vitro, field-stimulated contractions involving cholinergic nerves, of the circular muscle strips, from the canine terminal ileum were inhibited by opioids (Daniel,1982; Fox,1987), an action similar to that seen in various studies using the guinea pig ileal preparations (mentioned previously). However, in isolated perfused segment of canine small intestine, μ- and δ-agonists produced excitation of the circular muscle (Burks,1982; Manaka,1989).

Therefore, the responses of opioids in the small intestine depend on the study environment (*in vivo* or *in vitro*), the state (quiescent or active) and the species. A portion of the excitatory response in the canine small intestine definitely appeared to involve the action of opioids on nerves. All the inhibitory responses also appeared to be due to the action of opioids on nerves. The TTX-insensitive response was suggested to be due to a direct action of opioids on the smooth muscle. Therefore, the presence of opioid receptors both on the nerves and

the smooth muscle cells of the small intestine was postulated.

Further evidence supporting the postulate of opioid action on the smooth muscle came from the isolated cell system from the circular smooth muscle cells from guinea pig and human jejunum (Bitar,1985). These studies demonstrated that opioids contracted these cells with dynorphin being most potent. Furthermore, the longitudinal smooth muscle cells failed to respond to any of the opioids tested. Such an action of opioids on circular muscle cells came as a surprise, since dynorphin is mainly an inhibitory opioid and in canine small intestine, although μ- and δ-selective opioids were able to elicit contraction, excitation to dynorphin could not be elicited (Daniel,1982; Fox,1987). Furthermore, dynorphin was the most potent opioid tested in inhibiting the stimulated gut, an action that involved no direct action on smooth muscle (Daniel,1982; Fox,1987). The contraction of isolated cells by opioids may indicate species differences (although opioids are inhibitory in guinea pig ileum longitudinal muscle/myenteric plexuses), or it may be a reflection of some modification of the cellular responses when subjected to the process of digestion and isolation.

Therefore, the actual locus of the opioid action within the gastrointestinal tract is unclear. One way to clarify this uncertainty is to obtain relatively purified membranes for various smooth muscle cells and various nerves form the gastrointestinal tract and then perform comparative binding studies. Although several binding studies have been performed in the gastrointestinal tract

(mainly from guinea pig), none has addressed the question of the actual localization of the receptors within the gut in detail, except for one study by Pert and Snyder (1973). In that particular study, no opioid binding was observed on the longitudinal smooth muscle of guinea pig when this layer was dissected out from the myenteric plexus. Although, the neural action of opioids within the gastrointestinal tract may involve an action on the myenteric neurons, deep muscular neurons or on both, no attempt has ever been made to examine the distribution of opioid receptors on the deep muscular plexus. Additionally, the studies on the opioid receptor subtypes in the gastrointestinal tract have now become inadequate owing to the recent availability of ligands more selective for various subtypes of opioid receptors (Chang,1983,1984; Takemori,1986; Mosberg,1983).

4.1.4.2. ACTION ON WATER AND ION TRANSPORT

The role of opioids in water and electrolyte transport across the mucosa has, until recently, received much less attention than their role in the motility of the gastrointestinal tract. Although, the effects of opioids on the gastrointestinal transit and on gut water content have long been known, such effects were attributed secondary to the inhibition of the intestinal motility. Relatively recently it has been realized that opioids directly increase active intestinal electrolyte absorption.

Opioids enhance water and electrolyte absorption and inhibit the

secretion induced by VIP and other agents in several species such as rats, guinea pigs, rabbits and dogs (see Keast,1987). Binder *et. al.* (1985) observed that D-Ala_2-methionine-enkephalinamide (DALAMIDE) increased electrolyte absorption in rabbit ileum *in vitro*. TTX, although it did not alter the secretion induced by VIP, abolished the absorption induced by DALAMIDE. Moreover, no binding sites for [^{3}H]enkephalin were observed on the rabbit ileal enterocytes. These observations suggest a neural mechanism in the action of opioids on the water and electrolyte absorption. Fogel and Kaplan (1984) observed a decrease in the water and electrolyte absorption after intraluminal administration of opioid antagonists, naloxone and diprenorphine in rat intestine. The action of naloxone but not of diprenorphine was abolished by atropine. The action of naloxone through disinhibition of the acetylcholine release and of diprenorphine through the disinhibition of the release of some other mediator/s was proposed. No other nerve action blocker was used to confirm these findings. However, other workers failed to observe such an effect after systemic administration of opioid antagonists (Keast,1987). Dobbins *et. al.* (1980) also observed that enkephalins altered the basal absorption in rabbit ileum. The effect of enkephalins was abolished by TTX, indicating an indirect action of opioids on the water and electrolyte absorption.

The neural site of action of opioids is consistent with the electrophysiological studies in the guinea pig caecum, which demonstrated the presence of δ-opioid receptors on the submucosal neurons (Mihara,1986).

Submucosal plexus neurons have been implicated in the mucosal electrolyte transport function since no response to field stimulation could be demonstrated in the aganglionated tissue, but since TTX blocked these responses in ganglionated tissues (Andres,1985; Carey,1985). In rat colon, TTX decreased the short circuit current only when the submucosa was present, implying that *in vitro* submucosal secretomotor neurons are tonically active. The interconnections between the myenteric and the submucosal plexus suggest that these two plexuses may influence one another functionally with more nerve traffic flowing from the myenteric to the submucous plexus.

No studies have been performed to discern the biochemical properties of the submucosal plexus. Electrolyte transport studies are indicative of the presence of the opioid receptors on the submucous plexus neurons. Electrophysiological studies have demonstrated the presence of δ-subtype of opioid receptors on the submucosal neurons (in the guinea pig caecum). No such studies have been performed in the canine small intestine. Therefore, it was of interest to perform the biochemical studies on the submucosal plexus neurons to investigate the presence and the nature of opioid receptors on this plexus of neurons.

4.2. MATERIALS

The labelled compounds [^{3}H]etorphine (42 Ci/mmol) and [^{3}H]diprenorphine (36.7 Ci/mmol) were obtained from Amersham. [^{3}H]ethylketocyclazocine was from New England Nuclear. [D-Pen^2-D-Pen^5]enkephalin (DPDPE) was from Armand-Frappier, Laval, Canada. [N-Me-Phe^3-D-Pro^4]morphiceptin (PLO17) and Dynorphin-[1-13] were from Peninsula Laboratories, Belmont, CA). U-50488H {*trans*-(+)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide methanesulfonate hydrate} was a gift from Upjohn (Calamazoo, MI). Captopril was a gift from Squibb (Princeton, NJ)

4.3. METHODS

4.3.1. OPIOID BINDING ASSAYS

Binding of tritiated etorphine, diprenorphine and ethylketocyclazocine to the membrane fractions was performed in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 0.2% bovine serum albumin (BSA) and 50 μM of captopril. The reaction was carried out at 37 °C for 60° with 0.3-0.5 nM [^{3}H]diprenorphine and the separation of bound from free ligand was done by filtration using a Millipore filtration apparatus and Whatman GF/F filters (approximate pore size 0.7 μm). The filters were presoaked in the incubation buffer for at least 3 hours before use. The reaction, unless otherwise specified, was always terminated by dilution of the incubation sample (1 ml) with 3 ml of ice cold incubation buffer, and immediate filtration through the filters under reduced pressure (20 microns). The tubes and the filters were washed twice with 3 ml incubation buffer. The radioactivity on the filters was counted in a liquid scintillation counter (Beckman LS 6800). Nonspecific binding was defined as that not displaced in the presence of excess (1 μM) unlabelled etorphine. All the experiments were carried out in triplicates for total and nonspecific binding. The specific binding was defined as the difference between the mean total binding and mean nonspecific binding. No specific binding on the filters was observed *i.e.*, the background counts on the filters in the absence or in the presence of excess of unlabelled etorphine were equal.

4.3.2. PROTEIN DEPENDENCE OF LIGAND BINDING

Varying amounts of protein (0 - 200 µg per assay tube) were incubated with 0.3-0.5 nM labelled ligand in the incubation mixture. After 60 minutes the incubation mixture was diluted with cold buffer and filtered immediately as described earlier.

4.3.3. ASSOCIATION AND DISSOCIATION EXPERIMENTS

Synaptosomal membranes from DMP (20 -50 µg protein per assay tube) were incubated at 37 °C. The association was started by the addition of ^{3}H-diprenorphine. At various time intervals the reaction was stopped by dilution with 3 ml ice-cold incubation buffer and filtered. After 60 minutes of association, the dissociation was initiated by the addition of excess (1 µM) unlabelled etorphine in an additional set of tubes.

4.3.4. EQUILIBRIUM BINDING EXPERIMENTS

The membranes (20 - 50 µg protein per assay tube) were incubated with increasing concentration (0.01 - 10 nM) of the ^{3}H-diprenorphine in a total volume of 1 ml containing 50 µM Captopril at 37 °C for 60 minutes. The reaction medium was then filtered as described as above. For the saturation experiments with [^{3}H]diprenorphine an [^{3}H]ethylketocyclazocine, usually 8-10 concentrations between

0.1-10 nM were used. For the saturation experiments with [^{3}H]ethylketocyclazocine, where the shielding of the μ- and δ-receptors was desired, the incubation was carried out in the presence of 0.5 μM each of DPDPE and PLO17.

4.3.5. COMPETITION EXPERIMENTS AND EFFECTS OF VARIOUS IONS

The membranes were incubated at 37 °C in the presence of varying concentrations of the competing agents or various ions. Competition experiments for displacing ligands were performed in quadruplicates at each unlabelled ligand concentration. To determine the effects of various ions, triplicates for total and non specific binding were used at each ion concentration. When the effect of sodium (0 to 150 mM) was studied, 1 μM etorphine plus 1 μM naloxone as displacing agent were used to define non specific binding.

4.3.6. COMPUTER ANALYSIS

The saturation and competition binding data were analyzed by the computer program EBDA/LIGAND and CDATA87 and the kinetics of the binding experiments were analyzed using KINETIC program with an IBM PC/XT microcomputer (Munson,1980, McPherson,1983). Computer fitting of saturation and displacement curves were analyzed using statistical comparison (F-test) of the residual squares of the curve fitting.

4.4. RESULTS

4.4.1. DISTRIBUTION OF OPIOID BINDING

The distribution of the binding of the non-selective opioid ligand [^{3}H]diprenorphine together with the [^{3}H]saxitoxin binding, VIP-immunoreactivity and the specific activity of the smooth muscle plasma membrane marker 5'-nucleotidase,to the fractions from the circular muscle layer of the canine small intestine is illustrated in the fig. 28. It is apparent from the figure that the opioid binding demonstrated a unimodal pattern, being parallel to the binding of the neuronal marker saxitoxin and the VIP-immunoreactivity. Accordingly, the highest binding was observed in the fraction P4, which was also highest in the saxitoxin binding and the VIP-IR. The fraction M2, enriched in the smooth muscle plasma membrane marker enzyme 5'-nucleotidase, demonstrated extremely poor opioid binding; the level of binding in this fraction represented approximately 1% of that obtained in the fraction P4.

The recovery of the opioid binding sites in each of the fractions obtained upon differential centrifugation and sucrose density gradient is listed in the table XII. Among the differential centrifugation fractions, highest recovery was obtained from the fraction Mit I, which contained approximately 45% of the total binding sites (PNS taken as 100%). This value corresponds closely to the recovery of the saxitoxin binding sites in this fraction (47%, table I). Among the gradient fractions,

Figure 28. Distribution of the [^{3}H]diprenorphine (3H-DPR) binding to the fractions from the circular muscle homogenate. The distribution of the [^{3}H]saxitoxin binding (3H-STX), 5'-nucleotidase (5'-ND) and the VIP-immunoreactivity (VIP-IR) is also illustrated in the figure. The distribution experiments were generally performed with 0.3-0.5 nM [^{3}H]diprenorphine.

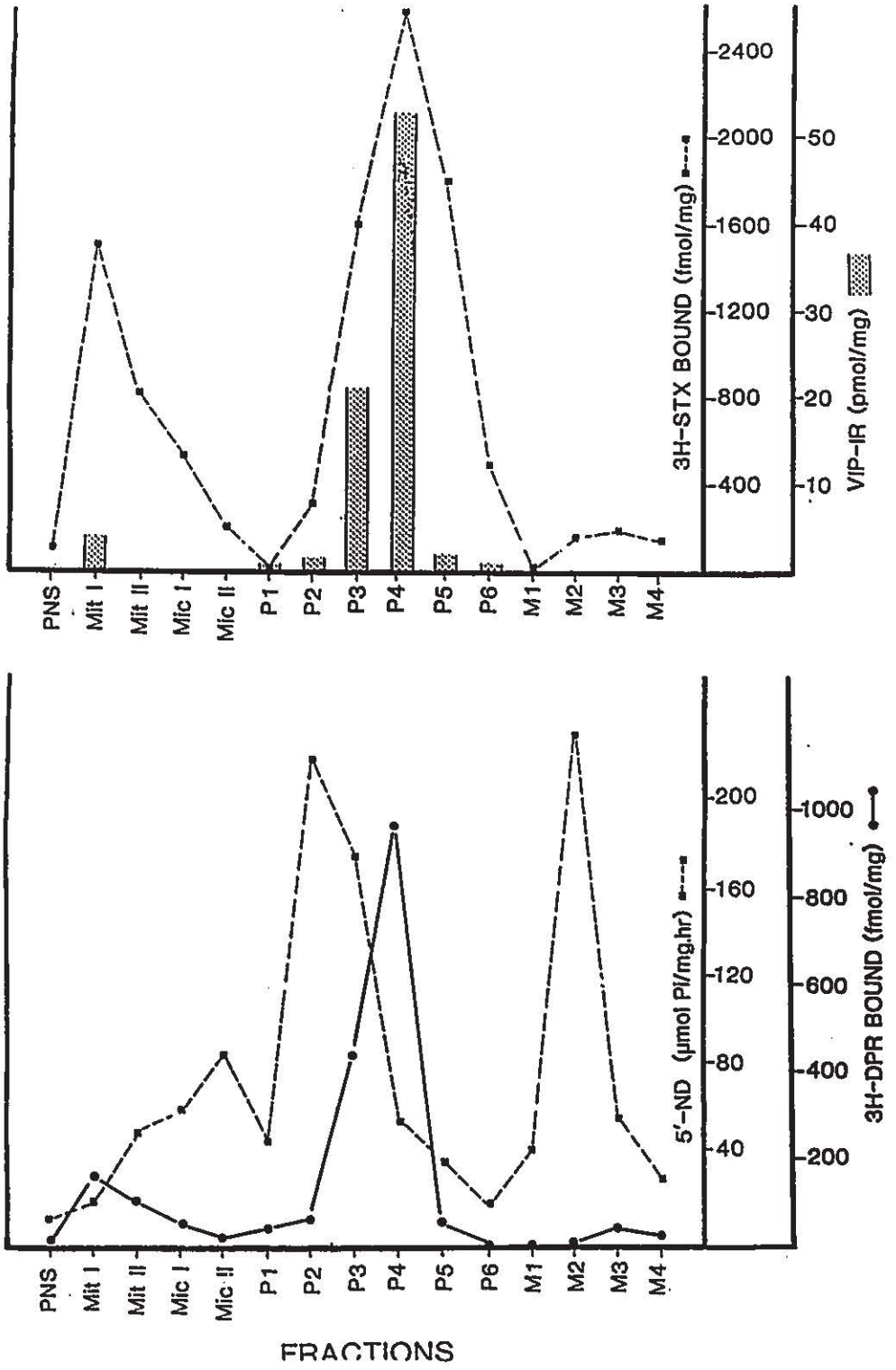

3H-STX BOUND (fmol/mg)
VIP-IR (pmol/mg)
2400
2000
1600
1200
800
400
50
40
30
20
10
PNS
Mit I
Mit II
Mic I
Mic II
P1
P2
P3
P4
P5
P6
M1
M2
M3
M4
5'-ND (µmol Pi/mg.hr)
3H-DPR BOUND (fmol/mg)
200
160
120
80
40
1000
800
600
400
200
FRACTIONS

TABLE XII

RECOVERY OF OPIOID BINDING SITES IN CIRCULAR MUSCLE FRACTIONS

FRACTIONS	% RECOVERY
PNS	100
Mit I	45.57 ± 14.6
Mit II	10.82 ± 4.11
Mic I	15.64 ± 5.71
Mic II	2.22 ± 0.85
P1	0.52 ± 0.15
P2	0.67 ± 0.30
P3	15.04 ± 4.27
P4	29.20 ± 7.59
P5	2.72 ± 1.37
P6	0.40 ± 0.10
M1	0.02 ± 0.01
M2	0.25 ± 0.07
M3	0.71 ± 0.11
M4	0.25 ± 0.18

Results are the mean ± sd from 3-5 experiments performed in triplicate. Results are experssed as the percentage recovery compared to post-nuclear supernatant (PNS) taken as 100 %.

highest recovery was observed in the fraction P4, which contained approximately 30% of the binding sites (compare with 26% recovery of saxitoxin binding sites). The smooth muscle plasma membrane enriched fraction M2 contained 0.25% opioid and 0.44% saxitoxin binding sites. The fraction P4 is enriched in the neuronal membranes from the deep muscular plexus, while the fraction M2 is the fraction enriched in the circular smooth muscle plasma membranes. These results therefore strongly suggest the presence of the opioid binding sites in the deep muscular plexus neurons and their absence from the circular smooth muscle.

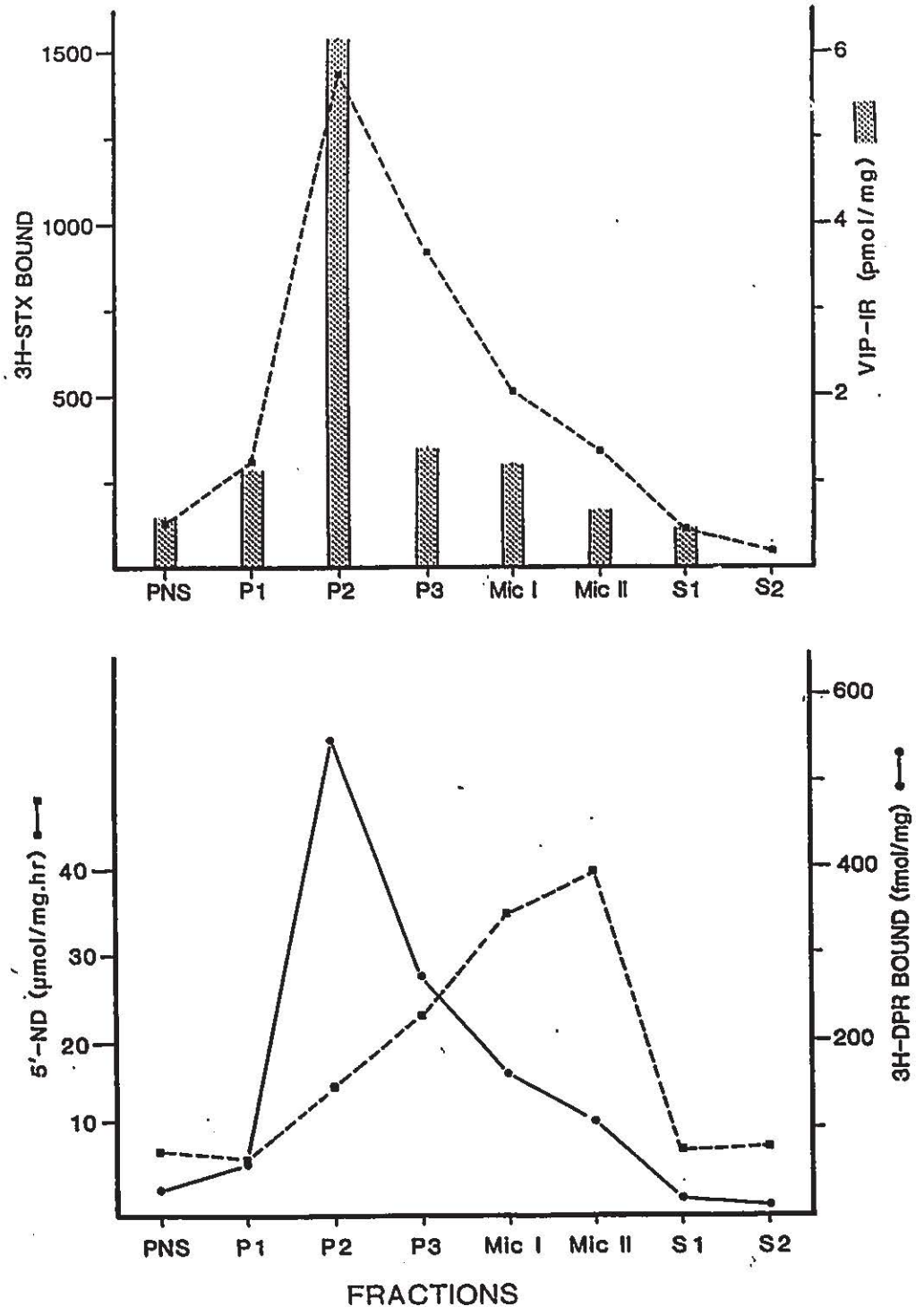
The distribution of the diprenorphine and saxitoxin binding and the specific activity of 5'-nucleotidase in the longitudinal muscle\myenteric plexus fractions is illustrated in the fig. 29. Again an excellent correlation between the saxitoxin and the opioid binding was observed. The purified synaptosomal fraction S2 contained the highest binding levels for diprenorphine and for saxitoxin. The fraction M2, enriched in the longitudinal smooth muscle plasma membrane, had very low levels of saxitoxin as well as diprenorphine binding. Therefore, in this preparation, the binding was confined to the neuronal membranes originating from the myenteric plexus.

The distribution pattern for the markers and the opioid binding to the fractions from the submucous plexus is illustrated in the fig. 30. Highest opioid binding was present in the fraction P2, which contained the highest saxitoxin binding and VIP-IR. The fraction Mic II, which was relatively enriched in the

Figure 29. Distribution of the [^{3}H]diprenorphine (0.3-0.5 nM) binding to the fractions obtained upon the centrifugation of the longitudinal smooth muscle/myenteric plexus of the canine small intestine. See legend to fig. 28 for further explanation.

3H-Diprenorphine binding (fmol/mg)
500
400
300
200
100
0
4500
4000
3500
3000
2500
2000
1500
1000
500
0
3H-Saxitoxin Binding (fmol/mg)
45
40
35
30
25
20
15
10
5
0
5'-Nucleotidase (umolPi/mg.h)
PNS
P1
P2*
S1
S2
S3
P3
Mic
II
M 1
M 11
M 2
M 21
M 3
M 4

Figure 30. Distribution of the [^{3}H]diprenorphine (0.3-0.5 nM) binding to the fractions obtained from the submucosa of the canine small intestine. For further explanation, see legend to the fig. 28.


1500
1000
500
3H-STX BOUND
6
4
2
VIP-IR (pmol/mg)
PNS
P1
P2
P3
Mic I
Mic II
S1
S2
40
30
20
10
5'-ND (μmol/mg.hr)
600
400
200
3H-DPR BOUND (fmol/mg)
PNS
P1
P2
P3
Mic I
Mic II
S1
S2
FRACTIONS

specific activity of 5'-nucleotidase, contained approximately 5 times less binding as compared to the fraction P2.

The recovery of the opioid binding in the fraction from submucous plexus is listed in the table XIII. More than 60% of the opioid binding was recovered in the fraction P2. The recovery of saxitoxin binding in this fraction was also comparable (~50%, cf.table II). The fraction Mic II contained about 15% of the opioid binding sites. Since this fraction contained about 15% of the saxitoxin binding sites, the opioid binding in this particular fraction can be ascribed to the contaminating neuronal membranes. In this preparation as well, the opioid binding sites are confined to the neuronal membranes (of submucous plexus), no binding to the smooth muscle membranes was observed.

The distribution pattern of the opioid binding in all three above mentioned preparations suggest that the opioid binding in the canine small intestine is confined to the neuronal membranes from the three plexuses i.e. the deep muscular plexus (DMP), the myenteric plexus (MP) and the submucous plexus (SMP). Under the conditions employed, the membranes from smooth muscle did not demonstrate the specific binding.

TABLE XIII

RECOVERY OF OPIOID BINDING IN SUBMUCOUS PLEXUS FRACTIONS

FRACTIONS	% RECOVERY
PNS	100
P1	8.00 ± 1.95
P2	61.26 ± 14.70
P3	15.79 ± 9.67
Mic I	32.94 ± 5.04
Mic II	15.27 ± 3.62
S1	80.78 ± 7.60
S2	34.39 ± 4.93

See legend to the table XII for explanation.

4.4.2. CHARACTERIZATION OF THE OPIOID BINDING SITES

4.4.2.1. PRELIMINARY CHARACTERIZATION

The working range of the protein concentration in the incubation for the binding was examined using the DMP synaptosomes. The result is illustrated in the fig. 31. There was linear relationship between the specific binding of [^{3}H]diprenorphine and the amount of protein used in the reaction up to 200 µg membrane protein per tube. The specific binding to the filters in the absence of membranes was negligible.

The binding of ^{3}H-diprenorphine to DMP showed a clear time dependency, reaching over 90 % of the equilibrium binding at 60 min. Fig. 32 shows a representative association- dissociation experiment of 3H-diprenorphine to DMP. The Kd value for DMP calculated from the kinetic experiments (k_1= 2.20 ± 0.86 x 10^6; k_{-1}= 1.63 ± 0.29 x 10^{-2}; n=3) was 0.09 nM.

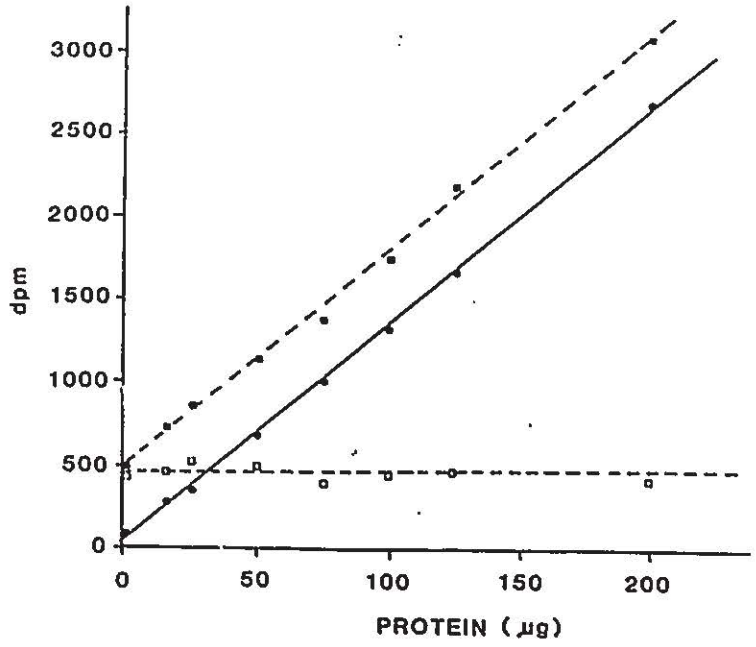
In all subsequent experiments, the protein used was within the range of usually 30 - 80 µg/tube, and the reaction was carried out for 60 min.

4.4.2.2. FURTHER CHARACTERIZATION OF THE BINDING

The concentration dependence of the binding of [^{3}H]diprenorphine to the DMP membranes is illustrated in the fig. 33. The specific binding increased with the increasing concentration of the radiolabel. Saturation was achieved at about

Figure 31. Protein binding profile. The increase in the binding with increasing concentration of the deep muscular plexus synaptosomes when incubated with 0.3 - 0.5 nM [^{3}H]diprenorphine. The filled squares represent the total binding, open squares are the nonspecific binding and the filled circles represent the specific binding.

Figure 32. The time course of association and dissociation of the [^{3}H]diprenorphine binding to the deep muscular plexus synaptosomes. The membranes were incubated with the radiolabelled ligand in the absence (for the total binding) or in the presence (for the nonspecific binding) of 1 μM unlabelled etorphine. At indicated times, the reaction was stopped by dilution and immediate filtration as described in the Methods. The arrow indicates the initiation of the dissociation by the addition of excess (1 μM) unlabelled etorphine. The values given are the values of rate constants of association, dissociation and the Kd value as computed by the Kinetic program. The figure is representative of three such experiments performed in duplicate.


3000
2500
2000
1500
1000
500
0
dpm
0
50
100
150
200
PROTEIN (µg)

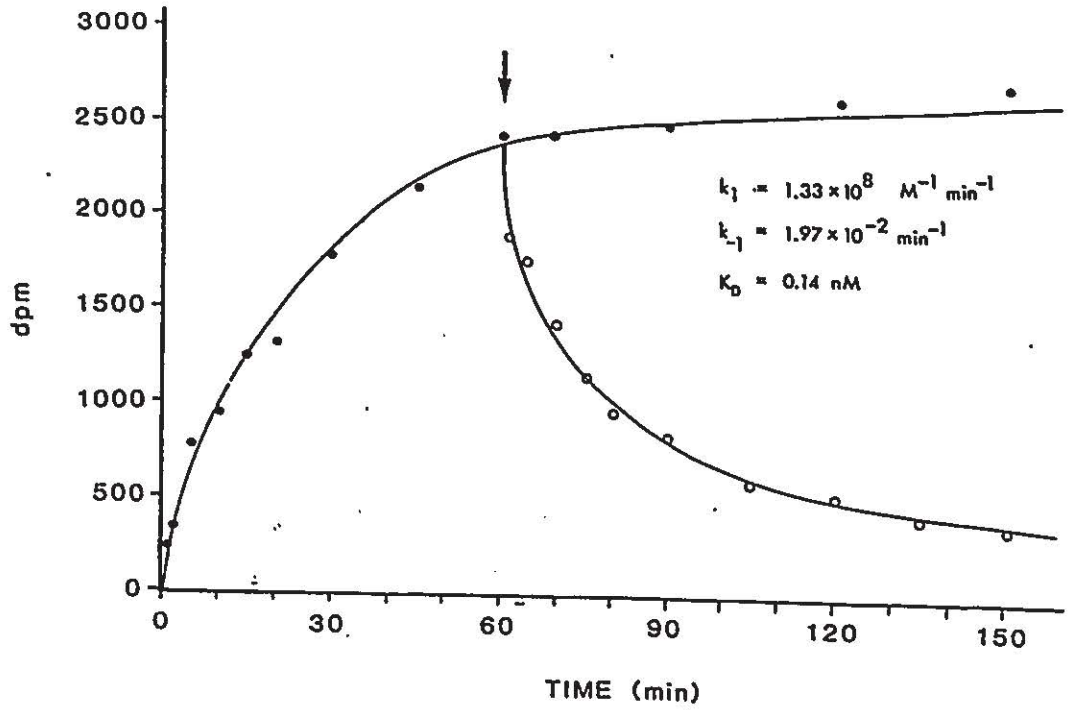

3000
2500
2000
1500
1000
500
0
dpm
0
30
60
90
120
150
TIME (min)
$k_1 = 1.33 \times 10^8$ M^{-1} min^{-1}
$k_{-1} = 1.97 \times 10^{-2}$ min^{-1}
$K_D = 0.14$ nM

Figure 33. Binding of [^{3}H]diprenorphine as a function of increasing concentration of the ligand to the deep muscular plexus synaptosomes. Increasing concentrations of the radioligand (generally 0.02 - 10 nM) were incubated with the membranes under the conditions described in the Methods. The inset shows the computer-generated Scatchard plot of the data. The plot is a representative of three such experiments.

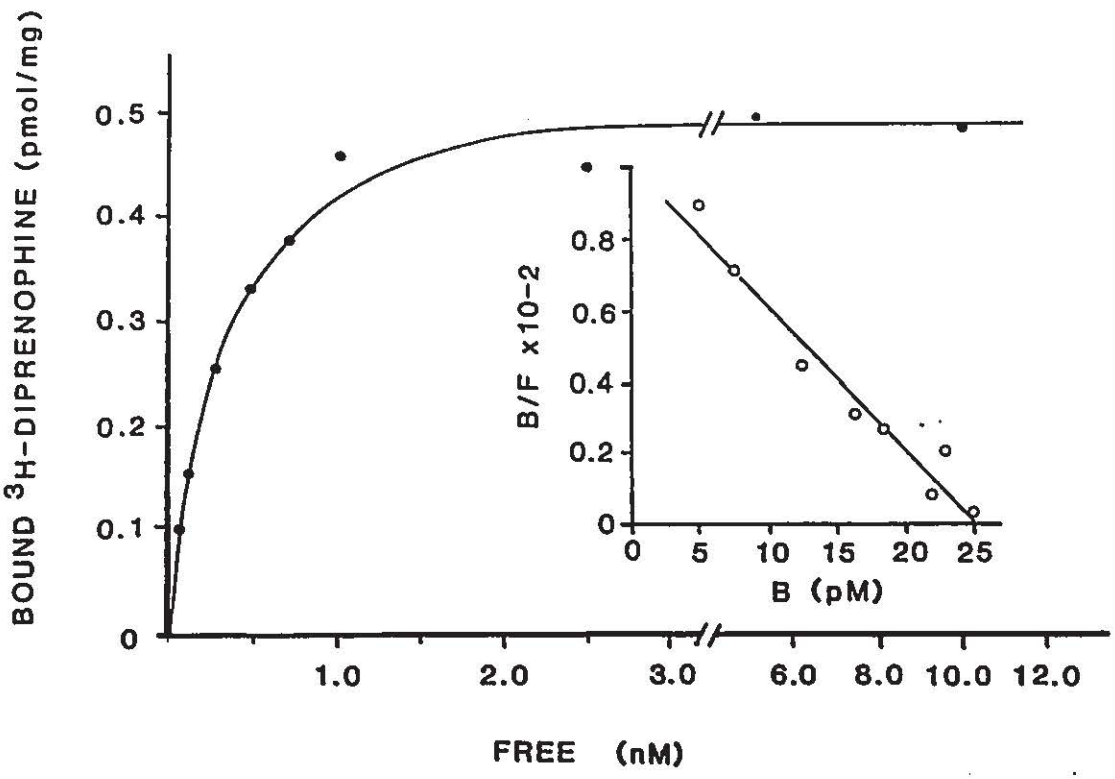

BOUND ^{3}H-DIPRENOPHINE (pmol/mg)
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
6.0
8.0
10.0
12.0
FREE (nM)
B/F x10-2
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
B (pM)

TABLE XIV

EQUILIBRIUM BINDING PARAMETERS FOR [^{3}H]DIPRENORPHINE BINDING

	Kd (nM)	Bmax (fmol/mg)	nH
DMP	0.18 ± 0.04	492 ± 20	0.71 ± 0.12
MP	0.12 ± 0.05	400 ± 21	0.75 ± 0.03
SMP	0.09 ± 0.03	683 ± 44	0.63 ± 0.15

Results are mean ± sd of 3 experiments each. The data was analysed on the computer program EBDA and LIGAND.

2 nM ligand concentration. The inset shows the computer generated Scatchard plot of the data. The analysis of the binding data yielded a linear Scatchard plot indicative of the a single population of the binding sites for all three membranes. The binding parameters for all three plexuses (DMP, MP, and SMP) are summarized in the table XIV.

In competition experiments unlabelled etorphine was used as a non-specific competing ligand. For μ-receptors, the morphiceptin analogue (N-MePhe-D-Pro-morphiceptin, PLO17) (Chang,1983,1984) and for δ-receptors, the dipenicilline substituted analogue of met-enkephalin D-Pen2-D-Pen5-enkephalin, DPDPE) (Mosberg,1983), were used to compete for the specific diprenorphine binding. These highly specific ligands were also used in a concentration of 0.5 μM for specific protection of the respective binding sites. For the characterization of k-receptors the less selective ligands dynorphin 1-13 and the compound U-50488-H (Takemori,1986) were used for competition studies.

Competition experiments on DMP, MP and SMP, using PLO17, DPDPE, dynorphin and U-50488-H, yielded biphasic competition curves (illustrated for DMP in fig. 34) *i.e.*, the computer analysis, using non-linear curve fitting, fitted a two site model significantly better than a single site model when the residual squares where compared. The summary of the results of the competition experiments for all three types of membranes is given in the table XV.

To further confirm the presence of the high affinity k-binding sites,

Figure 34. Competition of [^{3}H]diprenorphine by various opioid ligands from the deep muscular plexus synaptosomes. Membranes were incubated with the radioligand (0.3-0.5 nM) and increasing concentrations of the competitors. After 60 min, the reaction was terminated as usual. The results are expressed as the percentage of binding with that in the absence of any competitor as 100%. The data were analyzed with the EBDA/LIGAND and CDATA87 programs. The points are the average of three independent experiments performed in quadruplicate. For the value of the sd see table XV.

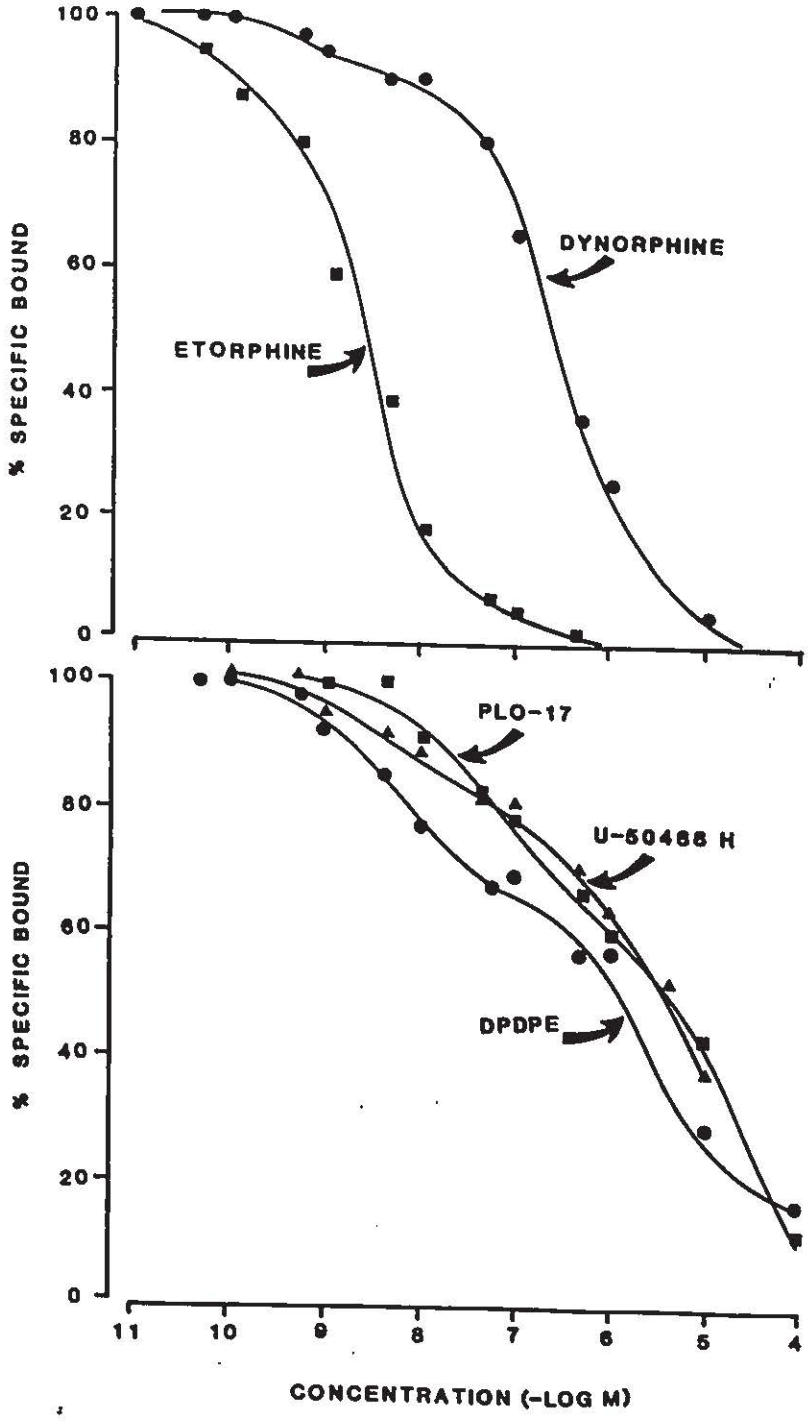

100
80
60
40
20
0
% SPECIFIC BOUND
DYNORPHINE
ETORPHINE
100
80
60
40
20
0
% SPECIFIC BOUND
PLO-17
U-50488 H
DPDPE
11
10
9
8
7
6
5
4
CONCENTRATION (-LOG M)

TABLE XV

SUMMARY OF THE COMPETITION DATA

COMPETITOR	HIGH AFFINITY		LOW AFFINITY	
	IC_{50} (nM)	%	IC_{50} (μM)	%
DMP				
PL0-17	43.6 ± 12.1	42 ± 8	24.0 ± 5.6	58 ± 8
DPDPE	7.7 ± 2.5	39 ± 8	9.8 ± 0.2	61 ± 3
U50488 H	1.0 ± 0.2	21 ± 4	8.7 ± 0.5	79 ± 12
Etorphine	1.6 ± 0.1	100	-	-
Dyn.1-13	1.2 ± 0.1	9 ± 1	0.3 ± 0.1	91 ± 8
MP				
PLO-17	25.0 ± 7.2	39 ± 5	14.0 ± 4.1	48 ± 5
DPDPE	8.1 ± 0.5	42 ± 4	37.0 ± 11.0	51 ± 3
U50488 H	3.1 ± 0.2	16 ± 1	9.0 ± 0.2	84 ± 8
SMP				
PLO-17	95.2 ± 58	63 ± 7	27.0 ± 23.0	40 ± 5
DPDPE	20.8 ± 16	24 ± 12	9.2 ± 4.0	78 ± 13
U50488H	19.0 ± 9	11 ± 10	4.1 ± 1.0	93 ± 6

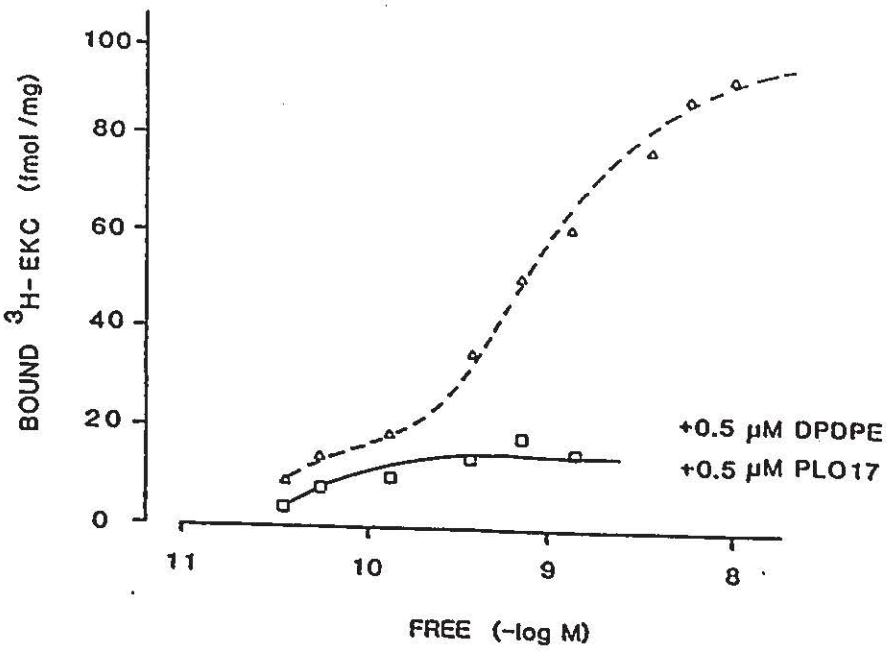
The results are mean ± sd from 3-4 experiments performed in quadruplicate. The binding was performed in the presence of 0.3-0.5 nM [^{3}H]diprenorphine. DMP = deep muscular plexus; MP = myenteric plexus and SMP is the submucous plexus. The results were analyzed with the computer programmes EBDA, LIGAND and CDATA87.

saturation experiments with [^{3}H]ethylketocyclazocine (EKC) were performed in the absence and in the presence of 0.5 μM each of PLO-17 and DPDPE to shield selectively the μ and δ receptors respectively. The result for the deep muscular plexus synaptosomes is illustrated in the fig. 35 and the results for deep muscular plexus synaptosomes and myenteric plexus synaptosomes is summarized in table XVI. In the absence of μ and δ ligands EKC recognized a high and a low affinity sites. In the presence of μ and δ ligands to shield these receptors, low affinity sites were not observed, only high affinity sites were present. The Kd and Bmax values for the high affinity sites were similar under both conditions.

Sodium ions are known to modulate opioid agonist and antagonist binding differentially such that in the presence of sodium ions the agonist binding is decreased and the antagonist binding is enhanced. In the present studies too, the effect of sodium and some other ions on the opioid agonist (etorphine) and antagonist (diprenorphine) binding to the DMP synaptosomes was studied. Mg^{2+} ions had no effect either on the etorphine or the diprenorphine binding at the concentration up to 10 mM; at 100 mM, it caused 40-45% inhibition of the binding of both the ligands (fig. 36).

Diprenorphine binding was more sensitive to the inhibition by K^+ and Ca^{2+}. However, neither of these ions affected binding at near physiological pH. Na^+ ions had the expected differential effect on the agonist and antagonist binding. At 150 mM, etorphine binding was decreased by 30% while diprenorphine binding was increased by 46%.

Figure 35. The saturation experiment with [^{3}H]ethylketocyclazocine on the deep muscular plexus synaptosomes in the absence (triangles) and the presence (squares) of 0.5 μM each of DPDPE and PLO17 to shield μ- and δ-opioid receptors. B is the Scatchard transformation of the data. The plot is the representative of three such experiments performed in triplicate.


BOUND ^{3}H-EKC (fmol/mg)
100
80
60
40
20
0
11
10
9
8
FREE (-log M)
+0.5 μM DPDPE
+0.5 μM PLO17

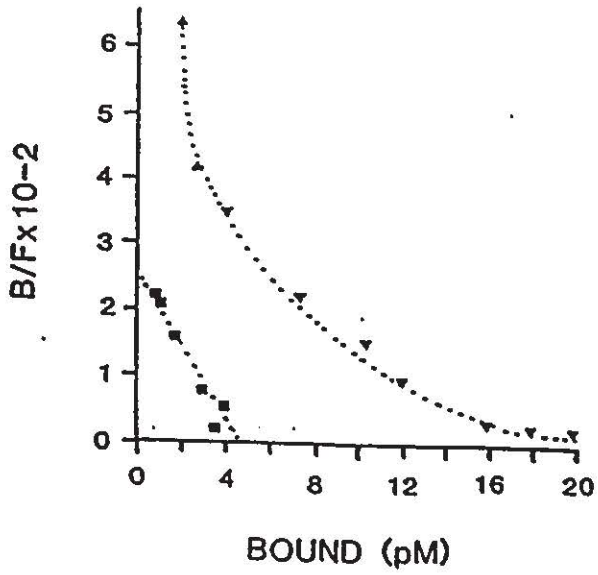

B/Fx10-2
6
5
4
3
2
1
0
0
4
8
12
16
20
BOUND (pM)

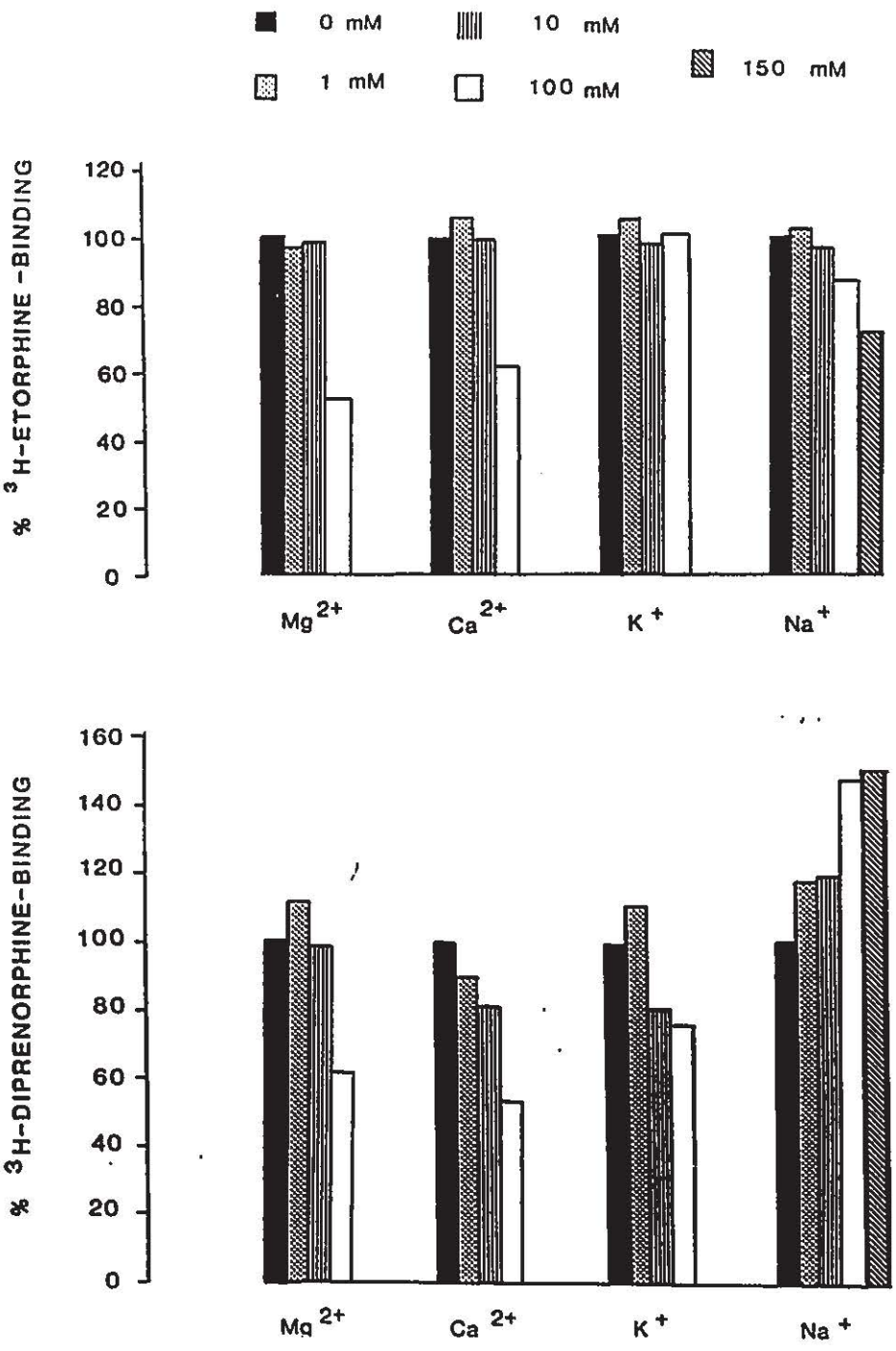
TABLE XVI

[^{3}H]ETHYLKETOCYCLAZOCINE BINDING WITHOUT AND WITH SHIELDING OF μ- AND δ-OPIOID RECEPTORS

	DMP		MP	
	Kd	Bmax	Kd	Bmax
WITHOUT SHIELDING				
Site I	0.05 ± 0.04	29 ± 14.3	0.08 ± 0.07	20 ± 5
Site II	0.92 ± 0.43	136 ± 9.0	1.13 ± 0.37	106 ± 33
WITH SHIELDING				
Site I	0.11 ± 0.05	28 ± 13.0	0.15 ± 0.09	23 ± 13
Site II	not detected	-	not detected	-

These are the results of the saturation experiments performed in control situation (without shielding) and in the presence of 0.5 μM each of PLO17 and DPDPE to shield the μ- and δ-opioid receptors. The results are mean ± sd of three experiments performed in triplicate for both total and the nonspecific binding.

Figure 36. Effect of cations on the specific binding of [^{3}H]etorphine and [^{3}H]diprenorphine (0.3-0.5) to the deep muscular plexus synaptosomes. The incubation was carried out in the presence of indicated ion concentrations and 0.2-0.4 nM radioligand. The results are expressed in percent with the specific binding in the absence of any added ions taken as 100%. The results are mean of three separate experiments performed in triplicate with the sd less than 10%.


0 mM
1 mM
10 mM
100 mM
150 mM
% 3H-ETORPHINE-BINDING
120
100
80
60
40
20
0
Mg 2+
Ca 2+
K +
Na +
% 3H-DIPRENORPHINE-BINDING
160
140
120
100
80
60
40
20
0
Mg 2+
Ca 2+
K +
Na +

4.5. DISCUSSION

4.5.1. LOCUS OF THE GASTROINTESTINAL OPIOID RECEPTORS

As mentioned in the introduction section, a number of studies have been performed in the gastrointestinal tract (mainly the guinea pig ileum). The majority of the studies localized the opioid receptors to the myenteric, or the submucosal plexus, or to the mucosal layer of the rat and guinea pig small intestine (see Introduction section). Autoradiographic studies by Nishimura *et. al.*. (1984,1986) suggested the opioid binding in the muscular layer of the gastrointestinal tract of rats and guinea pig. However, unfortunately, the light microscopic autoradiographic studies provide insufficient resolution to permit ascribing the binding to a certain cell type (neuronal or smooth muscle). In the present studies, the major conclusion regarding the locus of the opioid receptors in canine small intestine is that these receptors are confined to the neuronal membranes arising from each of the three main plexuses, i.e. the deep muscular, myenteric, and the submucous plexus. No binding to either the circular or the longitudinal smooth muscle plasma membranes was detected. These conclusions have been drawn from the following observations. The subcellular distribution of opioid and saxitoxin binding sites in all the three preparation was almost parallel. The fraction that was enriched in the opioid binding was also enriched in the saxitoxin binding. As a result of the parallel distribution, there was always a good correlation between

the opioid and the saxitoxin binding in all the three preparations (r = 0.82 - 0.85). On the other hand, the correlation between the opioid binding and the smooth muscle plasma membrane marker enzyme 5'-nucleotidase was extremely poor in these preparations (r = 0.06 - 0.3). The recovery of the opioid and the saxitoxin binding in the fractions obtained upon the differential centrifugation and the density gradient centrifugation also parallelled one another. For example, approximately 30% of the total opioid binding sites were recovered in the fraction P4, the recovery of the saxitoxin binding in this fraction was about 26%. Similarly, in the circular smooth muscle plasma membrane enriched fraction, the recovery of the opioid binding sites was 0.25% and that of the saxitoxin binding was 0.44%. In the fractions of submucosal plexus, a similar situation prevailed, where, the recovery of the opioid and saxitoxin binding was approximately 60 and 50% respectively. In the Mic II fraction, the recoveries of both the opioid and the saxitoxin binding sites were 15%. Apparently, the opioid binding in the fraction Mic II was owing to the presence of contaminating neuronal membranes, as suggested by the equal recoveries of the saxitoxin and the opioid binding sites.

4.5.2. PROPERTIES OF OPIOID RECEPTORS IN CANINE ENTERIC PLEXUSES

4.5.2.1. THE BINDING CONSTANTS

As summarized in the table XIV, the affinities of the opioid receptors in the three plexuses examined are similar. These values of the equilibrium

constants in the subnanomolar range are consistent with the equilibrium constants of other opioid receptor binding studies in the guinea pig ileum (Terenius,1975; Creese,1975). However, in these previous experiments, either crude homogenates of ileum were used, or no further characterization of the membranes was reported (Terenius,1975; Creese,1975). In the present studies, the saturation experiments using the non-specific antagonist ligand ^{3}H-diprenorphine revealed only a single, high affinity binding site.

4.5.2.2. OPIOID RECEPTOR SUBTYPES

The delta (DPDPE) and mu (PLO17) ligands used in the present studies have been shown to be highly selective. PLO17 was shown to be 1,000 - 10,000 times more selective for μ than for δ and k ligands (Chang,1983,1984). Also, DPDPE was found to be about 1,000 times more selective for δ than for other receptor subtypes (Chang,1983; Mosberg,1983). The biphasic competition curves for these highly selective ligands in displacing the non-selective ligand [^{3}H]diprenorphine suggest the existence of delta and mu receptors.

The lack of highly selective kappa receptor ligands complicates the displacement experiments for this receptor subtype. Two of the most selective ligands available at the time these experiments were performed were dynorphin 1-13 and the compound U-50488H (Chang,1983; Takemori,1986). In the present studies, these two compounds also showed a biphasic displacement pattern with a

high affinity and low affinity binding site. A small proportion of high affinity displacement by dynorphin 1-13 and U-50488H was consistently observed in the displacement studies. This suggested a small population of kappa receptors. The saturation experiments using the slightly kappa-selective ligand [^{3}H]ethylketocyclazocine in the absence and in the presence of 0.5 μM each of DPDPE and PLO17 clearly demonstrated the existence of a small proportion of high affinity kappa receptors which contributed 10-15% of the opioid receptors in all the three plexuses.

4.5.2.3. IONIC REGULATION

Opioid agonist and antagonist binding is differentially regulated by sodium ions. Agonist binding is decreased while antagonist binding is enhanced or not altered in the presence of sodium (Monferini,1981; Stefano,1982; Simon,1975; Pert,1974). Among other alkali metal ions, lithium, albeit to much lesser extent, also distinguished the antagonist versus agonist binding (Pert,1974). The discrimination of agonist/antagonist binding correlated impressively with their agonist/antagonist properties (Pert,1974). In the present studies, the effect of sodium and other mono- and di-valent ions on the agonist and antagonist binding to the DMP synaptosomes was examined. Consistent with the previous studies, sodium ions differentially regulated the agonist and antagonist binding. It has been suggested that the two conformations of opioid receptors i.e. agonist-binding and

antagonist binding are at any given time, in equilibrium, and that sodium ions shift the equilibrium further towards the antagonist-binding conformation (Simon,1975).

This study, using highly enriched synaptosomal fractions from the enteric plexus demonstrates that ionic regulation, dissociation constants and the maximum binding capacity of the peripheral neuronal opioid receptors from the dog gut are very similar to the opioid receptors on the synaptosomal fractions from the brain.

4.5.3. IMPLICATIONS IN RELATION TO THE FUNCTIONAL STUDIES

4.5.3.1. OPIOIDS AND THE MOTILITY OF THE SMALL INTESTINE

The main finding of the present studies on the aspect of the motility of the canine small intestine is the absence of these receptor sites from the smooth muscles and their presence on the neuronal plexuses in the gut. This implies that when considering the TTX-insensitive response to opioids (and other agents as well), one has to consider also the mechanisms other than direct action on the smooth muscle. A TTX-insensitive response does not by itself means the non-involvement of nerves in a given response. TTX-insensitive neural responses have been observed previously. In the opossum oesophagus, catecholamines, acting on dopamine D1 receptors caused hyperpolarization of the smooth muscle cells (Daniel,1987). This action was TTX-insensitive, but definitely involved action of nerves since the response was abolished after the treatment of the tissue with scorpion toxin, and probably involved the release of an inhibitory mediator. Therefore, the action of

opioids on contractility of the canine small intestine may involve such TTX-insensitive nerves. The action potential in some myenteric neurons (AH or Dogiel type II neurons) is also largely TTX-insensitive (Furness,1987).

Alternatively, the action of opioids may involve an inhibition of the tonic release of inhibitory mediator/s. One such candidate is VIP. Manaka *et. al.* (1988) have demonstrated that VIP is spontaneously released from the perfused canine small intestine *in vivo*. This release was inhibited by TTX accompanied by persistent phasic and tonic contractions of the smooth muscle as long as the effect on VIP release was present. However, the recovery of the spontaneous VIP release began and spontaneous contractions subsided (the experimental conditions under which the TTX-resistant effects were demonstrated; Daniel,1982; Fox,1987) before the recovery of the field-stimulated contractions. Opioids, like TTX, also decreased the spontaneous VIP release, met^5-enkephalin being 10 times more potent than dynorphin 1-13. Grider *et. al.* (1986) also have observed stretch induced, TTX-sensitive release on VIP from the rat and guinea pig isolated colonic segments. Therefore, it is possible that the *in vivo* contractile effects of opioids are partly due to the stimulation of the release of acetylcholine (atropine- and TTX-sensitive portion of the response) and partly due to the turn-off of the spontaneous release of VIP (TTX-insensitive portion of the response).

The inhibition of VIP release (Manaka,1988) as well as the contractile response *in vivo* (Daniel,1982; Fox,1987) appeared to be mediated by μ- and/or δ-

opioid receptors. In the present studies μ- and δ-subtypes of opioid receptors constituted more than 85% of the opioid receptors on the myenteric as well as on the deep muscular plexus. It has not been possible to demonstrate a contractile response to opioids *in vitro* (Daniel,1982; Fox,1987). A possible explanation for this *in vivo* and *in vitro* discrepancy may be the loss of the neuronal circuitry involved in the tonic release of VIP and/or other such inhibitory mediator/s.

The facts that the k-selective agonists were more potent inhibitors of the field-stimulated contractions and that in the present studies the k-receptors constituted only a small proportion of the opioid receptors may indicate that this subtype of opioid receptors are more restricted in distribution, and may be confined to certain neurons, where they inhibit the release of the excitatory mediator acetylcholine. Alternatively, there may be a larger pool of spare receptors of μ- and δ-subtypes than of the k-subtype of opioid receptors.

Surprenant and North (1984), using an intracellular current recording technique, reported the presence of μ-opioid receptors on neurons of the guinea pig myenteric plexus. We have, in the present studies obtained evidence for the presence of all three subtypes of opioid receptors in the canine small intestine. Beside the species differences, the lack of correspondence in these studies may have been due to the fact that μ-opioid receptors are present on the cell soma, therefore, accessible to the intracellular recording techniques, whereas δ- and k-opioid receptors are on the nerve endings, distant from the cell soma, therefore

may go undetected by intracellular recording techniques. Such a situation may be more pertinent for the case of the deep muscular plexus synaptosomes, since this plexus of neurons lacks the neuronal cell bodies. The fibres in the deep muscular plexus mainly originate from the myenteric plexus. Therefore, intracellular recordings performed in the myenteric plexus may detect the receptors on the cell bodies in the myenteric plexus but may not detect the receptors on the terminals in the myenteric plexus and in the deep muscular plexus.

4.5.3.2 OPIOIDS AND THE WATER AND ELECTROLYTE TRANSPORT ACROSS MUCOSA

As mentioned earlier, not many studies are present in the literature that elucidate the function of opioids in the water and electrolyte transport across the mucosa. The submucosal neurons are definitely involved in the mucosal function as suggested by the majority of the projections of submucosal neurons to the glands and villi of mucosa. These projections include dynorphin/enkephalin containing nerves in canine small intestine, suggesting the possible role of opioids in mucosal functions (Daniel,1987). As discussed earlier, opioids have been demonstrated to increase the water and electrolyte absorption. The most convincing binding studies to date did not observe any opioid binding site on the intestinal enterocytes from rats (Gaginella,1983).

Electrophysiological studies by Mihara and North (1986) have nicely

elucidated the action of opioids on δ-subtype of opioid receptors in guinea pig caecum submucous neurons. In the present studies, a majority of the opioid receptors were of μ-subtype. This may indicate species differences. Alternatively, it is possible that the μ-receptors are located on the axon terminals at a distance from the cell body. An electrophysiological response elicited at such a distance may have eluded the detection by a device implanted at the soma. The decrease in water and electrolyte absorption in the rat intestine by μ-receptors but not by δ-receptors was mediated by the release of acetylcholine (Fogel,1984). The modulation of the release of one mediator by another may occur at the soma, at the level of dendrite, or, more likely, at the release site i.e., the terminal. Therefore, it is tempting to postulate that the μ-receptors modulating the release of acetylcholine are situated at the terminal varicosity, while the δ-receptors are present at the soma.

As far as the k-receptors are concerned, no systematic study of this subtype has been performed on the submucous plexus neurons. We have found evidence for the presence of a small proportion of k-receptor subtype in the submucosal plexus neurons. It is possible, however, that these k-receptors are present on the contaminating membranes of the origin other than submucosal plexus, such as the blood vessels. Wolter (1985) found evidence that dynorphin-A 1-8-immunoreactive nerve fibres were present in close contact with submucosal blood vessels, but not in the submucosal plexus of rat duodenum. The detection of

k-receptor subtype on the canine submucosal plexus neurons, however, was not surprising since excess of enkephalin/dynorphin-immunoreactive nerves have been observed on this plexus in dogs (Daniel,1987). Further functional studies need to be performed to examine the possibility of the action of opioids through the k-receptor subtype in the submucosal plexus.

In summary, we have studied the localization and subtype distribution of opioid receptors in the canine small intestine. Opioid receptors were localized exclusively on the neuronal structure of the myenteric, deep muscular, and the submucous plexus. No receptors could be demonstrated on either the circular or the longitudinal smooth muscles. The properties of the opioid receptors on the myenteric and deep muscular plexus were similar with similar proportion of the three receptor subtypes (40-45% μ- and δ- and 10-15% k-subtype) while in the submucous plexus, the μ-subtype dominated. The opioid receptors in the canine small intestine were similar to those observed in other central and peripheral structures in terms of the affinity and ionic regulation.

CHAPTER 5

CONCLUDING REMARKS

Ligand binding studies provide extremely valuable information regarding the receptor, the ligand, and the ligand-receptor interaction. However, extreme caution should be exercised in interpretation of the results of the binding studies. The term receptor encompasses two basic phenomena. The binding of the ligand to the binding sites. If that binding site is then to be classified as the receptor, its functional significance should be established *i.e.* its coupling to a physiological response should be demonstrated. Therefore, the demonstration of the binding in a tissue, cell or membrane does not suffice for that particular binding site to be classified as the receptor, unless its physiological significance has been established.

In previous chapters, the term receptors, rather than the binding sites has been used freely. These studies were initiated to complement previous *in vivo* and *in vitro* studies which have established the role of both neurotensin and opioids in the physiology of the gastrointestinal tract, therefore the use of the term receptors was not entirely unreasonable. In the case of neurotensin receptors, we have demonstrated their presence on the circular smooth muscle, the deep muscular plexus and the submucous plexus. Functional studies also advocate both the neuronal and non-neuronal actiona of neurotensin on the canine small intestine. Furthermore, we have observed the presence of several classes or affinity states of these receptors in the canine small intestine. Which ones of these are the functional receptors is still unknown, however, based on the available data, some speculations have been made.

In the case of opioid receptors, binding was observed on all three plexuses. Again three major subclasses of opioid receptors were detected. Although functional studies do suggest the presence of opioid receptors on the smooth muscle (although other explanations are possible, discussed previously), we could not detect any opioid receptors on either the circular or the longitudinal smooth muscles. It is, however, possible that the opioid receptors on the smooth muscle are extremely labile, and therefore get inactivated upon the procedures of homogenization and membrane fractionation. We have not been able to resolve this issue. The starting material contained both the circular smooth muscle and the deep muscular plexus (or myenteric plexus and the longitudinal smooth muscle). The only avenue for the separation of the membranes from these was the extensive fractionation using differential and the density gradient centrifugation. A better separation was acheived only after the density gradient centrifugation. Therefore, extensive separation procedure was unavoidable. However, if these receptors are so extremely labile, they will have to be entirely different from any other previously identified opioid receptors in any system; a situation not impossible but highly unlikely.

5.1 FUTURE DIRECTIONS

The present work, by no means, is complete. Further biochemical studies need to be performed to discern the nature of the neurotensin, opioid and other receptors and ion channels in these membranes. The next step should be the solubilization, purification and further work on the molecular biology of neurotensin and opioid receptors in the gastrointestinal tract.

The isolation of the purified membranes from the circular and longitudinal smooth muscles and the three plexuses now provide an immense opportunity to further study the function of the gastrointestinal tract at biochemical and molecular levels.

The availability of synaptosomes from three plexus will also provide a unique opportunity to study the presynaptic regulation of the neurotransmitter content and release.

Further work in this areais in progress in our laboratory and in the laboratory of our collaborators. Some progress has already been made, a list of some of the published work is presented in the appendix.

CHAPTER 6

APPENDIX

6.1 RADIOLIGAND BINDING TECHNIQUE

Within the past two decades, rapid advances have been made in the study of the receptors at cellular and subcellular levels mainly due to the advent of direct radioligand receptor binding studies coupled with the availability of a variety of agonists and antagonists for use as radioligands with high specific activity and high selectivity or as competitors to such ligands. These powerful techniques make possible, the studies of receptors at the molecular levels. The basic goal of the radioreceptor binding assay is to study the first step in the receptor activation, the ligand binding to the receptor sites, and determine the quantitative parameters, which define the ligand, the receptor and the ligand-receptor interaction. Perhaps the most important factor in rendering the ligand binding studies so popular and common is the simplicity of their execution. A radioactive tag (usually ^{3}H or ^{125}I) is attached to the ligand which is then incubated with tissue slices, isolated cells, purified biological membranes, or solubilized preparation under appropriate conditions. Bound ligand is then separated from unbound ligand by centrifugation, filtration, simple washing (in the case of tissue slices), or by column chromatography etc. and radioactivity of the bound ligand is measured in a counter. Specificity, reversibility, saturability and other quantitative parameters of the binding sites for the ligand, in general, can easily be ascertained using this simple technique. The method of radioligand binding relies on the assumption that the radioligand will bind reversibly and stereospecifically to the receptors according to the law of mass action, in a stoichiometric and concentration-dependent manner, and the receptor sites will eventually be saturated by the radioligand. The detection of some binding does not automatically define the receptors; rather, the properties of the particular binding sites must fulfil certain criteria before these binding sites qualify to be designated as the receptors. These criteria are the following:

a) The radioligand used for the binding assays must be pharmacologically active at the receptor in question, i.e. the process of radiolabelling must not alter its pharmacological profile.

b) The number of binding sites must be limited i.e. it is saturable.

c) The binding must be reversible, to account for the reversibility of the action of the drug. However, certain drugs are chemically modified to render their action irreversible, in such cases, the binding may not be reversible.

d) Specificity of the binding is a very important criteria. Drugs belonging to a similar chemical or pharmacological class should be able to compete for the binding sites, while those belonging to different classes should not.

e) The rank order of potency of the related drugs in binding experiments should correlate with the pharmacological rank order of potency. This is usually strictly true for antagonist ligands but for agonists ligands, the potency is affected by intrinsic efficacy as well as by affinity for the receptor.

f) Regional distribution, tissue specificity and the cellular and subcellular distribution of the binding sites should correlate with the function of the receptor in the tissue.

The simplest model of the ligand receptor interaction assumes a homogeneous species of ligand and a single non-interacting population of binding sites. This situation is similar to the simple second order enzymatic reaction and can be represented as :

$$L + R = LR \quad \text{----------(1)}$$

where L is the ligand and R is the receptor, K_1 and K_{-1} are the rate constants for association and dissociation.

At equilibrium,

$$(L)(R)/(LR) = K_d = 1/K_a \quad \text{----------(2)}$$

where (L) is the concentration of the free ligand, (R) is the concentration of the free receptor sites, (LR) is the concentration of the ligand-receptor complex and the K_d and K_a are the equilibrium dissociation and association constants.

The two most useful methods of analyzing equilibrium saturation binding data are the Scatchard plot (Scatchard, 1949) and the Hill plot (Hill, 1913). Both of these plots are the transformation of the data obtained when the amount of the binding is determined as a function of the concentration of the unbound ligand. For the transformation of the data, equation (2) can be modified to give the Scatchard equation:

$$(LR)/(L) = [(R_t) - (LR)]\ K_d^{-1} \quad \text{----------(3)}$$

where the value R_t is the total density of the receptors (or the Bmax). If the radiolabelled drug binds to a single class of non-interacting binding sites, a plot of the ratio of the bound to free ligand (LR)/(L), versus the concentration of the bound ligand (LR) should give a straight line with a slope of $-1/K_d$, and the intercept on the abscissa provides a measure of the concentration of the binding sites (R_t), also known as the maximum number of the binding sites (Bmax). The parameter has meaning for a particular membrane of a particular cell only if that membrane is pure or its contamination by other membranes is known.

Once the value of the Bmax is known, then the saturation curve can be transformed into the Hill equation (Hill,1913):

$$(LR)/(R_t) = (L)^n/ [K_H + (L)^n] \quad \text{----------(4)}$$

which is transformed to a logarithmic form for convenience in plotting as follows:

$$\log (LR)/[(R_t)-(LR)] = n\log (L) - \log K_H \quad \text{----------(5)}$$ or in a simple version:

$$\log B/(Bmax-B) = n\log (L) - \log K_H \quad \text{----------(6)}$$

In this equation n is the Hill coefficient and K_H is the Hill binding dissociation constant.

Thus a plot of log B/(Bmax-B) versus log (L) has a slope of n and the

intercept on the abscissa is a measure of K_H. When the reaction follows the principle of mass action in the case of a simple non-interacting population of receptors, n equals 1.0 and K_H equals Kd. The value of Hill coefficient less than 1.0 indicates the possibility of radioligand binding to a heterogeneous population of the binding sites, or the occurrence of negatively cooperative interactions.

6.2. STRUCTURE OF THE WALL OF SMALL INTESTINE

The structure of the wall of small intestine is diagrammatically represented in Fig. 1. The diagram was constructed on the basis of observations in guinea pig small intestine, but represents reasonably well, the arrangements in the small intestine of all mammals (Furness, 1987).

The wall is composed of several well defined and some not so well defined layers. There are three distinct layers composed of smooth muscles. The outer longitudinal, middle circular, and the inner muscularis mucosal layer. In some species including the dog, the circular muscle is subdivided by a deep muscular plexus into two layers which have morphologically distinct characteristics (Duchon, 1974). There is a dispute over the organization of the cell bundles in circular and longitudinal layers. Some anatomists argue that, viewed from the oral end, the circular layer is wound counterclockwise in a helix, and that the longitudinal layer is composed of more open helices (Davenport,1985). Most believe that the circular layer is formed of closed rings of smooth muscle bundles and longitudinal layer is composed of longitudinal bands lying parallel to the axis of the gut (Davenport,1985). The muscularis mucosa is composed of both the longitudinally as well as circularly arranged fibres. Smooth muscle cells are densely packed, a transverse section shows them to be grouped into bundles containing hundreds of cells in a single bundle (Gabella, 1987). However, in serial transverse sections of the visceral muscle layers, the muscle bundle appear to extend only to a few hundred micrometers along the muscle length and either split into two or more or merge with other bundles (Gabella, 1981). Therefore, generally no clear cut bundles are present in visceral muscles but groups of muscle cells appear to be continuous with each other (Gabella 1987).

THE SMOOTH MUSCLE CELLS

Smooth muscle cells are uninucleated, generally elongated and spindle shaped structures with two tapering ends (Gabella, 1987). These cells may sometimes have extremely uneven contours along their lengths (Burnstock, 1970). The lengths of smooth muscle cells may vary between species, tissue and the method of measurement and the variation in observed size may, at least partly, be due to the degree or the state of contraction. Visceral smooth muscle cells, at rest, are generally 400 - 700 μm in length and 2 - 10 μm in diameter (Burnstock,1970; Gabella,1987). The volume of the visceral smooth muscle cell is about 3500 μm^2 and is more consistent between species (Gabella, 1987).

Invaginations or inpocketings in the cell membrane are abundant throughout the surface of the cells. These inpocketings, called caveolae are generally arranged, as arrays of 2 - 4 caveolae wide, parallel to the long axis of the cell (Gabella,1981 and references therein). Each caveolus measures about 70 nm across and 120 nm in length (Gabella,1987). Guinea pig taenia coli contains a total of 170,000 caveolae per cell thus increasing the cell surface membrane by 50 - 70% (Gabella,1976,1987). Caveolae appear to be associated with sarcoplasmic reticulum, which lie close to the base of caveolae, especially in intestinal muscles (Gabella,1981). The functional significance of caveolae are unknown, it has, however, been suggested that they may be involved in calcium transport across the membrane (Popescu,1974a,b), or these structures may be the site for the control of cell volume (Daniel,1971; Garfield,1977a,b).

Occupying the space between caveolae, electron dense patches or the dense bands are present on the cytoplasmic side of the cell membrane. These dense bands are generally about 0.2 - 0.4 μm wide and may extend up to 1 - 2 μm or more along the longitudinal axis of the cell (Gabella,1987). Dense bands in one cell may match a similar structure in an adjacent cell, giving rise to an intermediate or adherens-type junction (Gabella,1987). Thin filaments (actin)

penetrate into the dense bands and intermediate filaments have also been observed to penetrate the dense bands (Pease,1960; Furness,1980; Gabella,1987).

Endoplasmic reticulum (ER) is rather scarce in smooth muscle cells compared to striated muscle cells. A developing smooth muscle cell generally contains more of the rough type of ER than an adult one (Gabella,1987). Some cisternae of ER are present near the nuclear poles and among the filaments, these are more of the rough ER (Gabella,1987). Most of the smooth ER lie in very close proximity (~ 10nm) to the plasma membranes (Gabella,1987). ER occupy about 2% of the cell volume (Popescu,1974; Devine,1972; McGuffy,1976). ER are considered as the major store of releasable intracellular calcium (for reviews on this topic see Somlyo,1971,1975).

Mitochondria are elongated and lie parallel to the long axis of the cells generally along the periphery of cells or near the nuclear poles (Gabella,1987). Mitochondria occupy 5 - 9% of the cell volume. Somlyo and Somlyo (1971, 1975, 1979) have demonstrated that mitochondria from smooth muscle can accumulate divalent cations including calcium. However, it has been suggested that mitochondria do not play any role in the regulation of intracellular calcium during physiological contraction, rather, they accumulate massive loads of calcium in pathological conditions (Somlyo,1979).

Three types of filaments are generally present in smooth muscle cells. Thin or actin filaments (~ 7 nm diameter), intermediate filaments (~ 10 nm diameter) and thick or myosin filaments (diameter 15 - 17 nm) constitute the filamentous structure of the smooth muscle (Gabella,1987). The arrangement of filaments in smooth muscle is less well understood than in striated muscles. The ratio of actin to myosin varies between tissues, in visceral smooth muscles this ratio is about 12:1 (Bois,1973; Nonomura,1976). As mentioned before, thin filaments, and sometimes intermediate filaments do appear to penetrate dense bands (Pease,1960; Furness,1980; Gabella,1987). Filaments are considered to be the structural basis for the force generating system (Hartshorne,1987). Function of the

intermediate filaments is less well understood and it is thought that they form a cytoskeletal network and function to connect the force generating units via dense bodies and to distribute tension throughout the cell (Hartshorne,1987).

Specialized structures, thought to be the means of electrical, mechanical and metabolic coupling between adjoining cells are present on the plasma membranes of various cell types including smooth muscle cells. The best known of these is the gap junction (also previously called as nexus). Gap junctions are oval shaped areas on the cell membranes where the membranes of two adjoining cells come in close proximity (2-3 nm) to each other (Garfield,1985). In each such oval shaped area, many small structures called connexons are present. Each connexon is composed of six protein monomers (with associated lipids) arranged in a rosette, which line the central channel and extend from cytoplasmic to the extracellular face of the membrane (Ravel,1984) and is apposed to a similar structure in neighbouring cell (Chalcroft,1970). Gap junctions are dynamic structures, under control by a number of physiological determinants and pharmacological manoeuvres (Spray,1985). However, these structures appear to be quite stable since they survive homogenization and fractionation procedures and can be frequently seen in purified membrane preparations (see fig. 9). Gap junctions are proposed to provide electrical and metabolic coupling between the cells. Since the presence of gap junctions does not always correlate with the extent of electrical coupling doubts have been cast over the role of visible gap junctions as necessary in electrical coupling (Garfield,1985; Gabella,1981,1987). This may be because some gap junctions are so small in size that they can not be visualized by the electron microscope. They have been referred to as the point contacts.

The intercellular gap between two adjacent cells is frequently reduced to about 20 nm at the site of dense bands such that two dense bands in adjoining cells are in close proximity to each other (Gabella,1987). Increased density of cytoplasm is present in these gaps as well (Garfield,1985; Henderson,1971). These contacts are known as intermediate junctions. The function of intermediate

junctions is obscure, however, it has been proposed that they may support and fasten cells together and provide a mechanical link between adjacent cells (Gabella,1981,1987).

Close contacts or simple appositions are the nomenclature used for the areas of close approximation of the membranes of adjoining cells without any specialized structures (Garfield,1985; Gabella,1981). The junctional gap between the membranes of adjoining cells is 10 nm or less (Garfield,1985). Again, the function of these structures is poorly understood. Such junctions or contacts have sometimes been observed between smooth muscle cells and nerve endings, their significance as well is not known (Gabella,1987). Finger-like projections of one cell may sometime protrude into invaginations of neighbouring cells. Such interdigations are sometimes accompanied by close apposition type or intermediate contacts between two cells; gap junctions have also been observed in such interdigations (Garfield,1985).

THE ENTERIC NERVOUS SYSTEM

The gastrointestinal tract is a highly innervated organ. Nerves affecting the gastrointestinal function can be broadly categorized as of extrinsic and intrinsic origin and together these neurons constitute the third component of autonomic nervous system, the *Enteric nervous system (ENS)*. The enteric nervous system is present throughout the gastrointestinal tract. Furness and Costa (1987) described the ENS as a system of neurons and supporting cells present within the wall of the gastrointestinal tract. Extrinsic neurons are the ones whose cell bodies lie outside the of the wall of gastrointestinal tract. The cell bodies of Intrinsic, or enteric neurons lie within the wall of the gastrointestinal tract (Furness,1987). Within the gastrointestinal tract, these neurons are organized in to various networks at various levels in the wall, such network of neurons are known as the plexuses.

6.2.2.1 THE ENTERIC PLEXUSES

Within the wall of the gastrointestinal tract, neuronal cell bodies and their processes constitute meshworks. Such interconnected networks of nerves are called the plexuses. Within the plexuses, collections of nerve cell bodies may be present, such structures are called the ganglia. Ganglia are not the essential features of all the plexuses in gastrointestinal tract, but are rather confined to two plexuses (discussed below), which are called the ganglionated plexuses. There are other plexuses, which are formed by neuronal processes arising from the nerve cells of the ganglionated plexuses, these plexuses lack the ganglia and are termed as aganglionated plexuses.

Pioneering morphological studies by Meissner, Billroth, Remack and Auerbach in the mid nineteenth century clearly described the presence of ganglionated plexuses in the gastrointestinal tract. The late nineteenth and early twentieth century saw the description of arrangement of plexuses by such morphologists as Henle, Drasch, Dogiel, Cajal, Hill, Schabadasch and Stohr (reviewed in Furness,1987). The basic arrangement of plexuses as described by these workers still holds true, with some further details obtained in more recent years.

The two ganglionated plexuses present in the gastrointestinal tract are the myenteric plexus, situated at the border of longitudinal and circular smooth muscle cells, and the submucosal plexus, situated in the submucosa between the circular muscle and the muscularis mucosae (Gabella,1987).

The meshwork formed by myenteric plexus is characteristic of the species and the tissue. However, it is continuous around the circumference of the gastrointestinal tract and along its length (Furness,1987). The number of cells in each myenteric ganglion may vary between tissues and species from only a few to over 150 (Furness,1987; Gabella,1987). Single cell bodies may occasionally be present outside the ganglia. The ganglia are interconnected with strands of nerves

known as the internodal strands (Furness,1987). Three levels of the networks in the myenteric plexus have been observed namely, primary, secondary and the tertiary myenteric plexus (Furness,1987). The ganglia and interconnecting internodal strands constitute the primary plexus. The secondary strands are constituted by finer nerve fibres arising from the primary plexus and run close and parallel to the circular smooth muscle layer (Furness,1987). The total number of neurons in myenteric plexus varies highly between the species and the part of the gut. Generally, the number of neurons correlate with the thickness of the adjacent musculature (Gabella,1987).

The second ganglionated plexus is the submucosal plexus, present at the submucosa beneath the circular smooth muscle layer. It is present throughout the length of the intestine and continues along the circumference. Henle (1871) and Goniaew (1875) observed that generally, the size of ganglia, the meshwork, and the interconnecting strands are smaller in the submucous as compared to the myenteric plexus. However, the absolute number of cells in submucous plexus may be more or less than those in myenteric plexus in different species (Gabella,1976; Okhubo,1936; Sauer,1946). Two levels of meshwork at the submucosal plexus have been observed in some species (Gunn,1968; Stach,1977), and some authors have referred to them as two separate plexuses, the Henle's plexus (Situated close to the circular musculature) and Meissner's Plexus, situated near the muscularis mucosa. Since there is a profuse interconnection between these two levels and there is no histologic and functional distinctions between them, others prefer not to subdivide the submucosal plexus (Furness,1987).

A plexus of nerve fibres is present deep within the layer of the circular smooth muscle, separating the outer and inner components of circular muscle. This plexus referred to as the Deep Muscular Plexus, is present in the small intestine and is an aganglionated plexus (Furness,1987; Taxi,1965; Li,1937; Gabella,1972,1974; Faussione,1984,1985). The processes of the deep muscular plexus originate mainly from the cells of the myenteric plexus (Furness,1987). The predominant orientation

of the deep muscular plexus is parallel to the circular smooth muscle.

The above mentioned plexuses represent three major plexuses in small intestine. However, some authors have observed some less well defined meshwork of processes and have categorised them as the separate plexuses. However, due to the lack of any clear morphological or functional distinction, a majority of workers have preferred not to categorize them as separate entities. For this reason, the discussion in the present chapter has been deliberately limited to the three major plexuses.

Studies in the nineteenth and early twentieth century by Auerbach (1864), Dogiel (1895,1899), Hill (1927), and Lawrentjew (1931) and others suggested the interconnections between the ganglia within a plexus and also between the plexuses. These observations have been confirmed more recently by specific immunochemical histochemistry and the consequence of lesions of pathways of intrinsic and extrinsic nerves of small intestine. These studies have clearly established that the myenteric plexus provides processes for a majority of deep muscular plexus neurons (Daniel,1987; Furness,1987). Wilson *et. al.* (1987) observed that after microsurgical removal of longitudinal muscle with the attached myenteric plexus and following recovery in the guinea pig small intestine, more than 99% of the processes within the circular muscle layer disappeared and the rest (~ 0.7%) disappeared after concomitant extrinsic denervation. The myenteric and submucous plexus are also interconnected with each other with more traffic flowing from myenteric to submucous plexus than in the opposite direction (Furness,1987). In this context, Jessen *et. al.* (1980) observed reciprocal projections between myenteric and submucosal plexuses by vasoactive intestinal polypeptide (VIP) and substance-P (SP) containing neurons.

To summarize, various plexuses within the gastrointestinal tract supply nervous connections in the gastrointestinal tract. The myenteric plexus supplies the longitudinal and circular muscles, deep muscular and the submucosal plexus and also the mucosa. The deep muscular plexus innervates the circular smooth muscle

and the submucosal plexus supplies the secretomotor neurons to mucosa and muscularis mucosa. The submucous plexus occasionally sends processes to the myenteric plexus as well. Intrinsic neurons are also connected to the branches of the sympathetic and parasympathetic nervous system.

6.2.2.2 EXTRINSIC NERVES IN THE GASTROINTESTINAL TRACT

Sympathetic and parasympathetic divisions of the autonomic nervous systems also innervate the gastrointestinal tract at various levels. Langley (1921) defined the sympathetic component of the autonomic nervous system as the one that emerges from the thoraco-lumbar parts of the spinal cord and influences visceral and muscular function. The majority of sympathetic fibres synapse at sympathetic ganglia and some send post ganglionic fibres to the gastrointestinal tract. Post-ganglionic sympathetic fibres from the coeliac plexus supply the stomach and parts of the small intestine; caecum, appendix, the ascending colon and transverse colon are supplied by the superior mesenteric ganglia. The rest of the colon receives sympathetic post ganglionic inputs from the inferior mesenteric ganglia and the rectum is supplied by the pelvic ganglia. Most of the post-ganglionic sympathetic fibres synapse at the myenteric and submucous ganglia but only a few go to the smooth muscles without synapsing. Sympathetic innervation accounts for the majority of the norepinephrine containing processes within the gastrointestinal tract.

The majority of the efferent extrinsic parasympathetic innervation to the gastrointestinal tract is via the vagus nerve, whose fibres follow the blood vessels and synapse in the myenteric plexus. Parts of the gastrointestinal tract which are innervated by the vagus are the stomach, small intestine, caecum, appendix, ascending colon and transverse colon. The rest of the colon receives the parasympathetic innervation from the pelvic nerves via the hypogastric plexus, which also end in the myenteric plexus (Davenport,1985). Electrical stimulation of the majority of the vagal or sacral efferent fibres increases the contractile activity

of the gut. However, some of the vagal fibres synapse at the inhibitory ganglion cells of the myenteric plexus, or, on the interneurons that activate inhibitory ganglion cells (in gastric cardia and in lower oesophageal sphincter), activation of these vagal fibres results in the inhibitory response (Wood,1987).

Thus, the gastrointestinal tract is innervated by the extrinsic as well as intrinsic neurons, the intricate balance of the activities of various nerves and hormonal influence serves to regulate the activity of the gut.

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