

**GALANIN RECEPTORS IN CANINE SMALL INTESTINE**

By

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## **GALANIN RECEPTORS IN CANINE SMALL INTESTINE**

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## ABSTRACT

Galanin, a 29 amino acid polypeptide, is widely distributed in enteric nerves of the gastrointestinal tract (GIT) in mammals. Previous functional studies demonstrated that galanin may function as neurotransmitter and neuromodulator in GIT. In canine small intestine, galanin induces TTX-insensitive inhibition of small intestinal circular muscle motility *in vivo* and *in vitro*, suggesting a direct smooth muscle action of galanin (Fox *et al*, 1986). Intraarterial infusion of galanin inhibits vasoactive intestinal polypeptide release from isolated perfused canine small intestinal circular muscle, indicating a neuronal action of galanin (Fox *et al*, 1988). We used <sup>125</sup>I-porcine galanin as a ligand to study the galanin receptors in the circular muscle and deep muscular plexus from the canine small intestine. The separation, purification and characterization of nerve and muscle membranes were carried out using the technique developed in our laboratory by Ahmad *et al* (1988). Specific binding sites for galanin were found in both nerve and smooth muscle membranes. The galanin receptor on synaptosomes showed some similar characteristics to that on smooth muscle membranes: 1). The equilibrium binding analysis showed a high affinity and high capacity binding sites in nerve ( $K_d = 1.1$  nM,  $B_{max} = 244$  fmol/mg) and in muscle ( $K_d = 0.58$  nM,  $B_{max} = 389$  fmol/mg); 2). The specific binding of <sup>125</sup>I-galanin was inhibited by galanin or N-terminal galanin fragments (galanin 1-16, 1-15, 1-11), but not inhibited by C-terminal fragment galanin 15-29, suggesting a

crucial role of the N-terminal region of the galanin molecule in receptor recognition. Computer analysis suggested a two-site model of galanin receptor; 3). The receptor-bound  $^{125}\text{I}$ -galanin was only partially dissociated by addition of excess ( $1\ \mu\text{M}$ ) unlabelled galanin. However, in the presence of a GTP analog,  $\text{GTP}\gamma\text{S}$ , the dissociation of bound  $^{125}\text{I}$ -galanin was accelerated and completed, implicating involvement of G proteins in binding of galanin to a two-site receptor. This was supported by the observation that  $\text{GTP}\gamma\text{S}$  abolished the high affinity galanin binding site, leaving only a low affinity binding site in competition studies; 4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cross-linked galanin-receptor complexes from synaptosomes and smooth muscle membranes revealed a similar radioactive band at an approximate molecular mass of 50,000 dalton. Although, the neuronal and muscular galanin receptors have the similar properties, studies on characterizing the G proteins involved in these two groups of receptors using bacterial toxins indicated that galanin-receptor interaction in nerves involves a pertussis toxin-sensitive G protein, while the receptor in smooth muscle plasma membranes is coupled to a cholera toxin-sensitive, pertussis toxin-insensitive G protein. We conclude that, in canine small intestine, galanin may act as a neurotransmitter and/or neuromodulator by interacting with a specific receptor subtype coupled by distinct G proteins on smooth muscle membrane as well as synaptosomes.

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# Contents

|   |    |
|---|----|
| <b>Chapter I. Introduction</b>                      | 1  |
| 1-1. Discovery of Galanin                           | 2  |
| 1-2. Distribution and Function of Galanin           | 3  |
| 1-2-1. <i>Central Nervous System</i>                | 3  |
| 1-2-2. <i>Pancreas</i>                              | 4  |
| 1-3. Distribution of Galanin in Small Intestine     | 5  |
| 1-4. Effects of Galanin on Small Intestine          | 6  |
| 1-5. Galanin Receptors                              | 8  |
| <b>Chapter II. Literature Review</b>                | 11 |
| 2-1. Anatomy of the Small Intestine                 | 12 |
| 2-2. Structure of Smooth Muscle Cells in Gut        | 16 |
| 2-3. Control of Small Intestinal Smooth Muscle      | 17 |
| 2-3-1. <i>Myogenic Control</i>                      | 18 |
| 2-3-2. <i>Nerve Control</i>                         | 23 |
| 2-3-3. <i>Gastrointestinal Peptides</i>             | 29 |
| 2-4. Mechanisms in Ligand-Receptor Interaction      | 34 |
| <b>Chapter III. Peptide Receptor Identification</b> | 39 |
| — Ligand Binding Study                              |    |
| 3-1. Introduction                                   | 40 |

|  |   |           |
|--|---|-----------|
| 3-2.   | Ligand-Receptor Interaction                       | 40        |
| 3-3.   | Criteria for Receptor Identification              | 42        |
| 3-4.   | Analysis of Binding Data                          | 43        |
|  | 3-4-1. Saturation Study                           | 43        |
|  | 3-4-2. Kinetic Study                              | 44        |
|  | 3-4-3. Competition Study                          | 45        |
|  | 3-4-4. Two-state model                            | 45        |
| <b>Chapter IV. Hypothesis and Objectives</b> |   | <b>47</b> |
| 4-1.   | Hypothesis  | 48        |
| 4-2.   | Objectives  | 48        |
| <b>Chapter V. Methodology</b>                |   | <b>49</b> |
| 5-1.   | Membrane Preparation                              | 50        |
|  | 5-1-1. Introduction                               | 50        |
|  | 5-1-2. Materials                                  | 52        |
|  | 5-1-3. Tissue Selection and Handling              | 52        |
|  | 5-1-4. Membrane Preparation                       | 53        |
|  | 5-1-5. Analysis of Markers for Membrane Fractions | 54        |
| 5-2.   | <sup>125</sup> I-galanin Binding Assay            | 55        |
|  | 5-2-1. Materials                                  | 55        |
|  | 5-2-2. <sup>125</sup> I-galanin Binding Assay     | 55        |
| 5-3.   | Characteristic Study of Galanin Receptors         | 56        |



|   |    |
|---|----|
| 5-3-1. <i>Tissue Linearity</i>  | 56 |
| 5-3-2. <i>Equilibrium Binding Study</i>   | 56 |
| 5-3-3. <i>Time-course of Galanin Binding</i>  | 57 |
| 5-3-4. <i>Competitive Binding Study</i>   | 57 |
| 5-3-5. <i>Analysis of Molecular Weight of Galanin Receptor</i>  | 58 |
| <b>Chapter VI. Results</b>  | 59 |
| <b>6-1. Distribution of <sup>125</sup>I-galanin Binding Sites</b>   | 60 |
| 6-1-1. <i>Distribution of <sup>125</sup>I-galanin in Tissue Fractions Prepared by<br/>Differential Centrifugation</i> | 60 |
| 6-1-2. <i><sup>125</sup>I-galanin in Purified Nerve and Smooth Muscle Membranes</i>                                   | 60 |
| <b>6-2. Characterization of <sup>125</sup>I-galanin Binding Sites on Synaptosomes</b>                                 | 66 |
| 6-2-1. <i>Tissue Linearity</i>  | 66 |
| 6-2-2. <i>Equilibrium Binding Study</i>   | 66 |
| 6-2-3. <i>Time-course of <sup>125</sup>I-galanin Binding</i>  | 71 |
| 6-2-4. <i>Effect of GTP<math>\gamma</math>S on Dissociation of Bound <sup>125</sup>I-galanin in<br/>Synaptosomes</i>  | 71 |
| 6-2-5. <i>Competitive Binding Study</i>   | 76 |
| 6-2-6. <i>Effect of GTP analogue on Galanin Binding</i>   | 76 |
| 6-2-7. <i>Effect of Pertussis Toxin on <sup>125</sup>I-galanin Binding to<br/>Synaptosomes</i>                        | 76 |
| <b>6-3. Characterization of <sup>125</sup>I-galanin Binding Sites on Plasma Membrane of</b>                           |    |

|  |            |
|--|------------|
| Smooth Muscle  | 84         |
| 6-3-1. <i>Tissue Linearity</i>   | 84         |
| 6-3-2. <i>Equilibrium Binding Characteristics</i>  | 84         |
| 6-3-3. <i>Time-course of <sup>125</sup>I-galanin Binding</i>   | 89         |
| 6-3-4. <i>Competitive Binding of <sup>125</sup>I-galanin with Unlabelled Galanin, or Galanin Fragments</i> | 89         |
| 6-3-5. <i>Effect of Guanine Nucleotide</i>   | 94         |
| 6-3-6. <i>Effect of Cholera Toxin</i>  | 99         |
| 6-3-7. <i>Molecular Weight of Galanin Receptor</i>   | 99         |
| <b>Chapter VII. Discussion</b>   | <b>104</b> |
| 7-1. <b>Localization of Galanin Receptor</b>   | <b>105</b> |
| 7-2. <b>Binding Properties of Galanin Receptor</b>   | <b>107</b> |
| 7-3. <b>Structure-Activity in Receptor Recognition</b>   | <b>112</b> |
| 7-4. <b>G Protein Coupled Galanin-Receptor Interaction</b>   | <b>115</b> |
| 7-5. <b>Mechanisms of Neuronal Action of Galanin</b>   | <b>118</b> |
| 7-6. <b>Possible Effector in Mediation of Myogenic Action of Galanin</b>                                   | <b>120</b> |
| 7-7. <b>Galanin: a Neurotransmitter and/or Neuromodulator?</b>   | <b>121</b> |
| <b>Chapter VIII. Concluding Remarks</b>  | <b>124</b> |
| 8-1. <b>Conclusions</b>  | <b>125</b> |
| 8-2. <b>Future Directions</b>  | <b>125</b> |
| <b>References</b>  | <b>127</b> |

## List of Figures

|  |    |
|--|----|
| <b>Figure 1:</b> Distribution of [ <sup>125</sup> I]-galanin, [ <sup>3</sup> H]-saxitoxin and 5'-nucleotidase in crude membrane fractions  | 63 |
| <b>Figure 2:</b> Distribution of [ <sup>125</sup> I]-galanin, [ <sup>3</sup> H]-saxitoxin and 5'-nucleotidase in tissue fractions obtained by discontinuous sucrose gradient of synaptosomes and smooth muscle plasma membranes. | 65 |
| <b>Figure 3:</b> Tissue linearity binding of [ <sup>125</sup> I]-galanin in synaptosomes   | 68 |
| <b>Figure 4:</b> Equilibrium binding of [ <sup>125</sup> I]-galanin to synaptosomes  | 70 |
| <b>Figure 5:</b> Time-course of the binding of [ <sup>125</sup> I]-galanin to nerve membranes  | 73 |
| <b>Figure 6:</b> Effect of GTP $\gamma$ S on dissociation of [ <sup>125</sup> I]-galanin binding in synaptosomes   | 75 |
| <b>Figure 7:</b> Competitive binding of [ <sup>125</sup> I]-galanin with unlabelled galanin or galanin fragments in nerve membranes  | 79 |
| <b>Figure 8:</b> Effect of GTP $\gamma$ S on competitive binding of [ <sup>125</sup> I]-galanin with unlabelled galanin  | 81 |
| <b>Figure 9:</b> Effect of pertussis toxin on competitive binding of [ <sup>125</sup> I]-galanin with unlabelled galanin molecule  | 83 |
| <b>Figure 10:</b> Tissue linearity binding of [ <sup>125</sup> I]-galanin in smooth muscle plasma membrane   | 86 |
| <b>Figure 11:</b> Equilibrium binding of [ <sup>125</sup> I]-galanin in plasma membrane  | 88 |
| <b>Figure 12:</b> Kinetic study of binding of [ <sup>125</sup> I]-galanin in muscle membrane   | 91 |

|   |     |
|---|-----|
| <b>Figure 13:</b> Effect of GTP $\gamma$ S on the dissociation of [ <sup>125</sup> I]-galanin in plasma membrane              | 93  |
| <b>Figure 14:</b> Competitive binding of [ <sup>125</sup> I]-galanin with unlabelled galanin or galanin fragments             | 96  |
| <b>Figure 15:</b> Effect of GTP $\gamma$ S on competitive [ <sup>125</sup> I]-galanin binding in muscle membrane              | 98  |
| <b>Figure 16:</b> Effect of cholera toxin on competitive [ <sup>125</sup> I]-galanin binding in plasma membrane               | 101 |
| <b>Figure 17:</b> SDS-PAGE analysis of molecular weight of galanin receptor in synaptosomes and smooth muscle plasma membrane | 103 |

## List of Tables

|                 |   |     |
|-----------------|---|-----|
| <b>Table 1:</b> | Gastrointestinal peptides involved in regulation of gut motility  | 33  |
| <b>Table 2:</b> | Comparison of galanin receptor in nerve and smooth muscle plasma membrane preparations from canine small intestinal circular muscle/deep muscular plexus. | 108 |
| <b>Table 3:</b> | Summary of characterization of galanin receptors in various tissue sources  | 109 |
| <b>Table 4:</b> | Inhibitory constants ( $K_i$ ) of galanin and galanin fragments for galanin receptor in synaptosomes and smooth muscle plasma membranes                   | 114 |
| <b>Table 5:</b> | Classification of G proteins.   | 117 |

## List of Schemes

|  |    |
|--|----|
| <b>Scheme 1:</b> Structure of the small intestinal wall.                             | 15 |
| <b>Scheme 2:</b> Myogenic control of small intestine motility                        | 22 |
| <b>Scheme 3:</b> Relationship of enteric nervous system and extrinsic nervous system | 28 |
| <b>Scheme 4:</b> Modes of regulatory peptides acting on the target cells             | 32 |
| <b>Scheme 5:</b> Interaction of receptors, G protein and effectors                   | 38 |

## List of Abbreviations

**CCK:** cholecystokinin

**CNS:** central nervous system

**CTX:** cholera toxin

**DAG:** Diacylglycerol

**DMP:** deep muscular plexus

**DST:** disuccinimidyl tartarate

**DTT:** dithiothreitol

**ENS:** enteric nervous system

**ER:** endoplasmic reticulum

**GIT:** gastrointestinal tract

**GTP:** guanosine triphosphate

**GTP $\gamma$ S:** guanosine-5'-O-(3-thiotriphosphate)

**HEPES:** N-(2-hydroxyethyl) piperazine-N<sup>1</sup>-(2-ethanesulfonic acid)

**ICC:** interstitial cells of Cajal

**IP<sub>3</sub>:** Inositol-1,4,5-triphosphate

**IR:** immunoreactive

**MOPS:** 3-[-morpholino] propanesulfonic acid

**NO:** nitric oxide

**PNS:** post nuclear supernatant

**PKC:** protein kinase C

**PTX:** pertussis toxin

**SDS-PAGE:** sodium dodecyl sulfate-polayacrylamide gel electrophoresis

**SMB:** sucrose MOPS buffer

**STX:** saxitoxin

**TCA:** trichloroacetic acid

**TTX:** tetrodotoxin

**VIP:** vasoactive intestinal peptide

**VSSC:** voltage-sensitive sodium channel



## **Chapter I. Introduction**

## 1-1. Discovery of Galanin

It is known that many biologically active peptides contain an aminated carboxyl group, (i.e.  $-\text{CONH}_2$ ). Realizing the possibilities inherent in this C-terminal amino acid  $\alpha$ -amides structure, Tatemoto and Mutt developed a technique whereby C-terminal  $\alpha$ -amide amino acid residues are enzymatically cleaved off and identified by chromatography (Tatemoto and Mutt, 1978). Using this assay technique, they discovered several previously unknown peptides with the C-terminal aminated, one of them is galanin. In 1983, Tatemoto, *et al* found that the extracts of porcine intestine contain a previously unknown peptide with the C-terminal alanine-amide structure. They isolated the polypeptide and the amino acid sequence was determined using the chemical technique they devised. They named the peptide **GALANIN** designated from the N- and C-terminal residues, glycine and alanine. The porcine galanin is composed of 29 amino acid residues which are arranged as followed:

|   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1   | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 |
| Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-            |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 16  | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |    |
| Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH <sub>2</sub> |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

Sooner after galanin was first isolated, it was found that galanin is present in many animal species, *e.g.* rat, cow, dog, guinea-pig, rabbit, as well as in humans. The amino acid sequence of galanin from porcine, bovine, rat and human is identical in the first 15 N-terminal residues but differs at C-terminal residues (Tatemoto *et al*, 1983, Kaplan *et al*, 1988, Rokaeus and Carlquist, 1988, Bersani *et al*, 1991). Human galanin consists of 30, instead of 29, amino acid residues and is not aminated at the C-terminal end (Bersani

*et al*, 1991). Amino acid 1-22 of rat and porcine galanin are completely conserved. The amino acid residues only differ at position 23, 26, and C-terminal amidation in these two kinds of animals (Tatemoto *et al*, 1983). The sequence of galanin in dog has not been reported.

## **1-2. Distribution and function of Galanin**

Galanin has been suggested to be a new neuropeptide since it has been found, so far, only in the nervous system, including both the central and peripheral nervous systems, *e.g.* pancreas and gastrointestinal tract, and other peripheral tissues, such as the genito-urinary tract and the adrenal medulla (Melander *et al*, 1986, Bauer *et al*, 1986 b,c).

### **1-2-1. Central nervous system (CNS)**

Galanin-containing neurons are widely distributed throughout the spinal cord and the brain. In the spinal cord, it is most prominent in the dorsal horn and the lumbosacral region (Rattan, 1991). In the brain, a large number of galanin neurons are found in the medulla, locus caeruleus, midbrain, thalamus, hypothalamus and in the dorsal raphe nucleus (Ch'ng *et al* 1985). The highest concentration of galanin immunoreactivity, as well as the density of galanin binding sites, has been found in the hypothalamus and in the median eminence (Hokfelt *et al*, 1987, Melander *et al*, 1986b). In many of these areas, galanin coexists with several other neurotransmitters, *e.g.* acetylcholine, catecholamine, GABA, and serotonin (Melander *et al*, 1986b).

Several effects of galanin in CNS have been reported. It was shown that galanin modulates some neurotransmitter release, such as it inhibits dopamine, vasoactive intestinal polypeptide (VIP) and acetylcholine release (Ottlecz *et al*, 1988, Tsuda *et al*, 1992). Galanin increases the plasma level of growth hormone in human (Bauer *et al*, 1986) and rat (Ottlecz *et al*, 1988) when injected intravenously. Furthermore, it was reported that galanin induces a feeding response in rat. It was also demonstrated that galanin increased the consumption of high-fat containing food when it was injected into the hypothalamic paraventricular nucleus (Tempel *et al* 1988).

### ***1-2-2. Pancreas***

Several reports have been shown the occurrence of galanin in the pancreas of several species, *e.g.* rat, dog, pig, and humans (Dunning *et al*, 1986, Messell *et al*, 1990, Rokaeus, 1987, McDonald *et al*, 1992). The distribution of galanin immunoreactive (IR) nerve fibres in pancreas is different among these species. In dog and rat, galanin-IR nerve fibres form a dense network within the islets. However, in pig and human, there are no galanin-IR fibres associated with the islets, but instead, the galanin-IR nerves ramify throughout the exocrine pancreas (Rokaeus, 1987, McDonald *et al*, 1992).

In consonance with galanin's distribution in pancreas, a role of galanin in controlling pancreatic function has been suggested. One of the most extensive studied and important function of galanin is that it inhibits insulin secretion in some animal species. It was demonstrated that galanin decreased basal level of plasma insulin and produced

hyperglycaemia when it was given intravenously to dogs or rats (Fatemoto *et al*, 1983). Galanin also suppressed insulin secretion response to parenteral glucose, cholecystokinin (CCK), oral glucose or a mixed meal (McDonald *et al*, 1986). Stimulation of autonomic pancreatic nerves resulted in the inhibition of insulin secretion and increase in galanin concentration in a pancreas preparation studied *in situ* in anaesthetized dog (Dunning and Taborsky, 1989). In contrast, galanin has no effect on insulin release in humans (Gilbey *et al*, 1989, Ahren, 1990), whereas in the perfused pig pancreas, discrepant results, *i.e.* both inhibition and stimulation of insulin release, were reported (Messell *et al*, 1990). These studies suggest that the location of galanin containing nerves in relation to pancreatic islet cells is of importance in terms of potential physiological function of galanin in pancreas.

The effect of galanin on glucagon secretion is variable and seems dependent on experimental condition and species. *In vivo*, galanin stimulates glucagon secretion in dog and mice (Lindskog and Ahren, 1987) but inhibits basal glucagon secretion in rat (Lindskog and Ahren, 1989). *In vitro*, galanin inhibits glucagon secretion in the perfused dog pancreas (Hermansen, 1988), but stimulates the peptide secretion in the perfused pig pancreas (Messell *et al*, 1990). The reason for these discrepancies is not known.

### **1-3. Distribution of galanin in small intestine.**

Galanin neurons are present in intrinsic nerves of the enteric nerve plexus since extrinsic nerve denervation by surgical or chemical method does not affect their distribution (Bishop *et al*, 1986). Galanin nerve fibres project anally into all the layers

of the intestinal wall, with most in muscle layers and only a few in mucosa. There are some species differences regarding the distribution of galanin neurons in the gut. In the rat, mouse, pig and guinea-pig, galanin-containing nerve cells are present predominantly in the submucous plexus and, with less amount, in the myenteric plexus (Ekblad *et al*, 1985, Furness, 1987). Galanin-IR fibres are found in circular muscle layers in rat, mouse and pig, but are absent in guinea-pig (Melander *et al*, 1985). In humans, galanin fibres are absent in the myenteric plexus (Bishop *et al*, 1986). In canine, there is a higher density of galanin nerve cell bodies in the submucos plexus than in the myenteric plexus and more galanin immunoreactive neurites are present in the circular muscle of the intestine than in the longitudinal muscle layer (Gonda *et al*, 1989). However, it should be mentioned that in several of these species, the antibodies used were not against the galanin in that species and their use may have caused false negative findings.

In many animal species, *e.g.* porcine, dog, most galanin, if not all, coexists with VIP in cell bodies of the submucous plexus and in fibres of the muscle layers (Ekblad *et al*, 1985, Bishop *et al*, 1986, Gonda *et al*, 1989).

#### **1-4. Effects of Galanin on the Small Intestine**

It has been shown that galanin has effects on both the secretion and the motility of small intestine and these effects vary in different animal species.

*Effect on secretion:* Galanin regulates the release of a number of gastrointestinal (GI) peptides. Galanin inhibits gastrin release and gastric acid output in rat and dog (McDonald *et al*, 1992). In humans, galanin potently inhibits the postprandial release of

glucagon, neurotensin, somatostatin, and pancreatic polypeptide (Bauer *et al*, 1986a, 1989).

*Effect on motility:* Despite the wide distribution of galanin in the enteric nervous system, the biological action of galanin is not as yet known. Functional studies, *in vivo* or *in vitro*, suggest that galanin may have dual functions in gut, *i.e.* as neurotransmitter and/or as neuromodulator.

The effect of galanin on smooth muscle contraction is variable and species-dependent. Galanin contracts the longitudinal muscle layer in rat, pig and circular muscle layer in pigs (Ekblad *et al*, 1985a, Brown *et al*, 1990, Delvaux *et al*, 1991, Messell *et al*, 1992). In contrast, galanin inhibits contraction of circular muscle in dogs (Fox *et al* 1986). These effects, either excitation or inhibition, are tetrodotoxin (TTX)-insensitive, suggesting a direct smooth muscle action by galanin. However, in guinea-pig, galanin does not alter the basal tone of smooth muscle but instead, inhibits the neurally evoked contraction (Kuwahara *et al*, 1989). In dog intestine, too, there are neuronal effects (see below). It was shown that galanin depressed acetylcholine release induced by electrical stimulation or caused by substance P, indicating that galanin might act at the presynaptic level to modulate neurotransmitter release, thus indirectly affecting motor activity of the smooth muscle (Kuwahara *et al*, 1989). The role of galanin as a neuromodulator was also suggested by other studies. Fox *et al* (1991) demonstrated that intraarterial infusion of galanin into isolated perfused canine ileal segments was associated with dose-dependent inhibition of VIP release.

Evidence has been presented showing that N-terminal galanin fragments, *e.g.*

galanin 1-20, 1-15, are equipotent to the parent molecule in eliciting the actions of this neuropeptide. In contrast, the C-terminal fragments, *e.g.* 15-29, 20-29, have no, or only a weak effect (Fox *et al*, 1988). Therefore, the effect of galanin on small intestine motility may require the presence of the N-terminal portion of galanin. In guinea-pig, the whole molecule is essential for full potency (Kuwahara *et al*, 1990), while in rat jejunum, galanin 1-29 and galanin 1-10 evoke an effect to a similar extent (Ekblad *et al*, 1985, Fox *et al*, 1988).

### **1-5. Galanin Receptors**

In accordance with the wide distribution of galanin neurons, specific galanin binding sites have been demonstrated in CNS, pancreas, and small intestine.

A population of high-affinity ( $K_d = 0.6 \sim 0.9$  nM) binding sites for galanin has been found in rat brain, dorsal spinal cord, and hippocampus (Hedlund *et al*, 1992, Bedecs *et al*, 1992, Consolo *et al*, 1991, Fisone *et al*, 1989). Studies have shown that the binding requires the presence of an N-terminal portion and is coupled to a G-protein.

Binding sites for galanin are identified and characterized in membranes from hamster pancreatic  $\beta$ -cell tumour and rat pancreatic cell lines Rin m5F and Rin 14B (Amiranoff *et al*, 1987, 1989a, 1991). In these cells, a single class of galanin receptors was revealed with high affinity ( $K_d = 0.3 \sim 1.5$  nM) and binding capacity of 44  $\sim$  88 fmol/mg protein. By using chemical cross-linking technique, the galanin receptor was found to be a glycoprotein with molecular weight of 54 KDa (Amiranoff *et al*, 1989b).

Studies with fragments of galanin to elucidate the recognition properties of the



galanin receptor at the pancreatic cell lines demonstrated that it is the N-terminal portion of galanin that is critical for binding (Langny-Pourmir *et al*, 1989b, Gallwitz *et al*, 1990). This corresponded with the functional studies in which the inhibition of insulin release required the existence of N-terminal amino acid residues (McDonald *et al*, 1992). However, other evidence showed that the C-terminal portion is of importance for the full biological activity of the peptide based on the less potency of galanin fragments, *e.g.* 1-15, 1-11, to evoke the response as compared to the entire galanin molecule (Kuwahara *et al*, 1990).

There are several lines of evidence indicating that the galanin receptor in the pancreas is functionally coupled to a pertussis toxin-sensitive regulatory G-protein (Amiranoff *et al*, 1989, Lagny-Pourmir *et al*, 1989b): 1) GTP analogues, *e.g.* GTP $\gamma$ S, Gpp(NH)p, reduce <sup>125</sup>I-galanin binding to the cell membrane and increase the rate of dissociation 2) pretreatment of the insulin-secreting cells with pertussis toxin, which is suggested to ADP-ribosylate the G $\alpha$  subunit of the G-protein thus uncoupling the receptor from G proteins, dramatically reduces <sup>125</sup>I-galanin binding to the receptors.

The signal transduction pathway for galanin-receptor induced inhibition of insulin release has been studied in tumoral insulin-secreting cell line Rin m5F and in normal islet  $\beta$  cells (Amiranoff *et al*, 1989, Lindskog and Ahren, 1991). Multiple pathways have been proposed to be involved in the inhibition of insulin release, including: 1) hyperpolarization of the cell membrane by activation of ATP-sensitive K<sup>+</sup> channels and decrease in cytoplasmic free Ca<sup>2+</sup> concentration (Dunning *et al*, 1989); 2). inhibition of adenylate cyclase and subsequent decrease in cAMP production (Amiranoff *et al*, 1991);

3). inhibition of protein kinase C activity (Sharp *et al*, 1989); and 4). in Rin m5F cells, galanin may inhibit insulin release by directly affecting the exocytotic process (Ullrich and Wollheim, 1989).

Galanin binding sites, by receptor autoradiography, have been found in the myenteric plexus and longitudinal muscle layers of small intestine in a rat, rabbits, guinea-pig, and human (King *et al*, 1989). A single binding site for galanin was reported in a rat jejunal smooth muscle membrane preparation using a radioligand binding technique (Rossowski *et al*, 1990). This study suggested that the C-terminal but not the N-terminal portion of galanin is crucial for binding. There have been no reports on the signal transduction mechanism for galanin-receptor induced action on gastrointestinal tract (GIT). The receptor for galanin in GIT of dog has yet to be identified.

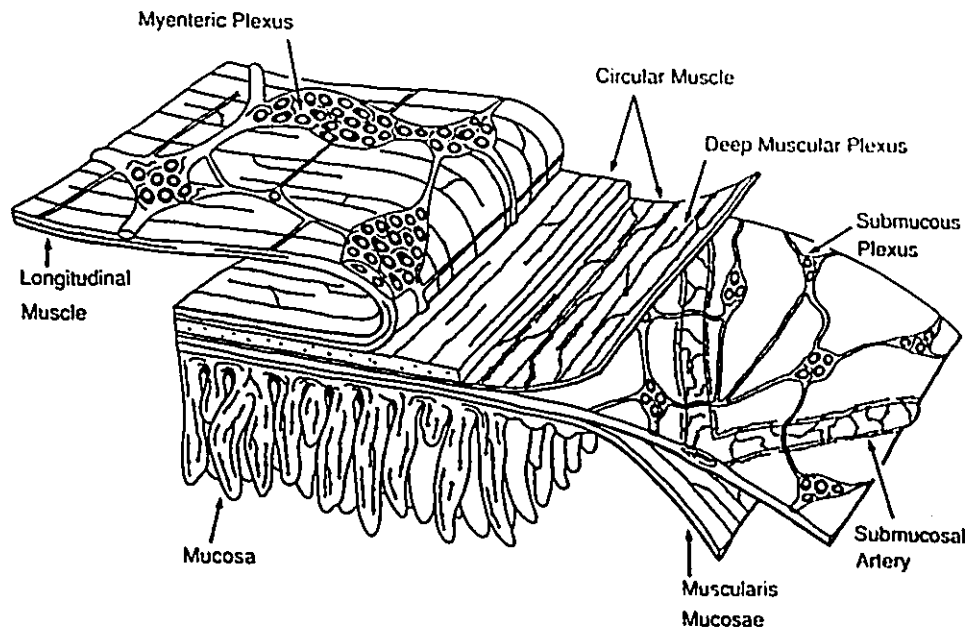
## **Chapter II. Literature Review**

## 2-1. Anatomy of the Small Intestine

The small intestine is a long tubular structure that connects stomach and colon. Its length varies between different species. In humans, it is about 18-24 feet long (Wood, 1981), while in dog, it is usually 10 ~ 15 feet in length. The wall of the small intestine is composed (from cavity to the outer layer) of: mucosa, submucosa, circular muscle layer, myenteric plexus layer, and longitudinal muscle layer (Scheme 1) (Furness and Costa, 1987). The epithelium of the mucosa comes into contact with the foodstuffs and functions in digestion and absorption. Outside the mucosa is the submucosa composed of submucous plexus, connective tissue, lymphatic and vascular tissues. The two muscle layers are oriented at 90-degree angles to each other to form an outer longitudinal layer and an inner circular layer separated by the myenteric plexus layer. In mammals, most nutrients, electrolytes and water are absorbed by the small intestine as they are moved along the gastrointestinal tract (GIT). Contraction patterns of the smooth muscle cells of the two muscle layers of small intestine are organized to bring about mixing the food stuffs with digestive enzyme and movement of the contents into contact with the absorptive cells of the mucosa and to transport the residues into large intestine (Hasler, 1991). It is believed that the circular layer plays the major role in mixing and propulsion of intestinal contents because circular contractions can cause occlusion of the lumen and displacement of gut contents. Although longitudinal muscle contraction has little effect on mixing and propulsion, some physiological functions of this muscle layer have suggested, *e.g.* increase luminal diameter to facilitate the passage of a large bolus, and shorten the gut to transit chyme longitudinally (Macagno *et al*, 1975). It also may

provide a pathway controlling the spread of intestinal activity along the axis of the organ. The enteric nervous system, which consists of a number of interconnected plexuses of nerves, is found within the wall of small intestine. The major plexuses include: submucous plexus which is within the submucosa, and myenteric plexus which is located in between the longitudinal and circular muscle layers. In some species, such as human and dog, a plexus named deep muscular plexus is located between submucosa and circular muscle layer (Furness and Costa, 1987b). It lacks nerve cell bodies, but contains nerve fibres and interstitial cells of Cajal (Furness and Costa, 1987b).

**Scheme 1:** Schematic representation of the components of small intestine wall (from Furness and Costa, 1987b).



## 2-2. Structure of Smooth Muscle Cells in the Gut

The smooth muscle cells in the GIT are arranged in bundles. Each bundle of muscle fibers is separated from each other by connective tissue, but at some points the bundles fuse with each other. Single smooth muscle cells are spindle-like structure with up to 400  $\mu\text{m}$  length and 5  $\mu\text{m}$  wide when fully relaxed (Makhlouf, 1991). The plasma membrane of smooth muscle cells has specialized structures, known as caveolae. The caveolae, 70 nm wide and 120 nm deep sacs, are invaginations of the membrane and arranged in clusters lined up along the surface of the cells. There are about 15,000 ~ 17,000 caveolae per cell and they increase its surface area by one third. It is believed that caveolae may be functionally involved in calcium transport across the membrane based on the evidence that the cytoplasmic side of the caveolae are closely associated with an abundant endoplasmic reticulum (ER) which controls the release and storage of calcium in smooth muscle (Gabella, 1987).

Clusters of caveolae are separated, in the cytoplasmic side of the cell membrane, by electron dense patches, called dense bands, 1 ~ 2  $\mu\text{m}$  long and 0.2 ~ 0.4  $\mu\text{m}$  wide (Gabella, 1987). These dense bands are attached to thin actin filaments which, at the other end, interdigitate with thick myosin filaments. The dense bands are connected with each other by intermediate thick filaments within the cell, or are juxtaposed to adjacent cells at locations called intermediate junctions which are narrowed intercellular spaces (30 ~ 40 nm wide) (Gabella, 1987). Therefore, adjacent smooth muscle cells are linked by these intermediate junctions which couple the contractile apparatus of the muscle cells and transmits force from one cell to the next (Hasler, 1991). In some regions within the



muscle layer, patches of the plasma membrane of adjacent cells are closely apposed, known as gap junctions or nexuses (Gabella, 1987). The space between gap junctions is less than 3 nm. The gap junction functions as a channel to permit movement of ions and small molecules, *e.g.*  $\text{Ca}^{2+}$ , cAMP, between the cells, thus propagating the signal from one cell to the other (Gabella, 1987). Therefore, the whole muscle layer, longitudinal or circular, functions as a syncytium when an action potential is elicited. Dense bands and contractile filaments occupy about 80% of the content of cytoplasm, the rest is occupied by various organelles, including ER (2%), mitochondria (5 ~ 9%), Golgi apparatus, and lysosomes (Makhlouf, 1991). Evidence shows that the ER is the site of  $\text{Ca}^{2+}$  storage and release (Daniel *et al*, 1985), while the mitochondria may be involved in taking up excessive calcium only when cytosolic  $\text{Ca}^{2+}$  increases above physiological levels (1 ~ 5  $\mu\text{M}$ ) (Rayemakers *et al*, 1977, Burgess *et al*, 1983).

### **2-3. Control of Small Intestinal Smooth Muscle**

The major function of the small intestine is processing and absorption of nutrients. It requires both mixing of chyme with the bile and digestive enzymes and forward propulsion of the chyme in an aboral direction. Mixing and propulsion occur not only after a meal but under fasting conditions as well. The movements of the small intestine, termed "intestinal motility", are controlled by many factors, including: both intrinsic and extrinsic innervation of the small intestinal wall, circulating gastrointestinal hormones, and myogenic characteristics of small intestinal smooth muscle (Wood, 1981, Hasler, 1991).

### **2-3-1. Myogenic control of small intestinal smooth muscle:**

Unlike other type of smooth muscle, the smooth muscle of GIT has two basic types of electrical activity: slow waves and spike potentials as illustrated in figure 2-2 (for review, see Hasler, 1991, Makhlouf, 1991).

The slow wave is an ubiquitous oscillating fluctuation in membrane potential with intensity varying between 3 ~ 18 millivolts and the frequency ranges between 3 ~ 12 per minutes in human and 10 ~ 20 per minutes in dog. The frequency decreases aborally in human and canine intestine from 12 ~ 20 cycles/min in the duodenum to 7 ~ 10 cycles/min in the ileum. Intracellular electrodes record a typical slow wave consisting of a rapid depolarization, a partial repolarization, a prolonged plateau phase of depolarization followed by a complete repolarization to resting membrane potentials (normally -50 ~ -60 mv) (Scheme 2. A). Evidence has been assembled suggesting that slow waves originate in the interstitial cells of Cajal (ICC) located at the junction of the two smooth muscle layers (Thuneberg, 1989). Morphologically, ICC have some distinct characteristics as compared to other types of cells: a single large nucleus, an abundance of processes, surface caveoli, mitochondria, rough endoplasmic reticulum and intermediate filaments. ICC are extensively innervated and form close contacts, often gap junctions, with smooth muscle cells and other ICC (Komuro, 1989). Slow waves, generated by ICC, propagate within the circular muscle layers through the abundant low-resistance gap junctions. ICC are also located in the deep muscular plexus and may form a secondary set of pacemaker generators (Barajas-Lopez *et al*, 1989). However, the structure responsible for electrical coupling in longitudinal layers is still unknown since

few or no gap junctions have been found by electron microscopic examination in this layer (Gabella, 1987). The ionic mechanism for generation of slow waves is not as yet known although some studies suggest it might be caused by a decrease in conductance of  $K^+$  ions. It is possible that initiation of slow waves depends on metabolic events in the ICC. The slow waves do not necessarily cause muscle contraction because they may not reach a critical level of depolarization that triggers the action potentials (Wood, 1981). However, it has recently been shown that in some cases the slow wave *per se* can initiate  $Ca^{2+}$  entrance and muscle contraction (Daniel *et al*, 1985). The slow waves control the appearance of intermittent spike potentials which actually cause muscle contraction. Spike potentials, which are action potentials, occur during the plateau potential (about -40 mv) of the slow wave, and, the higher the amplitude of the plateau potential, the greater the frequency of the spike potentials (Scheme 2. B). Usually the spike potentials have about 30 mv amplitude and durations vary within 10 ~ 100 milliseconds. Evidence showed that the depolarization phase of the spike potential is carried mainly by calcium. The calcium channels open and close more slowly than the  $Na^+$ -channels thus this may account for the longer duration of the spike potential compared to that of action potentials in nerve and striated muscles.

Contraction of small intestinal smooth muscle shows two major types: phasic and tonic (Hasler, 1991). In dogs, as well as in humans, most of intestinal contractions are phasic, and are under the control of spike potentials and slow waves. Tonic contractions, with periods from 10 sec. to as long as 8 minutes, are sometimes seen in the circular muscle layer. The mechanism involved in tonic contraction is not known.

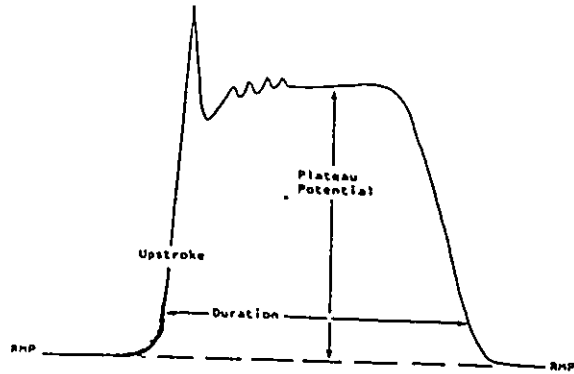
Stimulatory and inhibitory neurohormonal input influences the amplitude of plateau potential of slow wave and thus the frequency of spike potential, therefore the magnitude and occurrence of phasic contraction in the intestine is primarily controlled by neural and humoral factors (Scheme 2. B)

**Scheme 2:** Profile of a typical slow wave (panel A) and the relationship of slow wave (SW) and spike potential (SP) in smooth muscle (panel B). (Modified from Hasler, 1991, Makhlouf, 1991).

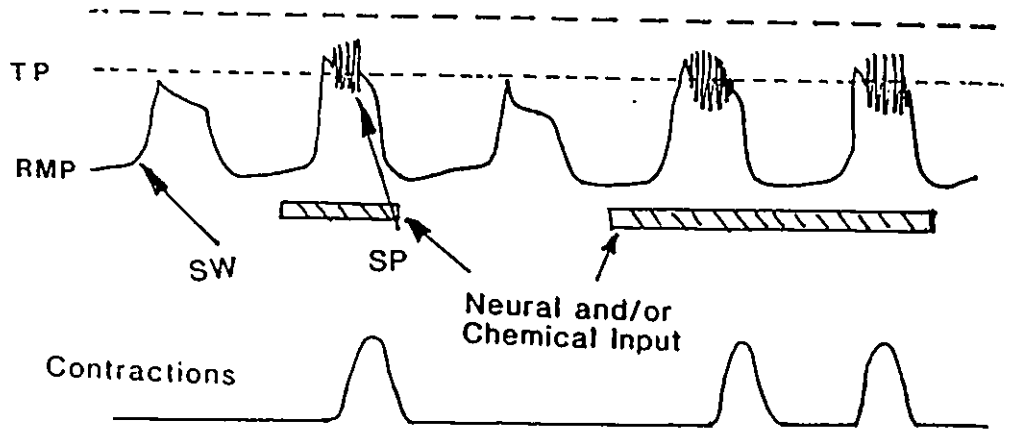
**A panel:** A slow wave is composed of a rapid upstroke followed by partial repolarization , then a plateau potential of variable duration, and a complete repolarization.

**B panel:** Without neural and/or chemical input, slow waves exhibit continuous oscillation that usually can not reach the threshold potential (TP), therefore no contraction occurs. Spike potentials are generated superimposed on plateau potential of SW by neural/chemical input and induce smooth muscle contraction. Note that, as shown in panel B, there are some slow waves which reach contraction threshold without action potentials. RMP: resting membrane potential.

A



B



### **2-3-2. Neuronal control of small intestinal smooth muscle:**

The muscle layers of the small intestine are extensively innervated by both intrinsic nerves, *i.e.* the enteric nervous system, and extrinsic nerves that have their neurons outside the gut (for review, see Furness and Costa, 1987b).

Intrinsic innervation: The enteric nervous system (ENS) consists of a large number of nerve cell bodies with their projections embedded in the wall of the GIT (scheme 3). The grouped nerve cell bodies form the enteric ganglia which are connected by nerve projections to form two major ganglionated plexuses, the myenteric (Auerbach's) plexus and the submucous (Meissner's) plexus. It is the myenteric plexus, located in between the longitudinal and circular muscle layers, that predominantly controls the motor activity of the small intestine. The submucous plexus plays a major role in regulation of nutrient and water absorption from the mucosa but is considered to have little role in motility. Neurons in the myenteric plexus project most of their fibres to other neurons in the plexus and the circular muscle layer. They also send processes to submucos and mucosa layer. There are a small number of nerve projections to the longitudinal muscle layer. In canine, as well as in human intestine, there is a thin dense plexus of nerve fibres near the submucosal border of circular muscle layer, termed deep muscular plexus (DMP) (scheme 1). DMP lacks nerve cell bodies and its nerves derive primarily from neurons of myenteric plexus. Thus they are involved in control of small intestinal smooth muscle activity (Furness, 1987). The nerve projections that innervate the muscle cells are arranged in bundles with each nerve axon surrounded by enteric glial cells except where axons swell to form multiple varicose structures packed with synaptic

vesicles. There are a few extrinsic nerves that send projections to prejunctional ganglia. Unlike the innervation of striated muscle cells, there is no special postjunctional specialization at junction of nerve ending connected with smooth muscle cells, in GIT (Furness, 1987). The vesicle containing nerve fibres simply come close to the muscle cells or ICC and the neurotransmitter released from axonal varicosities diffuses through the junction space (20 ~ 100 nm) to interact with the specific receptors on membranes of smooth muscle cells, other neurons, or ICC (Hasler, 1991).

Neurons in the myenteric plexus fall into two categories in relation to motor control: those that contain transmitters to cause muscle contractions are excitatory neurons, and those that contain transmitters to cause relaxation of the smooth muscle are inhibitory neurons. A peptide present in neurons of myenteric plexus must fulfill the following criteria to be identified as an neurotransmitter (Makhlouf, 1991):

- 1). immunocytochemical demonstration of the presence of the neuropeptide in nerve terminals.
- 2). synthesized by the neurons and released by physiological stimulation.
- 3). release of the peptide should elicit a biological effect that can be mimicked at a cellular level by exogenous application of the peptide and blocked by specific antibodies or antiserum or, antagonists.

Although it has been shown that the excitatory neurons of the myenteric plexus are primarily cholinergic, there are atropine resistant contractions of smooth muscle in response to nerve stimulation in many intestinal systems (Wood, 1981) and, current evidence suggests that neuropeptides, e.g. substance P or neurokinin A, may be the

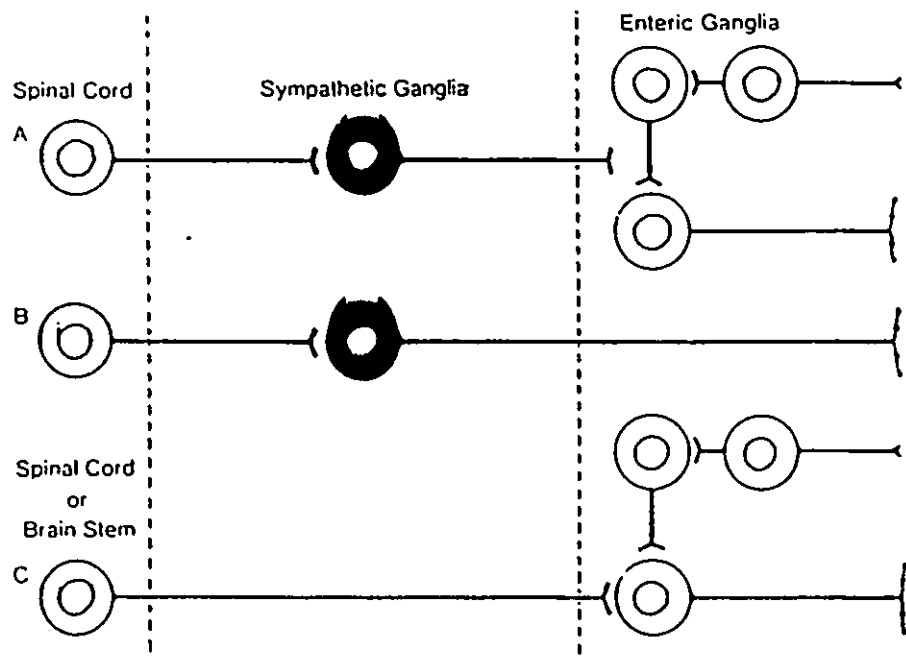


neurotransmitters for the atropine-resistant contraction (Sanders and Publicover, 1989). The inhibitory neurotransmitter has not been unequivocally identified. There are several lines of evidence which suggest that vasoactive intestinal peptide (VIP) might be the inhibitory neurotransmitter (Grider *et al*, 1985): 1) The wide distribution of VIP immunoreactive nerves and their projections are consistent with inhibitory reflexes in GIT; 2) The release of VIP by stimulation of inhibitory nerves; and 3) The ability of VIP to directly relax intestinal smooth muscle with the effect is prevented by VIP antagonists or antibodies. Moreover, in some regions these antagonists or antibodies reduce responses to stimulation of inhibitory nerves. However, the inhibitory neurons contain more than one transmitters. Many neuropeptides, *e.g.* neuropeptide Y (NPY), the opiate peptides, dynorphin, enkephalin and galanin, have been found to coexist with VIP to varying degrees in nerves projecting anally to other ganglia and to circular muscle (Makhlouf, 1991). The coexisting transmitters may be released together to elicit combined influences on the target cells, *i.e.* smooth muscle cells or adjacent neurons. Recently, evidence in support of nitric oxide (NO) as an inhibitory noncholinergic, nonadrenergic (NANC) neurotransmitter has been provided (Bult *et al*, 1990, Boeckxstaens *et al*, 1990, Murray *et al*, 1991). It was shown that electrical field stimulation (EFS) of NANC nerves caused relaxation of intestinal smooth muscle and this response was mimicked by exogenous NO. N<sup>G</sup>-nitro-L-arginine (L-NNA), a specific inhibitor of NO synthase inhibited the EFS-induced relaxation and the inhibitory effect was antagonized by L-arginine which is the substrate for NO synthase (Murray *et al*, 1991). The role of NO in control of smooth muscle activity in GIT and the relationship

of NO with other putative inhibitory transmitter, *e.g.* VIP, is under intense investigation and is far from clear. More recently, immunohistochemical studies demonstrated that NO synthase and VIP are co-localized in enteric nerves in guinea-pig small intestine (Costa *et al*, 1991) and in canine gut (Berezin *et al*, 1992), implicating that correlations may exist between NO production and VIP release and thus between their effects on smooth muscle cells.

Extrinsic innervation: The extrinsic innervation of the small intestinal smooth muscle includes parasympathetic, sympathetic and sensory nerves (Furness, 1987). Most, if not all, extrinsic nerve fibres terminate at the level of the myenteric or submucous plexus and form connections within the enteric ganglia cells which in turn supply the nerve projections to the smooth muscle cells. Scheme 3 shows the relationship of extrinsic nerve and ENS. Parasympathetic neurons project their cholinergic nerve fibres directly from the spinal cord or brain stem to the enteric ganglia and act on nicotinic receptors on ganglion cells within the myenteric plexus. Stimulation of parasympathetic nerves is the main cause of the smooth muscle contraction. The sympathetic innervation proceeding by the way of splanchnic nerve has its neuronal cell bodies outside the GIT. The preganglionic neurons in the spinal cord, which are cholinergic, project to the prevertebral ganglia from which the postganglionic neurons, which are noradrenergic, send the nerve processes, *i.e.* splanchnic nerves, to enteric ganglia. The noradrenergic stimulation generally elicits an inhibition of smooth muscle contraction which may be induced by inhibition of release of excitatory mediators.

**Scheme 3:** Diagrammatic representation of the relationship of the enteric nervous system and extrinsic sympathetic (A, B) and parasympathetic system (C) in gut (from Furness, 1987). Most sympathetic nerves have preganglionic neurons in the prevertebral ganglia, and postganglionic neurons project into enteric neurons (A). Some sympathetic neurons project directly into gut (B). The parasympathetic neurons send their nerve fibers directly from the spinal cord or brain stem to the enteric ganglia (C).



### 2-3-3. *Gastrointestinal peptides*

Since the first hormone was discovered in 1902 (Bayliss and Starling, 1902), there has been more than 20 hormones found in GIT. The word "hormone" is derived from the Greek word meaning "to arouse to activity". Studies to date have shown that almost all GI hormones are peptides. Therefore, the term "GI peptides" is often used instead of hormone, especially if a definitive physiological action has not been established. According to "classical endocrinology", a hormone is a chemical which is secreted by endocrine cells and released into the blood in response to a physiological stimulus and exerts its biological effect on distant target organs. However, GI peptides have several distinct aspects as compared to the "classical hormones" (McDonald, 1991): 1) relatively low tissue concentration in GIT; 2) present not only in GI but also in a number of other tissues, *e.g.* brain, adrenal medulla; 3) synthesis and release not only by endocrine-type cells, but also by neural structures as well, leading many GI peptides to be called neuropeptides; 4) transport to the target cells by four distinct mechanisms: endocrine, neurocrine, paracrine, and autocrine (Scheme 4) (Walsh, 1987).

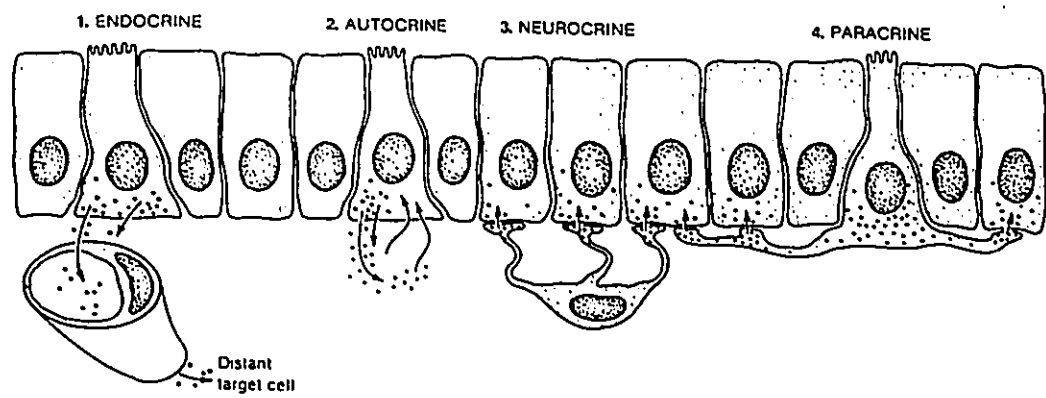
An endocrine peptide, such as gastrin, or secretin, acts in the fashion of a classical hormone. A neurocrine peptide, such as substance P, or CCK, is released from nerve endings and acts in the manner of a neurotransmitter (on smooth muscle) or neuromodulator (on other neurons). A paracrine peptide, *e.g.* somatostatin, is released to exert effects on cells in the immediate vicinity. Autocrine peptides, *e.g.* insulin-like growth factors 1 and 2, are released from either an endocrine cell or a nerve to act on a receptor on their cells of origin to modulate further release of the same peptide or other

peptides. The coexistence of neurotransmitters within the same nerve endings, such as VIP/galanin, substance P/ acetylcholine, may regulate the release of each other by autocrine mechanisms.

The nerves and smooth muscle of the small intestine are responsive and sensitive to a wide variety of GI peptides. Some of the GI peptides are exclusively present in neuronal structures, *e.g.* VIP, substance P, galanin, and function as neurotransmitters or neuromodulators (*i.e.* regulate other neurotransmitter release). It seems likely that these neuropeptides play an important role in the control of small intestinal motility. The effects of these GI neuropeptides on smooth muscle activity are either excitatory, inhibitory or both. Table 1 shows some of the GI peptides involved in regulation of motor functions of small intestine.

**Scheme 4:** Proposed modes of action of regulatory peptides on target cells (From Tayler and Mannon, 1991). See text for explanation.

## MECHANISMS OF ACTION OF GI PEPTIDES





**Table 1:** Gastrointestinal peptides in regulation of gut motility: (derived from Taylor, 1991, and Mannon, 1991).

| Peptides       | Cells of origin                                | Motor effect                          |
|----------------|--|---------------------------------------|
| Somatostatin   | endocrine "D" paracrine cells and nerves       | Inhibitory                            |
| CCK            | endocrine cells in duodenal and jejunal mucosa | excitatory                            |
| Substance P    | endocrine cells & nerves in the gut            | excitatory                            |
| VIP            | nerves throughout the gut                      | inhibitory                            |
| Motilin        | endocrine cells in duodenal mucosa             | excitatory                            |
| Neurotensin    | endocrine cells in ileum and neurons           | inhibitory or excitatory<br>in dogs   |
| Peptide YY     | endocrine cells in ileo-colonic mucosa         | inhibitory, excitatory<br>in dogs     |
| Galanin        | enteric nerves                                 | inhibitory or excitatory              |
| Opioids        | nerves throughout the gut                      | inhibitory, but excitatory<br>in dogs |
| Neuropeptide Y | enteric nerves                                 | vasoconstriction                      |

## 2-4. Mechanisms in ligand-receptor interaction

The external signals (*e.g.* neurotransmitter, hormone, or drug) need to be transduced into an internal signal to generate biological actions. The neuropeptide induced biological activity, such as neurotransmitter release or contraction/relaxation of smooth muscle, is achieved by the interaction of the ligand with a specific receptor which in turn activates the transducing systems. The transduction pathway, for most of ligand-receptor interactions, involves a guanosine triphosphate (GTP)-binding protein (G protein) which couples the receptor to a particular effector enzyme (second messengers) or directly to ion channels (Gilman, 1987).

**Receptor:** The receptor for peptides is a transmembrane protein consisting of external, membrane-spanning, and cytoplasmic domains with features that determine the recognition ability to a specific ligand. Binding of the ligand to its receptor is highly specific and, in the case of an agonist, will subsequently elicit biological response. The function of a receptor is to recognize a specific ligand and then convert this information into a internal signal which results in a physiological effect. Most peptide receptors are linked to heterotrimeric GTP-binding proteins that function as a signal transducer.

**G proteins:** G proteins are a large family of closely related guanine nucleotide-binding proteins that function as signal transducers (Casey and Gilman, 1988, Gilman, 1987). G proteins are heterotrimers, with subunits designated  $\alpha$ ,  $\beta$  and  $\gamma$  in order of decreasing mass. The differentiation of G proteins is determined by  $\alpha$  subunit which contains a single, high-affinity binding site for GTP and possesses GTPase activity.  $\beta$  and  $\gamma$  subunits are similar among the various G proteins. The steps in G protein coupled

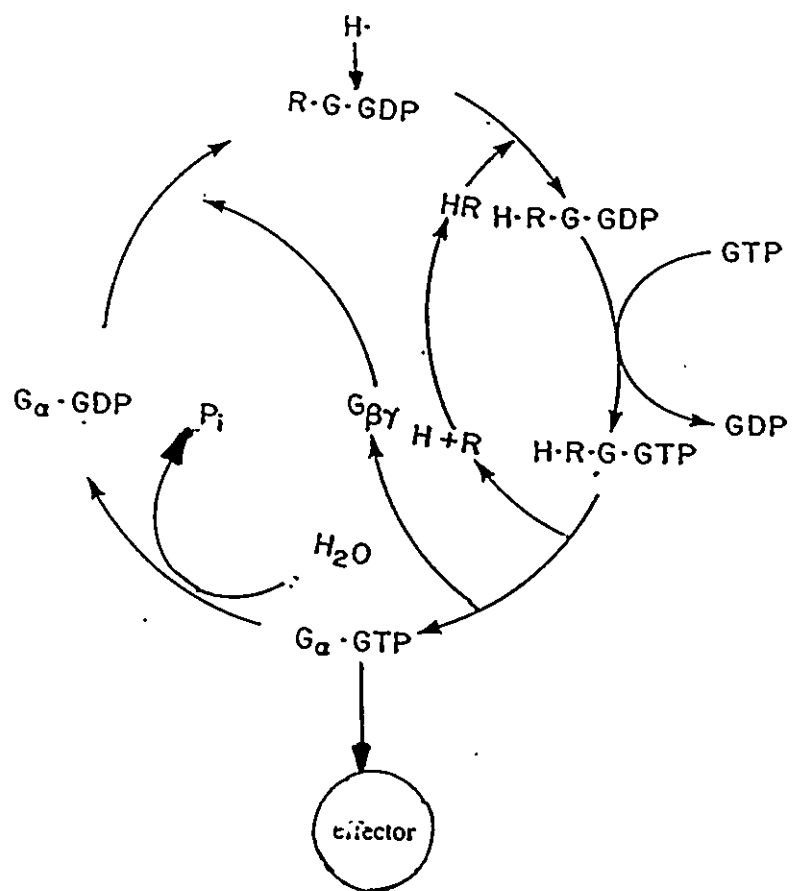
signal transduction are shown in Scheme 5. In the basal state, the  $\alpha$  subunit is tightly bound by guanine diphosphate (GDP). The binding of a ligand to its receptor stimulates the dissociation of GDP and association of GTP with the  $\alpha$  subunit. This causes: 1) a decrease in the affinity of receptor for the ligand and 2) dissociation of  $\beta\gamma$  complex from G protein to generate  $G\alpha$ GTP complex which is usually the actual unit to activate the effector enzyme. The intrinsic GTPase activity of the  $G\alpha$ GTP complex hydrolyses GTP to GDP and Pi, This terminates the activity of G proteins (i.e. deactivation) and enables the reassociation of the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. The receptor-G protein complex is then ready for another "activation-deactivation cycle" to deliver the signal of another molecule of agonist. Non-hydrolysable GTP analogues, such as  $GTP\gamma S$  and  $Gpp(NH)p$ , promote dissociation of oligomeric G proteins and generate a permanently activated state of G proteins ( $G\alpha$ GTP) thus enabling prolonged activation of the effector enzymes and lowered affinity for the ligand. It is the various G ( $G\alpha$ ) proteins that couple the receptor to different effector enzymes or ion channels to generate various effect of the ligand on target cells.

**Effector system:** G protein coupled effectors include: adenylate cyclase, cGMP-specific phosphodiesterase, and phospholipase C. The external signals (binding of the ligand to the receptors) are transduced by various G proteins which in turn activate or inhibit the effector(s) to elicit biological actions such as stimulation or inhibition of neurotransmitter release, smooth muscle contraction or relaxation.

It is important to note that signals do not always have to be transmitted by effectors. Recently, a direct action of G protein on ion channels, referred to G protein

gating ion channels, has been identified (Brown and Birnbauber, 1988, Yatani *et al*, 1987). Evidence has shown that the mechanism by which G proteins regulate some ion channels, such as atrial  $K^+$  channel and cardiac muscle  $Ca^{2+}$  channel, is independent of any phosphorylation events or of changes in cytoplasmic levels of second messengers such as cAMP,  $Ca^{2+}$  or inositol triphosphate (IP3). At present, G protein gating of ion channels has been proved for two kinds of ion channels, *i.e.*  $Ca^{2+}$  and  $K^+$  channels, but it is likely that many other ion channels may also be involved, such as  $Na^+$  and  $Cl^-$  channels (Brown and Birnbauber, 1988).

**Scheme 5:** Schematic demonstration of interaction of receptor (R) and ligand (H), G protein, GTP, and effectors (second messengers or ion channels). See text for explanations (Modified from Gilman, 1987).



## **Chapter III. Receptor Identification**

### **——Ligand Binding Study**

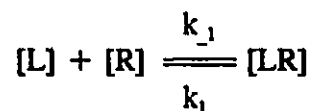
### 3-1. Introduction

The recognition stage of neuropeptide interaction with biological receptors can be studied *in vitro* by the binding of a radioactive ligand. The ligand-binding study was first introduced in 1978 by Bennet and the fundamental principles and methodology of receptor binding were first reviewed by Yamamura *et al* in 1978. In the binding assay, a radioactive ( $^3\text{H}$ ,  $^{45}\text{Ca}$ ,  $^{32}\text{P}$ , or  $^{125}\text{I}$ ) labelled ligand, such as a neuropeptide or neurotransmitter, hormone, or drug, is incubated with tissue slices, isolated cells, purified biological membranes, or solubilized preparations under appropriate conditions. The bound ligand is then separated from unbound ligand by centrifugation, filtration, or column chromatography, and the radioactivity of the bound ligand is counted.

This conceptually simple technique has resulted in an increase in our knowledge at the molecular level of the action of various neuropeptide, neurotransmitters on their target cells. The binding assay has also led to the discovery of new high-affinity drugs selective for the various receptors (for review, see Yamamura, 1978).

### 3-2. Ligand-receptor interaction

The assumption made in receptor binding studies is that the ligand-receptor binding is a reversible reaction that at equilibrium obeys the law of mass action:



where  $[\text{L}]$  represents the concentration of the free ligand,  $[\text{R}]$  is the receptor,  $[\text{LR}]$  is the concentration of ligand-receptor complex, and  $k_1$  and  $k_{-1}$  are the association and



dissociation rate constants, respectively.

The equilibrium dissociation constant ( $K_d$ ) is used as a measure of affinity of the ligand for the receptor and is defined by the law of mass action:

$$K_d = \frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_1} \quad (1)$$

At equilibrium,

$$k_1[R][L] = k_{-1}[RL] \quad (2)$$

The total number of receptors ( $B_{max}$ ) is the sum of the bound and the free receptors, therefore:

$$B_{max} = [R] + [RL] \quad (3)$$

Combine equations (1), (2), and (3):

$$\frac{[RL]}{[L]} = \frac{-1}{K_d} [RL] + \frac{B_{max}}{K_d} \quad (4)$$

Let  $B$  represent bound ligand receptor  $[RL]$ ,  $F$  represent free ligand concentration  $[L]$ , equation (4) becomes:

$$\frac{B}{F} = \frac{-1}{K_d} B + \frac{B_{max}}{K_d} \quad (5)$$

By measuring the specifically bound radioligand ( $B$ ) and knowing the concentrations of the free ligand ( $F$ ) under the binding conditions at equilibrium, a so called "Scatchard plot" can be obtained by plotting the ratio of bound and free ligand ( $B/F$ ) against the amount of bound ligand ( $B$ ). The equilibrium binding constant ( $K_d$ ) is then estimated from the plot as a negative reciprocal of the slope and the capacity of the

binding sites ( $B_{max}$ ) is given by x-intercept of the line.

### 3-3. Criteria for receptor identification

It has been realized since the initial studies that if a tissue is exposed to a radioligand, some binding of radioactivity will occur, "everything binds to everything". Thus the nature of the binding must be established before we define a receptor. A biological receptor is a molecule or molecular complex which is capable of recognizing and selectively interacting with a ligand to generate an internal signal that triggers the physiological, pharmacological response. Whether a given binding site represents the receptor for a ligand can be determined by a set of necessary but not sufficient criteria:

1) binding must be saturable and displaceable by nonradiolabeled ligand or drugs known to act at the receptor.

2) binding should show high affinity, *i.e.* the equilibrium dissociation constant ( $K_d$ ) value should be in the range of the ligand concentration required for physiological response.

3) binding should be present only in tissue where the receptor exists.

4) binding should show stereospecificity if the response of biological systems distinguish between stereoisomer of ligands.

These characteristics of a receptor can be identified from saturation, kinetic, and competitive binding studies which will be explained in the following sections. It should be mentioned that obtaining sufficient evidence of binding to a new receptor is rarely achieved since it probably requires isolation of the receptor based on ligand recognition

properties, characterization and its reconstitution in functional form. Thus, the characteristic studies for a receptor are focused on fulfilling the necessary requirements.

### **3-4. Analysis of binding data**

#### **3-4-1. Saturation study:**

According to the necessary criteria for a biological receptor, a receptor must have not only high affinity but be present in a limited amount. When more and more radioactive ligand is added, binding increases to the point where all sites are occupied and there is no net change in the concentration of [RL], *i.e.* it saturates.

In saturation binding studies, the equilibrium binding affinity constant ( $K_d$ ) and the maximum number of the binding sites ( $B_{max}$ ) can be obtained by plotting the binding data on Scatchard plots (Scatchard, 1949).

Scatchard plot: By measuring the specific ligand binding ( $B$ ) and knowing the concentration of the free ligand ( $F$ ) in the incubation medium at equilibrium, a Scatchard plot is obtained by plotting  $B/F$  against  $B$ . If only one receptor or receptor state is involved in ligand binding and no allosteric interactions are present, a Scatchard plot generates a straight line with the slope =  $-1/K_d$  and  $B_{max}$  = the x intercepts. It is not uncommon that some saturation binding data yield a nonlinear Scatchard plot. This would indicate the heterogeneity of receptor sites, *i.e.* more than one binding site with different affinity, or the cooperative interactions between distinct receptor binding site. However, the low affinity binding sites sometimes can not be obtained by saturation study because of some limitations: 1) the binding affinity is too low; 2) as the concentration of

radioligand increases, the nonspecific binding also increases; and 3) less capacity of the low affinity binding sites as compared to the high affinity binding sites. For further investigation of the different binding sites, other binding studies need to be done as explained below.

**Hill plot:** The presence or absence of cooperativity can be further determined from Hill analysis of binding data. The Hill plot can be obtained from saturation binding data by plotting the  $\log[F]$  against the ratio  $\log[B/B_{\max}-B]$ , where  $B_{\max}$  is established by Scatchard analysis. The slope of the line fitted by linear regression is the Hill coefficient ( $n_H$ ). A straight line with  $n_H$  close to 1.0 indicates the lack of cooperativity. The  $n_H$  greater than 1.0 suggests a positive cooperation, i.e. binding to one receptor facilitates binding to the other. The  $n_H$  less than 1.0 suggests negative cooperation, i.e. a decrease in the affinity of the remaining receptors as binding increases.

### **3-4-2. Kinetic study:**

The equilibrium constant ( $K_d$ ) can also be obtained from experiments in which binding is as the function of time:

$$K_d = k_{-1}/k_1$$

The dissociation rate constant ( $k_{-1}$ ) can be estimated from dissociation studies in which the tissue preparation is incubated with a fixed ligand concentration until equilibrium is reached, and then an excess (1000-fold) of nonradioactive ligand or, 100-fold excess of incubation buffer is added. The specific binding is measured at various time intervals and the half-life ( $t_{1/2}$ ) of loss of specifically bound radioligand is obtained from a plot of  $\log$

[B] against time, and  $k_{-1} = 0.693/t_{1/2}$ .

The association constant ( $k_1$ ) can be calculated as follows:

$$k_1 = \frac{k_{obs} - k_{-1}}{[L]}$$

where  $k_{obs}$  is the slope of the line when  $\ln (B_{eq}-B_t)/B_{eq}$  is plotted against time.  $B_t$  is the amount of bound ligand at various time intervals, and  $B_{eq}$  is the specific bound radioligand at equilibrium.  $k_{-1}$  is the dissociation rate constant,  $[L]$  is the free ligand concentration. This method for estimation of  $k_1$  can be used only when less than 10% of the radioligand is bound at equilibrium.

### **3-4-3. Competition:**

One of the important criteria for identification a binding site of a receptor is its pharmacological specificity. The binding affinity of nonradioactive ligand ( $K_i$ ) could also be obtained from competition studies in which a fixed concentration of radioligand is incubated with increasing concentration of unlabelled ligand.  $K_i$  is calculated as:

$$K_i = IC_{50}/(1 + [L]/[K_d])$$

where  $IC_{50}$  is the concentration of the unlabelled ligand required to inhibit 50% of the radioligand binding,  $[L]$  is the concentration of the free radioligand,  $K_d$  is the binding affinity of radioligand as determined from saturation. In practice, the concentration of the radioligand ( $[L]$ ) is usually kept near the  $K_d$  value.

### **3-4-4. Two-state model:**

The observation of high- and low-affinity sites for a ligand can be rationalized in terms of the two-state model (Hollenberg, 1985). In this model, it is hypothesized that some receptors, e.g. muscarinic receptor, have a "desensitized situation" which has a ligand affinity distinct from agonist state (T state) or antagonist state (R state). The implications for the existence of more than one binding sites can be obtained from several lines of evidence: 1) saturation studies showing  $nH < 1.0$ ; 2). the radioligand bound to the receptors can not be completely released by addition of excess amount of unlabelled ligand (1000-fold excess of unlabelled ligand); 3). different  $K_d$  values from saturation and kinetic studies. For further elucidation of the nature of the observed binding, some additional experiments will be helpful: 1). competitive binding curve fits two-sites model; 2). dissociation of the bound radioligand by dilution, i.e 100-fold excess of incubation buffer. If more than one binding site exists and the binding to the low affinity site inhibits dissociation, dilution should accelerate the dissociation rate; and 3). use of guanine nucleotides analogues which bind to the G proteins to decrease the affinity of the binding sites.

## **Chapter IV. Hypothesis and Objectives**

#### **4-1. Hypothesis**

The exact mechanism responsible for the modulatory effect of galanin on GIT is not known. Galanin could be considered as a neurotransmitter since it can directly act on the smooth muscle to regulate motility. On the other hand, galanin also might be a new neuromodulator by affecting neuropeptide or neurotransmitter release such as VIP, substance P, or acetylcholine. Therefore, there might be specific galanin receptors on both nerve and the smooth muscle cell plasma membrane in GIT to mediate the biological effects of galanin on GIT. We hypothesize that galanin interacts with its receptors on nerves to modulate neuropeptide (such as VIP) release or the receptors on muscle membranes to directly inhibit or excite motor activity of the GIT.

#### **4-2. Objectives.**

1. Localize the galanin receptors;
2. Characterize the recognition properties of the receptors, *i.e.* saturability, reversibility, specificity, selectivity;
3. Determine the structural requirements for the receptor recognition;
4. Assess the mechanisms involved in galanin-receptor mediated biological action;
5. Determine the molecular weight of the receptor.



## **Chapter V. Methodology**

## 5-1. Membrane Preparation

### 5-1-1. Introduction

The analysis of cell surface structure and its constituents requires separation and isolation of cell membranes from the cytoplasm and its sub-cellular organelles. The development of a simple procedure for membrane purification is necessary for studying membrane properties in relation to their function in communication between cells and cell responses, *e.g.* membrane receptors.

Membrane preparation involves cell disruption by means of tissue homogenization followed by several steps of differential, and/or density gradient centrifugation (Maeda *et al.*, 1983). The various membrane fractions obtained during this procedure can be characterized by electron microscopy and/or the analysis of specific marker enzymes. The use of a specific membrane receptor or enzyme activity as markers for plasma membranes has enabled us to develop a technique for the purification of different membranes, including both neuronal and smooth muscle plasma membranes.

Although the general schema for membrane preparation from various tissue sources is similar, a detailed technical procedure had to be established for individual tissues. In the gastrointestinal tract, different smooth muscle (longitudinal or circular) and neuronal components (myenteric or submucosal plexus) can be initially separated by simple dissection. Four different tissues can be obtained by dissection (figure 1): longitudinal muscle with myenteric plexus attached; circular muscle with deep muscular plexus attached; submucosa; and mucosa. Further separation of smooth muscle from neuronal components is essential and can be achieved by the membrane purification

technique which will be described in the following sections.

The markers for plasma membranes of smooth muscle and synaptosomes have been well described (Forsman and Gustafsson, 1985, Kostka *et al*, 1987, Ahmad *et al*, 1988). It has been shown that 5'-nucleotidase, which is an integral membrane enzyme which catalyses the nucleotide 5'-monophosphates to nucleosides, has its strongest activity in the plasma membrane of the smooth muscle cells, but the enzyme activity is almost absent in nerve membranes (Ahmad *et al*, 1988). 5'-nucleotidase is an easily measurable enzyme and has been proven to be highly specific and be more sensitive than some other marker enzymes, *e.g.* phosphodiesterase, it is thus a good marker for smooth muscle plasma membranes. In several tissues, there is also a soluble 5'-nucleotidase, normally lost in the supernatant and, thus not a problem in membrane characterization (Ahmad *et al*, 1988).

Evidence showed that the voltage-sensitive sodium channel (VSSC), which is responsible for eliciting action potentials in excitable tissues, *e.g.* nerves, skeletal muscle, can be blocked by saxitoxin (STX) and tetrodotoxin (TTX) which bind with high affinity to the same site in the channel. In contrast, the VSSC with high affinity for saxitoxin is absent on smooth muscle on which the action potential is carried by  $Ca^{++}$ . By using radioligand binding technique, Ahmad *et al* (1988) demonstrated that  $^3H$ -STX has turned out to be a highly sensitive marker for neuronal membranes in canine small intestine. It was shown that the nerve VSSC has a  $K_d$  for STX  $< 1$  nM, but the muscle plasma membrane VSSC has  $K_d$  values in the range of 20 -- 30 nM (Ahmad *et al*, 1988).

Enzyme markers for identification of subcellular organelle membranes are also

available. Cytochrome c-oxidase and NADPH cytochrome c-reductase have been demonstrated as good markers for mitochondrial inner membranes and sarcoplasmic reticulum membrane respectively (Daniel *et al*, 1982).

In summary, in canine small intestine, enriched plasma membranes of synaptosomes or smooth muscles can be obtained by tissue dissection followed by membrane preparation. Any mutual contamination of nerve and smooth muscle membranes can be assessed by the highly-specific and sensitive markers for neuronal and muscular plasma membrane, i.e.  $^3\text{H}$ -STX and 5'-nucleotidase, respectively.

#### **5-1-2. Materials:**

$^3\text{H}$ -saxitoxin (17 ~ 46 Ci/mml) was purchased from Amersham (Arlington Heights, IL). Sucrose, 3-(N-morpholine) propanesulfonic acid (MOPS), tetrodotoxin, tris (hydroxymethyl) aminomethane,  $\text{MgCl}_2$ , were all from Sigma Chemical Co. (St. Louis, MO).

#### **5-1-3. Tissue selection and handling**

Canine small intestinal circular muscle layer and the deep muscular plexus were selected for all the experiments since functional studies were carried out on circular muscle activity (Fox, 1986). The general structure of the canine small intestinal wall is shown in Chapter II, scheme 1. To obtain tissues, adult mongrel dogs were killed by an intravenous injection of pentobarbital sodium (100 mg/kg) by a procedure approved by the McMaster University Animal Care Committee. The whole intestine was removed and

suspended in ice-cold sucrose magnesium MOPS buffer (SMB) containing 25 mM MOPS, 10 mM MgCl<sub>2</sub>, 8% sucrose, pH = 7.4. The intestine was cut into pieces approximate one inch long and fat was removed. The intestinal piece was opened along the mesenteric border and pinned on a dissecting dish with the mucosal surface down. The longitudinal muscle layer with the myenteric plexus attached was peeled off using forceps leaving the circular muscle layer exposed. The circular muscle layer and the embedded deep muscular plexus were peeled off the submucosal membrane and placed in cold SMB immediately. The dissected circular muscle was blotted dry on filter paper and weighed before being resuspended in SMB (10 ml/g tissue). The tissue was minced finely by a scissors and homogenized by a Polytron PT 20 homogenizer for 20 seconds (2 x 10 sec) at ~ 15,000 rpm.

#### ***5-1-4. Membrane preparation***

The homogenized tissue was centrifuged at 1,000 g for 10 min. The supernatant (PNS), which was thus cleared of the nuclei and unbroken cells, was spun at 10,000 g for 10 min. The pellet (MitI) was saved and the supernatant was centrifuged at high-speed (170,000 g) for 60 min. The pellet (MicI) was resuspended in 2 ~ 4 ml SMB and spun at 10,000 g for 10 min to obtain the pellet (MitI) and the supernatant (MicII).

For further purification of the membrane fractions, MitI (resuspended in SMB) and MicII, 2 ml of each, were loaded on discontinuous sucrose gradient consisted of 2 ml each of 48, 40, 35, 25, 14, 8% (w/v) sucrose, or 48, 40, 33, 14, 8% sucrose, respectively. The sucrose gradients with the membrane loaded were centrifuged in a

Beckman SW 40 rotor at 30,000 rpm for 100 min. The protein bands at all the interfaces were collected and saved for the measurement of protein concentration, enzyme activity, and galanin binding. There were six fractions from Mit I, *i.e.* F1, F2, F3, F4, F5, F6, and 4 fractions from MicII, *i.e.* M1, M2, M3, M4, sucrose gradients.

### ***5-1-5. Analysis of markers for membrane fractions.***

#### **6-1-5-A. Determination of protein concentration:**

The measurement of protein concentration in various fractions was done by the method of Lowry using bovine serum albumin as standard.

#### **6-1-5-B. [<sup>3</sup>H]-saxitoxin binding assay:**

The binding of tritiated saxitoxin (STX) to the membranes was carried out in 250  $\mu$ l incubation mixture containing 50 mM Tris-HCl, 0.2% BSA, and 0.5 ~ 0.8 nM [<sup>3</sup>H]-STX. Samples were incubated at 25 °C for 15 min. The reaction was started by the addition of the membranes (20  $\mu$ g protein) to the incubation mixture. The total and nonspecific binding were determined in the absence or presence of 1  $\mu$ M TTX, respectively, the specific binding was defined as the difference between total (no TTX) and nonspecific binding (with TTX). After incubation for 15 min, the reaction was terminated by adding 3 ml ice-cold buffer and the samples immediately filtered through Whatman GF/F filters by vacuum millipore filtration apparatus. The filters and tubes were washed three times with 3 ml ice-cold incubation buffer. The radioactivity remaining on the filters was counted on a Beckman model LS 6800 b counter. All total and nonspecific bindings were performed in triplicate.

#### 6-1-5-C. 5'-Nucleotidase activity:

5'-nucleotidase activity was determined by measuring the amount of phosphate liberated from the membrane when incubated with 5'-AMP disodium salt (5 mM) in a medium containing 50 mM imidazole-HCl, 5 mM MgCl<sub>2</sub> in a final volume of 1 ml at 37°C for 30 min. The reaction was started by adding the membrane (20 ~ 30 µg protein) into the preincubated mixture (5 min, 37°C). The reaction was stopped by addition of 1 ml ice-cold 10% trichloroacetic acid (TCA) and the incubation mixture was centrifuged for 15 min. The 1 ml of supernatant was collected for the measurement of inorganic phosphate according to Taussky and Shorr (1953).

### **5-2. <sup>125</sup>I-galanin Binding Assay**

#### ***5-2-1. Materials:***

<sup>125</sup>I-galanin (porcine) (2200 Ci/mmol) was purchased from Dupont, Massachusetts, Boston. Unlabelled galanin (porcine) and galanin fragment 1-16 were from Peninsula Laboratories, Inc (Belmont, California). Galanin fragments 1-15, 15-29 were provided by Dr. T.J. McDonald. Sucrose, Mops, MgCl<sub>2</sub>, Tris-Base, bovine serum albumin (BSA), aprotinin, bacitracin, trypsin inhibitor, guanosine-5'-O-(3-thiotriphosphate) (GTPγS), pertussis toxin (PTX), cholera toxin (CTX), were all from Sigma Chemical Co. (St. Louis, MO). Disuccinimidyl tartarate (DST) was from Pierce Chemical Co. (Rockford, IL).

#### ***5-2-2. <sup>125</sup>I-galanin binding assay:***

Binding of  $^{125}\text{I}$ -galanin to various membrane fractions was performed in triplicate in an incubation mixture with total volume of 200  $\mu\text{l}$  containing of 25 mM Tris-Base, 2 mM  $\text{MgCl}_2$ , 1% BSA (w/v), 0.2 mg aprotinin, 0.4 mg bacitracin, 50  $\mu\text{g}$  trypsin inhibitor, 0.5 ~ 1 nM/L  $^{125}\text{I}$ -galanin and 15 ~ 20  $\mu\text{g}$  membrane fraction. Samples were incubated at 25<sup>o</sup> C for 25 min. The reaction was started by addition of the membranes to the incubation tube. The nonspecific binding was determined with 1  $\mu\text{M}$  unlabelled galanin in the assay. The percentage of nonspecifically bound counts varied between 20% and 30% of total binding. The bound and free radioligand were separated by vacuum millipore filtration and washing through Whatman GF/F filters (0.2  $\mu\text{m}$  pore size) with ice cold incubation buffer. The radioactivity of the bound ligand was determined by a Beckman 5500 gamma counter with counting efficiency of 30 ~ 37%.

### **5-3. Characteristic studies of Galanin Receptors**

#### ***5-3-1. Tissue linearity***

The binding of  $^{125}\text{I}$ -galanin to MitI or MicII was measured in increasing concentrations of membrane protein under the conditions described above.

#### ***5-3-2. Equilibrium binding study:***

The MicII or MitI fraction was incubated with increasing concentrations of radioiodinated galanin under the binding conditions above. The equilibrium dissociation constant (Kd), capacity of binding sites (Bmax) and Hill coefficient (nH) were calculated by the computer program EBDA, 1983 (McPherson, 1983).



### ***5-3-3. Time course of galanin binding:***

The association experiments were started by the addition of the MitII, or MicII membrane preparation at 25<sup>o</sup>C. At various time intervals, the reaction was terminated by the addition of 3 ml ice cold incubation buffer followed by filtration and washing as described above. For dissociation studies, membranes were incubated with <sup>125</sup>I-galanin in the incubation mixture until equilibrium was reached (25 min), then an excess of unlabelled galanin (1 μM) was added to initiate dissociation. Samples were collected at various time intervals to determine the membrane bound radioligand during dissociation kinetic studies. To study the effect of GTPγS on the rate of the dissociation, the membrane preparations were treated with the nucleotide at concentration of 10<sup>-4</sup>M and then added to the incubation mixture to initiate the reaction. After 25 min of association, dissociation was initiated by addition of excess (1 μg) unlabelled galanin. At various time intervals, the reaction was stopped by addition of ice-cold buffer.

### ***5-3-4. Competitive binding study:***

Synaptosomal or circular muscle plasma membranes were incubated with <sup>125</sup>I-galanin in the presence of increasing concentrations of unlabelled galanin or galanin fragments, i.e. galanin 1-16, 1-15, 1-11, or 15-19. After incubation at 25<sup>o</sup>C for 25 min, membrane-bound radioligand was determined as described above. The effect of the GTP analog, GTPγS, pertussis toxin (PTX), or cholera toxin (CTX) on galanin binding was studied. GTPγS was added directly to the membrane preparation (10<sup>-4</sup> M). PTX was activated by incubation with 25 mM dithiothreitol (DTT) for 20 minutes at 30<sup>o</sup>C. The

preactivated PTX (4  $\mu\text{g/ml}$ ) was incubated with the membrane in the presence of 1 mM ATP and 0.2 mM NAD for 30 minutes at 30°C. CTX was preactivated with 50 mM DTT at 30°C for 2 hours. The activated CTX (2 mg/ml) was incubated with the membrane at 30°C for 30 min in the presence of 1 mM ATP, 0.2 mM NAD, and 20 mM arginine.

***5-3-5. Determination of the molecular weight of the galanin receptor:***

The membrane fractions, *i.e.* MitI or MicII (0.2 ~ 0.4 mg), were incubated with 0.4 ~ 0.5 nM  $^{125}\text{I}$ -galanin in 0.5 ml assay buffer (20 mM HEPES, 5 mM  $\text{MgCl}_2$ , 0.1% (w/v) bacitracin, 1 mg/ml aprotinin), pH = 7.5, for 25 min at 25°C. The reaction was stopped by centrifugation at 20,000 x g for 15 min to obtain a pellet which was subsequently washed twice with 3 ml of the wash buffer (20 mM HEPES, pH 7.5). Nonspecific binding was determined in the presence of 1  $\mu\text{M}$  unlabelled galanin. The prewashed pellet was resuspended in 0.5 ml wash buffer containing a cross-linker DST which is a homobifunctional cross-linking agent by reaction with the amino groups on a ligand and the protein (receptor) to form stable amide bonds (1 mM, dissolved in 50 mM dimethyl sulfoxide). The cross-linking reaction was carried out on ice for 15 min and was terminated by centrifugation at 20,000 x g for 10 min. Samples (10  $\mu\text{l}$ ) were run on a 12% polyacrylamide slab gel with 4% stacking gel. After 1.2 hr electrophoresis (100 volt power), the gel was dried and exposed to a Kodak XM film for 14 days at -70°C. Gels were calibrated with a standard containing proteins with known molecular weight.

## **Chapter VI. Results**

## 6-1. Distribution of $^{125}\text{I}$ -galanin Binding Sites

### *6-1-1. Distribution of $^{125}\text{I}$ -galanin in tissue fractions prepared by differential centrifugation:*

The circular muscle containing the deep muscular plexus from canine small intestine was homogenized followed by differential centrifugation. During this membrane preparation, five fractions were obtained, *i.e.* PNS, MitI, MitII, MicI, and MicII. The binding of  $^{125}\text{I}$ -galanin and the distribution of the markers for plasma membrane of nerves and smooth muscle were analyzed. Figure 1. shows the  $^3\text{H}$ -saxitoxin ( $^3\text{H}$ -STX) binding, 5'-nucleotidase (5'-NTD) activity and  $^{125}\text{I}$ -galanin binding in membrane fractions obtained by tissue homogenization followed by several steps of differential centrifugation.  $^3\text{H}$ -STX binding is highest in MitI fraction, and 5'-NTD was enriched in MicII fraction. Subcellular distribution of  $^{125}\text{I}$ -galanin binding followed a bimodal pattern, *i.e.*  $^{125}\text{I}$ -galanin binding sites were enriched in both crude synaptosomes (MitI) and smooth muscle plasma membrane (MicII).

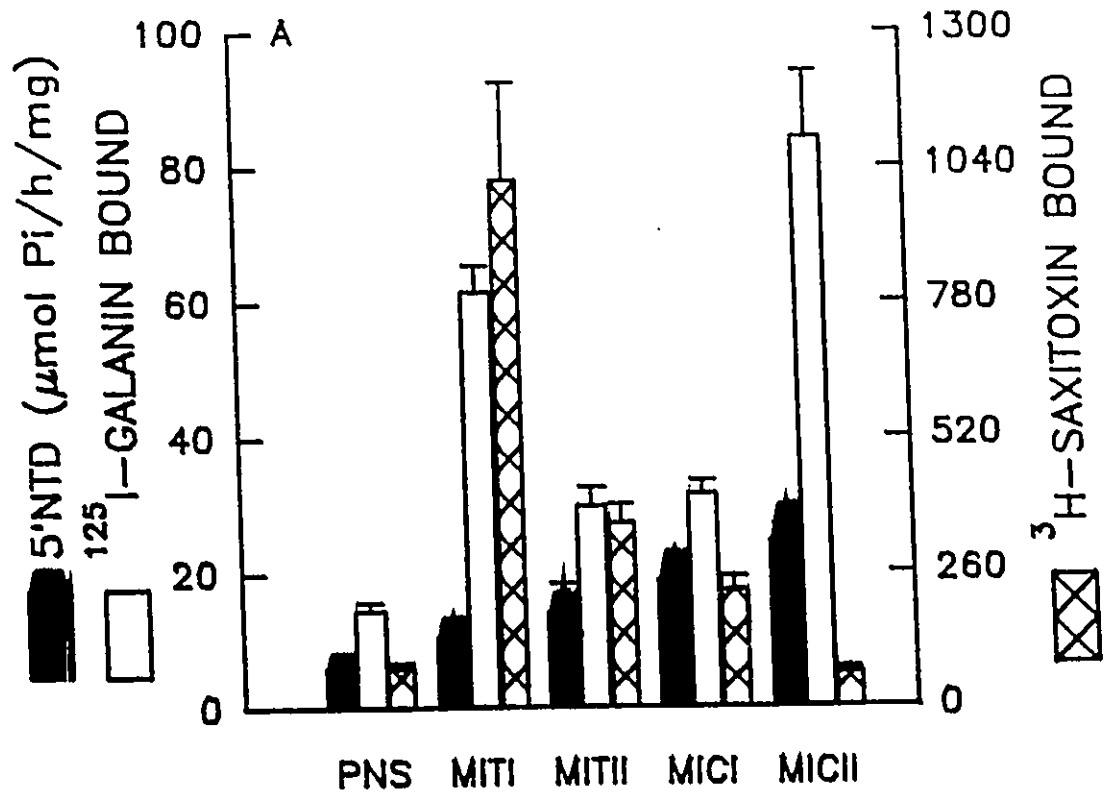
### *6-1-2. $^{125}\text{I}$ -galanin in purified nerve and smooth muscle membranes:*

The crude synaptosomes and smooth muscle membranes (MitI and MicII) were purified by discontinuous sucrose gradient centrifugation. Figure 2. A panel shows the distribution of markers and  $^{125}\text{I}$ -galanin binding in subfractions from MitI. Highest  $^{125}\text{I}$ -galanin binding was found in F3 and F4 which were enriched in  $^3\text{H}$ -STX binding. Figure 2. B panel shows the distribution of  $^{125}\text{I}$ -galanin binding in sucrose gradient fractions

from MicII. The  $^{125}\text{I}$ -galanin binding activity was highest in M2 fraction which also has the strongest 5'-NTD enzyme activity.

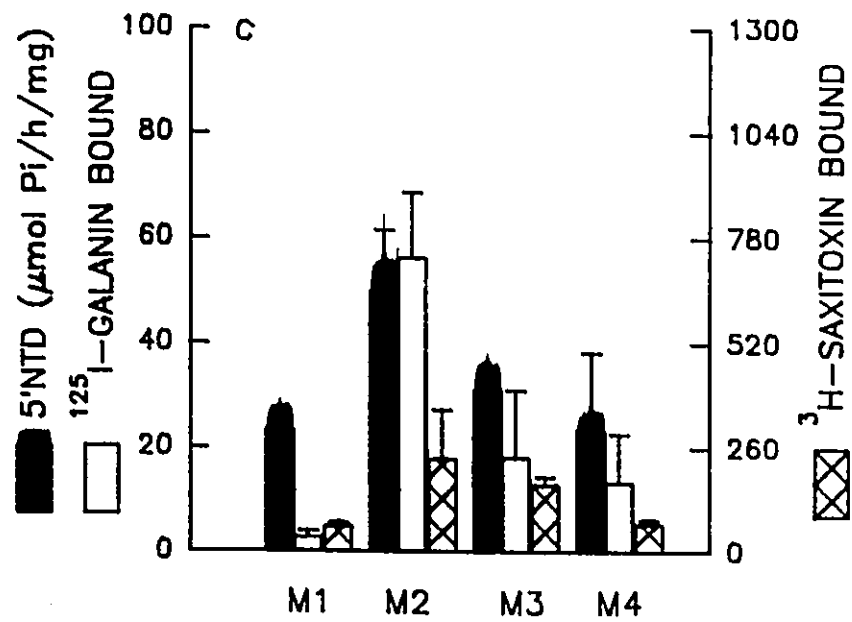
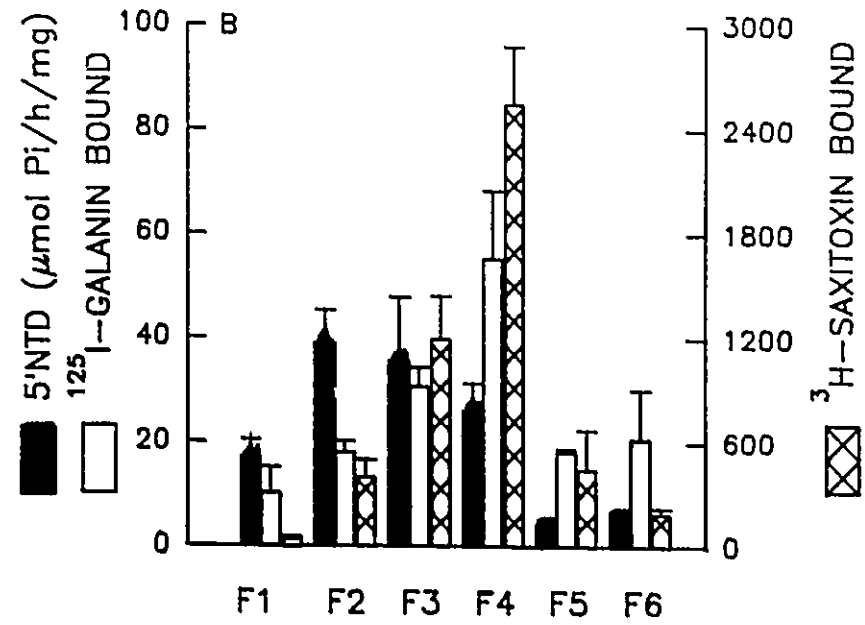
In summary, the results presented here indicated that the  $^{125}\text{I}$ -galanin binding sites are present in both synaptosomes and smooth muscle plasma membranes in canine small intestinal circular muscle/deep muscular plexus.

**Figure 1.** Distribution of  $^{125}\text{I}$ -galanin (0.1 ~ 0.2 nM) binding, marker enzyme 5'-nucleotidase, and binding of  $^3\text{H}$ -saxitoxin to the fractions obtained after differential centrifugation of the homogenate from circular muscle/deep muscular plexus of canine small intestine. Data plotted were means  $\pm$  SD of 3 ~ 4 separate experiments. Abbreviations: [ $^{125}\text{I}$ ]-Gal. = [ $^{125}\text{I}$ ]-galanin; [ $^3\text{H}$ ]-STX = [ $^3\text{H}$ ]-saxitoxin; 5'-NTD = 5'-nucleotidase.



**Figure 2.** Distribution of [<sup>125</sup>I]-galanin binding, [<sup>3</sup>H]-saxitoxin binding, and 5'-nucleotidase activity in the fractions obtained by discontinuous sucrose gradients of nerve membrane (Mit I) ( A panel), and of plasma membrane (MicII), ( B panel). Results are means  $\pm$  SD of 3 – 4 separate experiments.





## 6-2. Characterization of Galanin Binding Sites on Synaptosomes

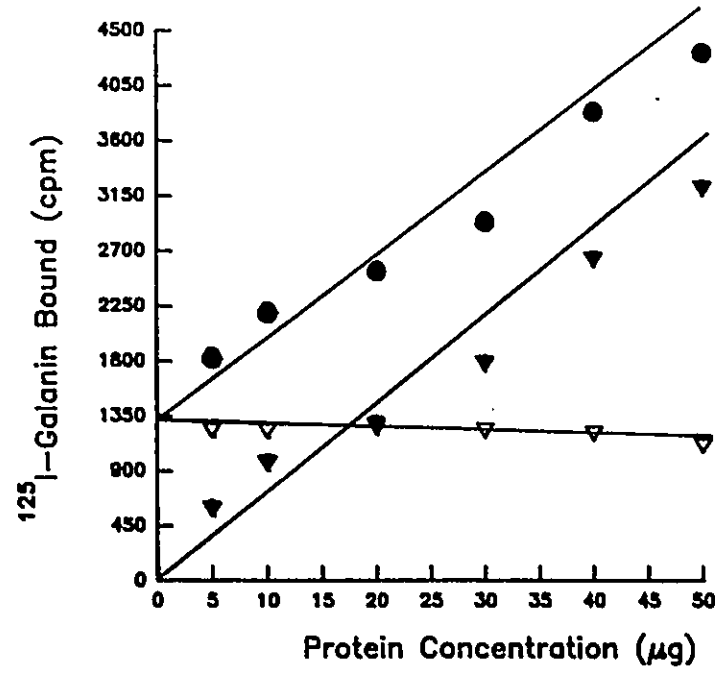
### 6-2-1. *Tissue linearity:*

The correlation between membrane protein concentration, over the range of 5 ~ 50  $\mu\text{g}$  per test tube, and  $^{125}\text{I}$ -galanin binding is shown in figure 3. The total and specific binding of  $^{125}\text{I}$ -galanin increased linearly with the increase in the membrane content. Non-specific binding to membrane was negligible and was located on the filter under these binding conditions. The protein concentration of 15 ~ 25  $\mu\text{g}$  was used in the following binding studies.

### 6-2-2. *Equilibrium binding study:*

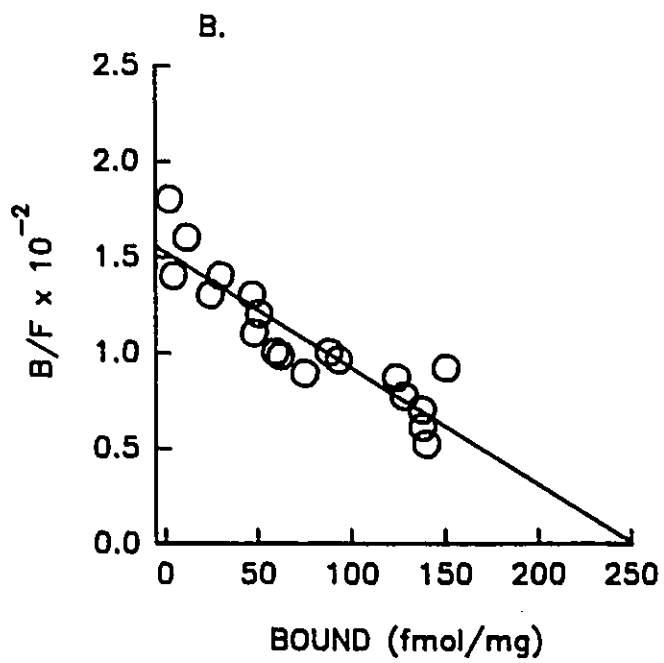
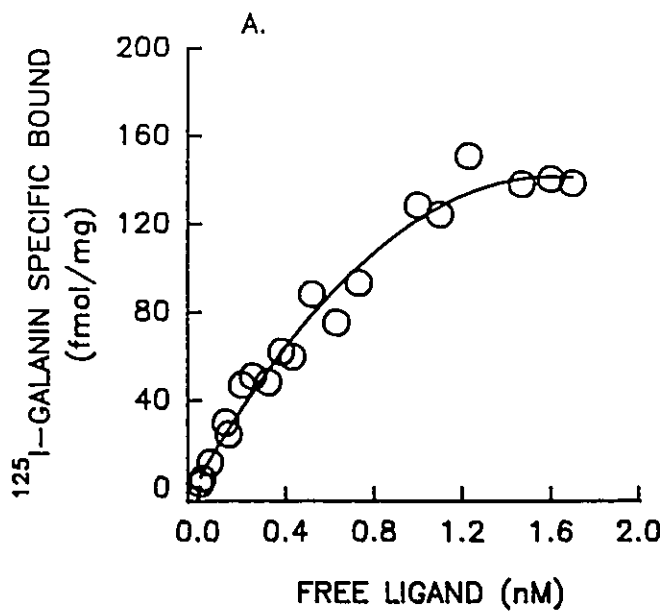
The amount of  $^{125}\text{I}$ -galanin bound to the nerve membranes increased with increasing concentrations of the iodinated peptide [figure 4]. The computer analysis of the binding data gave a linear Scatchard plot with (means  $\pm$  SD):  $K_d = 1.1 \pm 0.13$  nM,  $B_{\text{max}} = 244 \pm 2.1$  fmol/mg prot.  $nH = 0.99 \pm 0$ , (n=3).

**Figure 3.** Binding of  $^{125}\text{I}$ -galanin (0.1 nM) to increasing concentrations of crude synaptosomal membrane (MitI) in the range of 5 – 50  $\mu\text{g}$  per test tube. The experiment was repeated twice.



● Total Bound    ▼ Specific Bound    ▽ Nonspecific Bound

**Figure 4.** Steady state analysis of  $^{125}\text{I}$ -galanin binding to synaptosomal fraction MitI (100  $\mu\text{g}/\text{ml}$ ) as increasing concentration of the radioligand (upper panel). The lower panel: same data plotted by computer-generated Scatchard plot. Values from computer analysis were (means  $\pm$  SD):  $K_d=1.1 \pm 0.13$  nM,  $B_{\text{max}}=244 \pm 2.1$  fmol/mg protein,  $nH=0.99 \pm 0$ ,  $n=3$ .



### **6-2-3. Time-course of $^{125}\text{I}$ -galanin binding:**

The specific binding of  $^{125}\text{I}$ -galanin to the nerve membrane increased with time and reached equilibrium by 25 min. The half-time of association ( $T_{1/2}$ ) was 5 min with approximately 90% of the total binding achieved by 20 min. The specific binding at equilibrium did not change for up to 60 minutes of incubation [figure 5]. Pretreatment of the membrane with a GTP analogue, GTP $\gamma$ S, did not change the association of  $^{125}\text{I}$ -galanin with the receptors (figure 5).

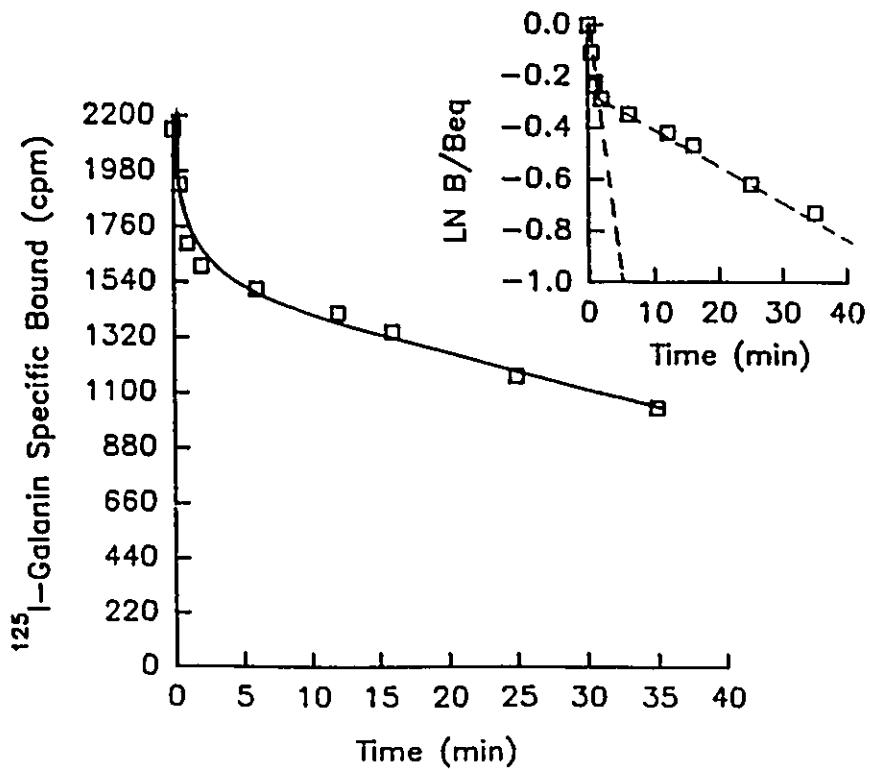
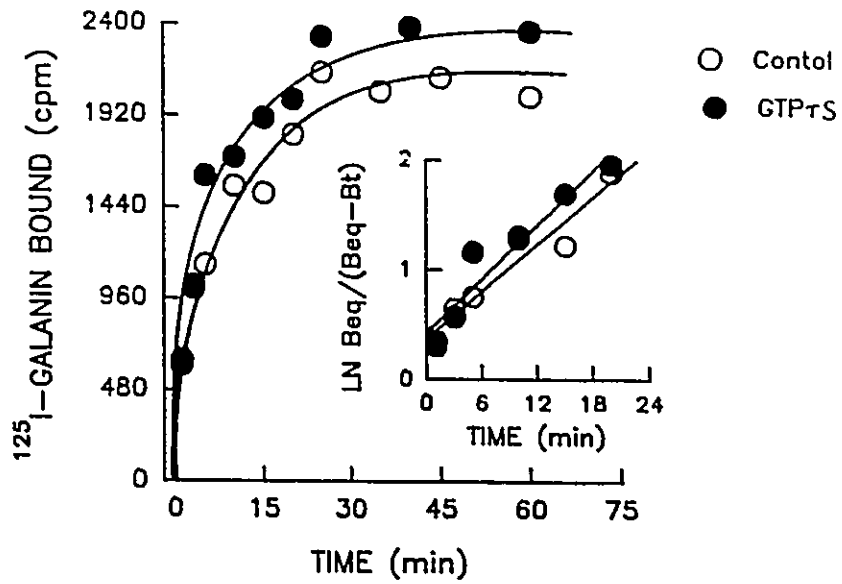
The binding of  $^{125}\text{I}$ -galanin to the nerve membrane receptor is reversible. Excess (1  $\mu\text{M}$ ) of unlabelled galanin was used to study dissociation of bound galanin from the receptor. The dissociation was incomplete after 60 min in incubation, *i.e.* 40% of the  $^{125}\text{I}$ -galanin was still bound at 60 min after adding excess unlabelled galanin (figure 5). The dissociation appeared to be biphasic, *i.e.* during the first few minutes, the dissociation rate was fast, but later it became slower. The calculated rate constant of dissociation were:  $k_{11} = 0.24 \pm 0.015 \text{ min}^{-1}$  and  $k_{12} = 0.012 \pm 0.007 \text{ min}^{-1}$ . These values led to rate constants of association of:  $k_{11} = 0.38 \pm 0.029 \text{ min}^{-1}$  and  $k_{12} = 1.1 \pm 0.1 \text{ min}^{-1}$ . The calculated  $K_d$  values were:  $K_{d1} = 0.6 \pm 0.07 \text{ nM}$  and  $K_{d2} = 0.012 \pm 0.007 \text{ nM}$ .

### **6-2-4. Effect of GTP $\gamma$ S on the dissociation of bound $^{125}\text{I}$ -galanin in synaptosomes:**

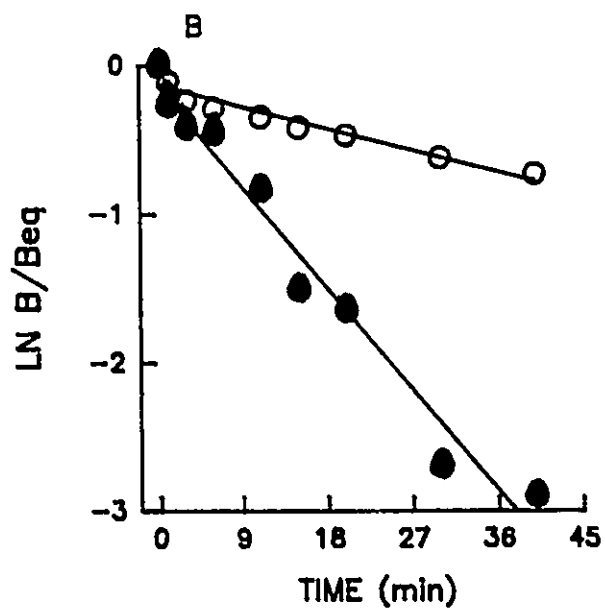
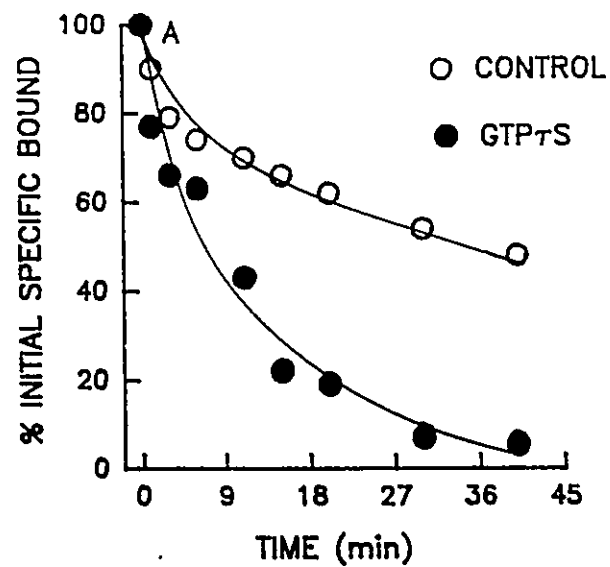
In the presence of GTP $\gamma$ S, the dissociation was rapid and complete within 45 min (figure 6). The rate constant of dissociation was  $0.08 \pm 0.006 \text{ min}^{-1}$ . The association rate constant was  $k_1 = 0.75 \pm \text{min}^{-1}$ . The calculated  $K_d$  was  $0.1 \pm \text{nM}$ .

**Figure 5.** Time-course of the binding of  $^{125}\text{I}$ -galanin to nerve membrane (MitI) in the presence and absence of  $\text{GTP}\gamma\text{s}$ . The equilibrium specific binding was reached after 25 min incubation and remained stable for up to 60 min (panel A). The dissociation was started by adding  $1\ \mu\text{M}$  unlabelled galanin (panel B). Insets: same data plotted according to first-order kinetic reaction for association and dissociation, where  $B_{\text{eq}}$  is the specific bound at equilibrium, and  $B$  is the specific bound at various time intervals. This figure is representative of three separate experiments performed in triplicate.





**Figure 6.** The effect of GTP $\gamma$ S on the dissociation of the  $^{125}$ I-galanin binding from nerve membrane (MitI). The synaptosomal fraction (MitI) was treated with the GTP $\gamma$ S by direct addition of the nucleotide at concentration of  $10^{-4}$ M. After the equilibrium binding was achieved by 25 min incubation,  $1 \mu$ M unlabelled galanin was added to start dissociation. The calculated dissociation rate constant in the presence of GTP $\gamma$ S was:  $k_{-1} = 0.08 \pm 0.006 \text{ min}^{-1} \pm 0.01$  The experiment was performed three times.



#### **6-2-5. Competitive binding study:**

Figure 7 shows the competitive inhibition of the  $^{125}\text{I}$ -galanin binding by galanin (1-29) and its fragments. The computer analysis, using nonlinear curve fitting, suggests a two-site model, a high affinity ( $K_i=0.02 \pm 0.005$  nM) and a low affinity ( $K_i=1.05 \pm 0.3$  nM) binding sites for galanin (1-29). The order of potency in competition of galanin and its fragments was galanin 1-29 > galanin 1-16 > galanin 1-15 > galanin 1-11, according to the  $K_i$  values (figure 7). The galanin fragment 15-29 was completely inactive up to 1  $\mu\text{M}$ .

#### **6-2-6. Effects of GTP analogue on galanin binding:**

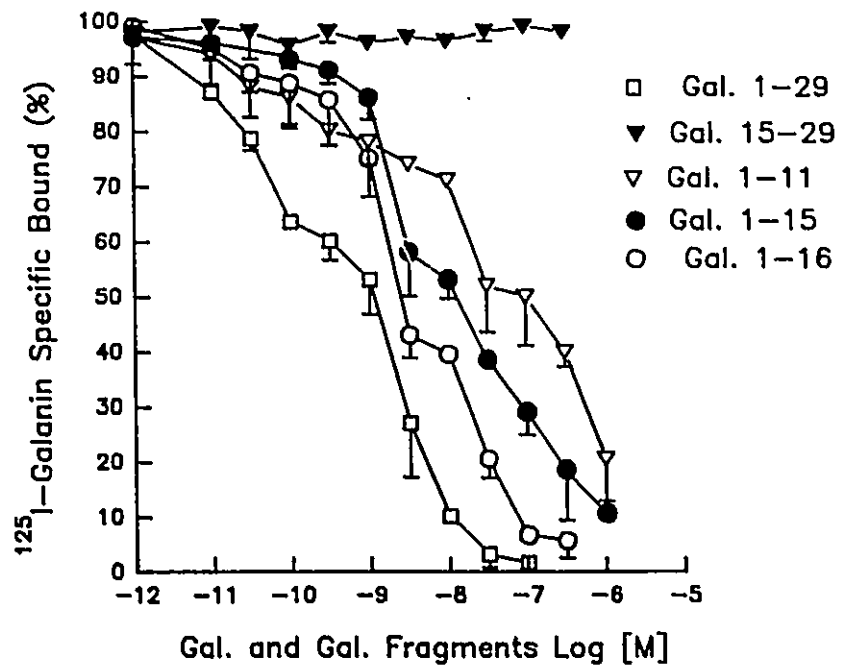
The effect of  $\text{GTP}\gamma\text{S}$  on galanin binding to the nerve membranes was studied by adding increasing concentrations of unlabelled galanin (figure 8). In the presence of the nucleotide ( $10^{-4}$  M), the upper part of the competition curve was shifted to the right compared to the control. Computer analysis, using nonlinear curve fitting, suggests a one-site model with  $K_i=1.2$  nM, slope ( $n_H$ )=0.96.

#### **6-2-7. Effect of pertussis toxin on $^{125}\text{I}$ -galanin binding to synaptosomes:**

Figure 9 demonstrates the competitive binding of galanin and  $^{125}\text{I}$ -galanin to the receptor in the presence of pertussis toxin (PTX). The effect of PTX on galanin binding was similar to that of  $\text{GTP}\gamma\text{S}$ . It occurred when the preactivated toxin (4  $\mu\text{g}/\text{ml}$ ) was added to the incubation buffer. The resultant  $K_i=1.4$  nM,  $n_H=0.95$  was similar to that after  $\text{GTP}\gamma\text{S}$  and that of the low affinity site in the control membrane. Cholera toxin (100

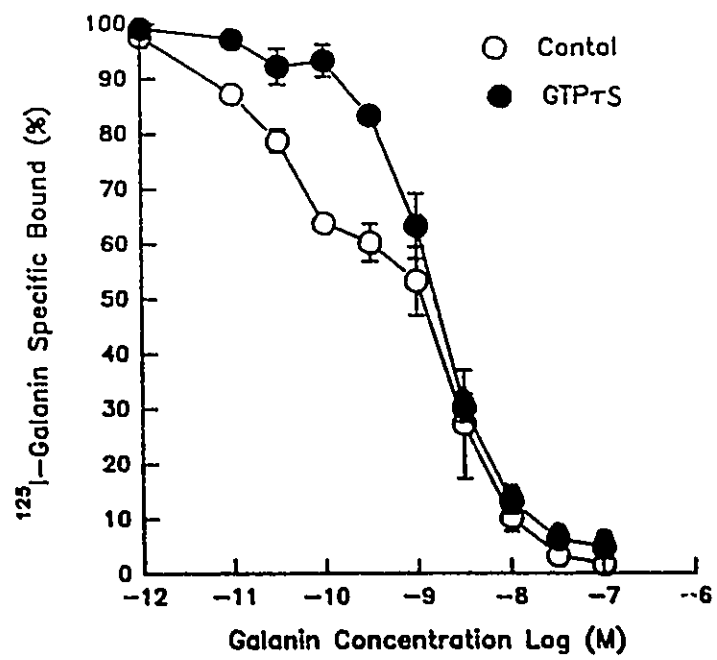
$\mu\text{g/ml}$ ) was inactive (data not shown).

**Figure 7.** Displacement of  $^{125}\text{I}$ -galanin from synaptosomal fraction MitI by various concentration of galanin (Gal. 1-29) and galanin fragments 1-16 (Gal. 1-16), 1-15 (Gal. 1-15), 1-11 (Gal. 1-11), and 15-29 (Gal. 15-29) under conditions described in text. Specific binding was expressed as a percentage of maximal specific binding. Values are means  $\pm$  SD of three similar experiments carried out in triplicate.

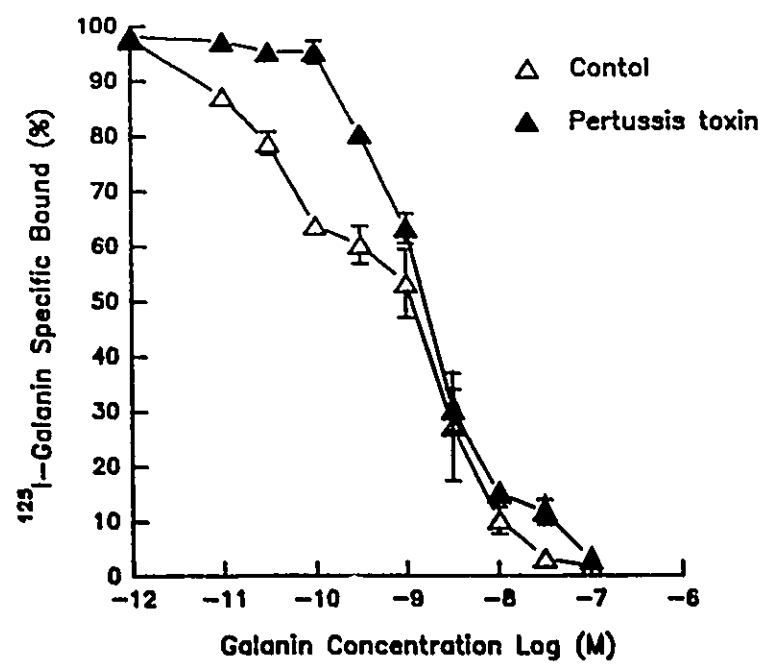


**Figure 8.** Effect of nonhydrolyzable GTP analog, GTP $\gamma$ S, on displacement of  $^{125}$ I-galanin from nerve membrane (MitI) by unlabelled galanin. The nucleotide was added directly to the membrane ( $10^{-4}$ M). Results are means  $\pm$  SD of three separate experiments.





**Figure 9.** Effect of pertussis toxin (PTX) on displacement of bound  $^{125}\text{I}$ -galanin from synaptosomes by unlabelled galanin ( $10^{-12} \sim 10^{-6}$  M). The PTX was activated by 25 mM dithiothreitol (DTT) for 20 min at  $30^{\circ}\text{C}$ . Then the preactivated pertussis toxin ( $4 \mu\text{g}/\text{ml}$ ) was incubated with the fraction in the presence of 1 mM ATP and 0.2 nM NAD for 30 min at  $30^{\circ}\text{C}$ . Values are means  $\pm$  SD of the experiments performed 3 times.



### 6-3. Characterization of $^{125}\text{I}$ -galanin binding sites on plasma membrane of smooth muscle

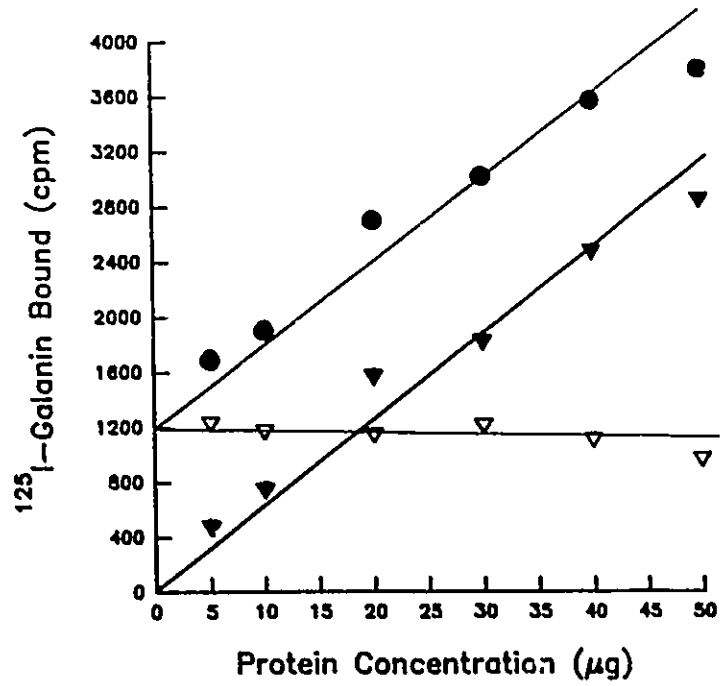
#### *6-3-1. Tissue linearity:*

The total and specific  $^{125}\text{I}$ -galanin binding to the plasma membrane increased linearly as the membrane protein concentration increased in the range of 5 ~ 50  $\mu\text{g}$  (figure 10). Non-specific binding did not change as the protein content increased. All the nonspecific binding was found on the filter.

#### *6-3-2. Equilibrium Binding characteristics:*

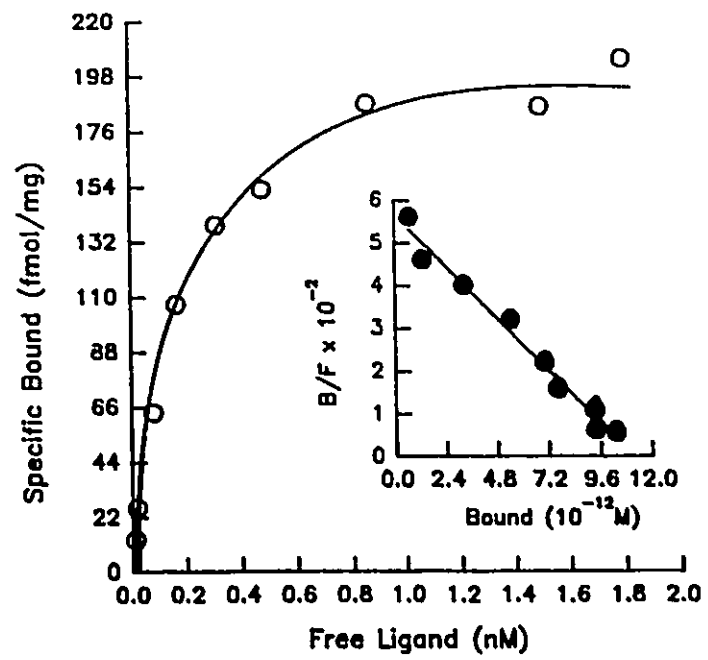
The specific binding of  $^{125}\text{I}$ -galanin to the plasma membrane increased with increasing concentration of the radioligand (figure 11). The computer analysis of the saturation binding data gave a linear Scatchard plot with  $K_d = 0.58 \pm 0.085$  nM,  $B_{\text{max}} = 389 \pm 29.9$  fmol/mg protein, and  $n_H = 0.99 \pm 0.017$  (n=3).

**Figure 10.** Binding of  $^{125}\text{I}$ -galanin to increasing contents of smooth muscle plasma membrane fraction (MicII). In the range of 5 ~ 50  $\mu\text{g/ml}$ , the total and specific binding were increased linearly with the increased membrane protein concentration. The non-specific binding was to the filter. The experiment was repeated twice.



● Total Bound    ▼ Specific Bound    ▽ Nonspecific Bound

**Figure 11.**  $^{125}\text{I}$ -galanin specific binding to plasma membrane fraction (MicII) as a function of increasing concentration of the radioligand under the standard binding conditions. *Inset:* the same data plotted by computer-generated Scatchard plot. Values from computer analysis were (means  $\pm$  SD):  $K_d=0.58 \pm 0.085$  nM,  $B_{\text{max}}=389 \pm 29.9$  fmol/mg protein,  $n_H=0.99 \pm 0.017$ . The figure is the representative of three separated experiments.





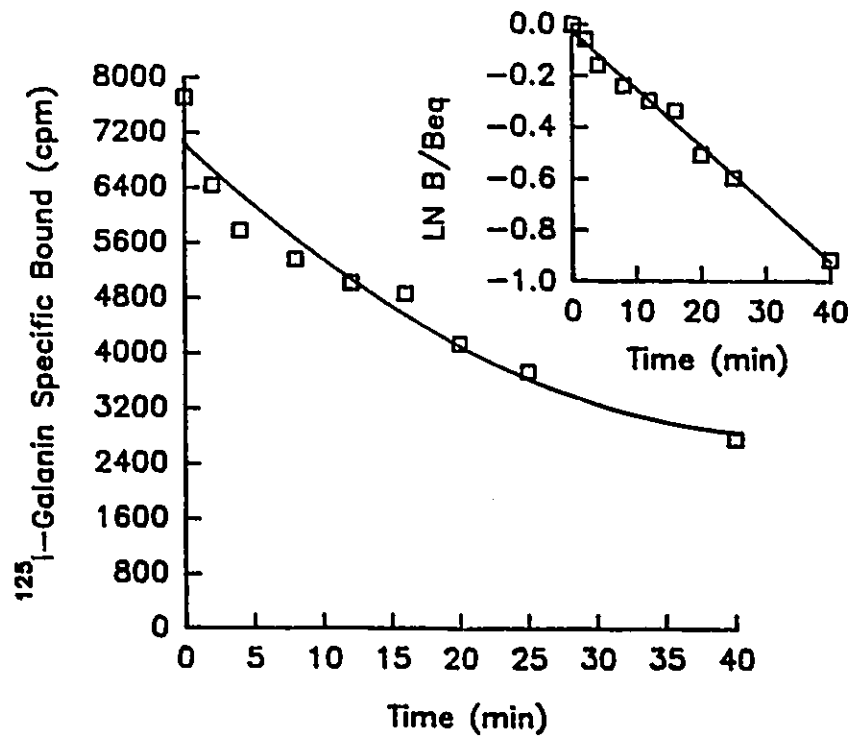
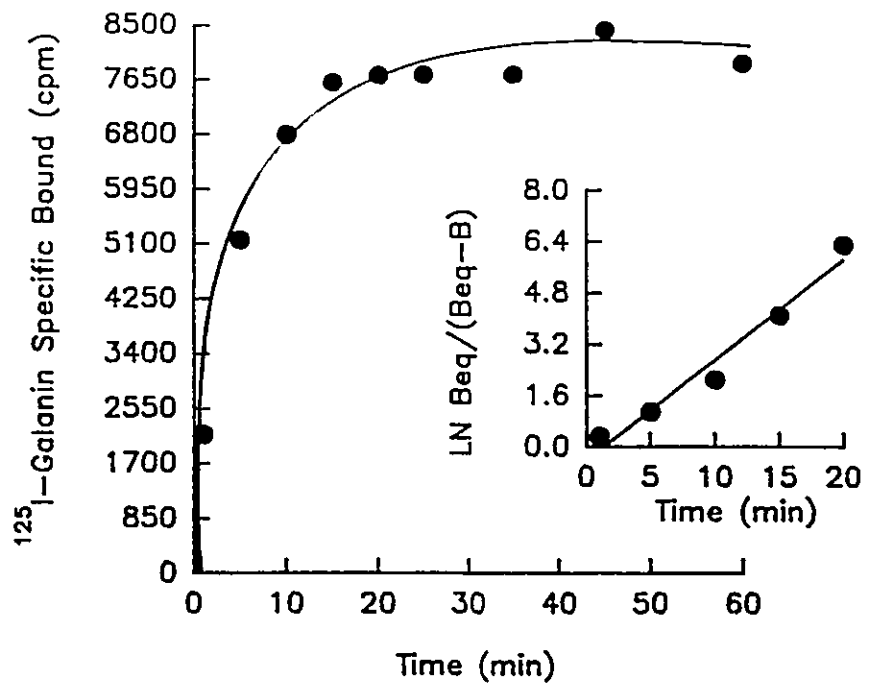
### *6-3-3. Time-course of $^{125}\text{I}$ -galanin binding to the smooth muscle plasma membrane:*

The specific binding of  $^{125}\text{I}$ -galanin to the plasma membrane was time-dependent (figure 12). At  $25^{\circ}\text{C}$ , the binding rapidly reached equilibrium by 25 min. The half-time of association ( $T_{1/2}$ ) was 3 min and about 90% of the total binding was achieved by 10 min. The level of specific binding did not change for up to 60 min of incubation (figure 12 A panel). The specifically bound radioactive galanin could be dissociated by the addition of excess ( $1\ \mu\text{M}$ ) of unlabelled galanin (figure 12 B panel). However, the dissociation was incomplete after 40 min in the presence of unlabelled galanin, leaving over 50 % of the  $^{125}\text{I}$ -galanin still bound. The calculated parameters were: dissociation rate constant ( $k_{-1}$ ) =  $0.024 \pm 0.005\ \text{min}^{-1}$ , association rate constant  $k_1 = 2.2 \pm 0.3\ \text{min}^{-1}$ ,  $K_d = 0.01 \pm 0.001\ \text{nM}$ .

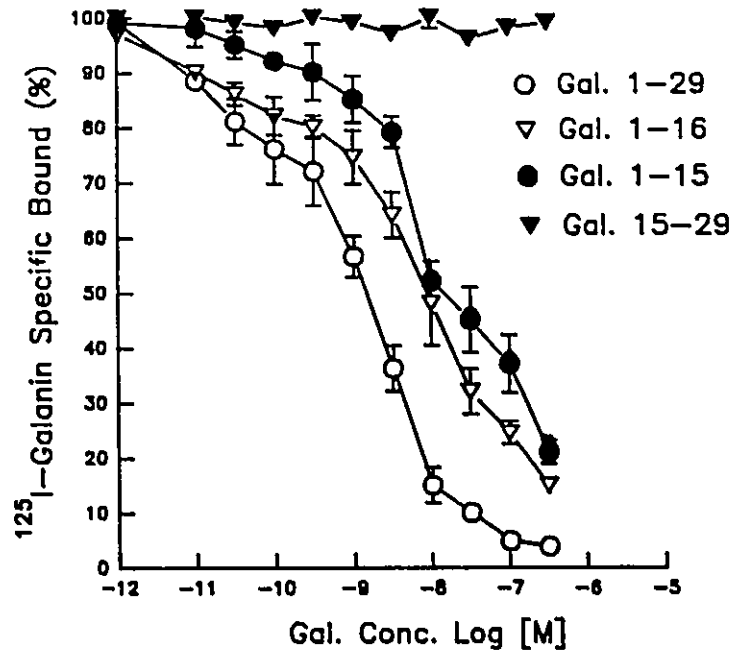
### *6-3-4. Competitive binding of radioiodinated galanin with galanin, or galanin fragments:*

Figure 13 shows the competitive inhibition of the  $^{125}\text{I}$ -galanin binding by the whole galanin molecule (1-29), and its fragments. The computer analysis using nonlinear curve fitting suggested a two-site model with a high affinity ( $K_i = 0.01 \pm 0.05\ \text{nM}$ ) and a low affinity ( $K_i = 2.8 \pm 0.3\ \text{nM}$ ) binding site for galanin. The N-terminal fragments, i.e. galanin 1-16, galanin 1-15, also competed with  $^{125}\text{I}$ -galanin for binding as the concentration of the galanin fragments increased (figure 13). The C-terminal galanin fragment 15-29 was completely inactive in competitive binding.

**Figure 12.** Time-course of the binding of  $^{125}\text{I}$ -galanin to plasma membrane (MieII). The specific binding of  $^{125}\text{I}$ -galanin reached equilibrium state by 15 min and remained stable for up to 60 min (panel A). The excess unlabelled galanin ( $1\ \mu\text{M}$ ) was added to the assay to start dissociation (panel B). The calculated rate of association  $K_{+1}=2.2 \pm 0.3\ \text{min}^{-1}$ , dissociation rate constant  $K_{-1}=0.024 \pm 0.005\ \text{min}^{-1}$ . *Insets:* same data plotted according to a first-order kinetic reaction for association and dissociation, where  $B_{\text{eq}}$  is the specific bound ligand at equilibrium and  $B$  is the specific bound ligand at various time intervals. The kinetic study was repeated three times.



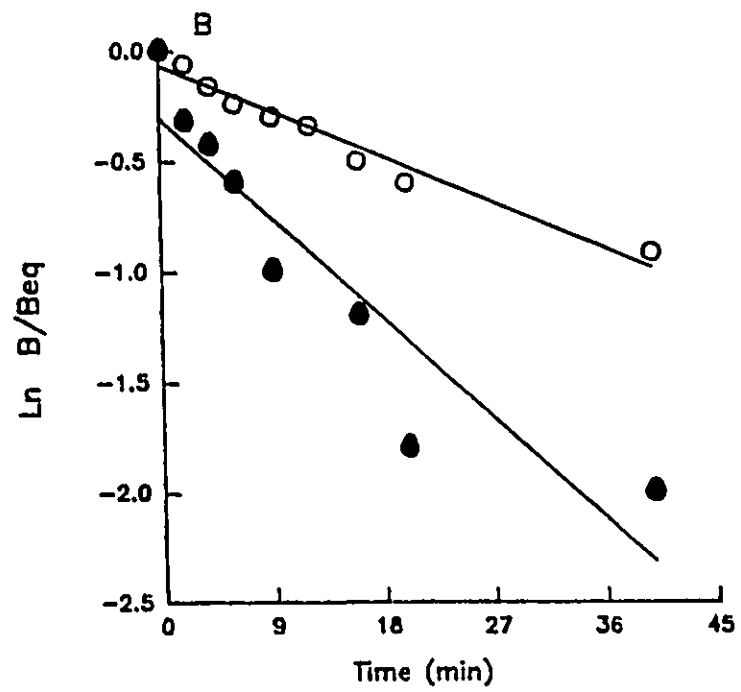
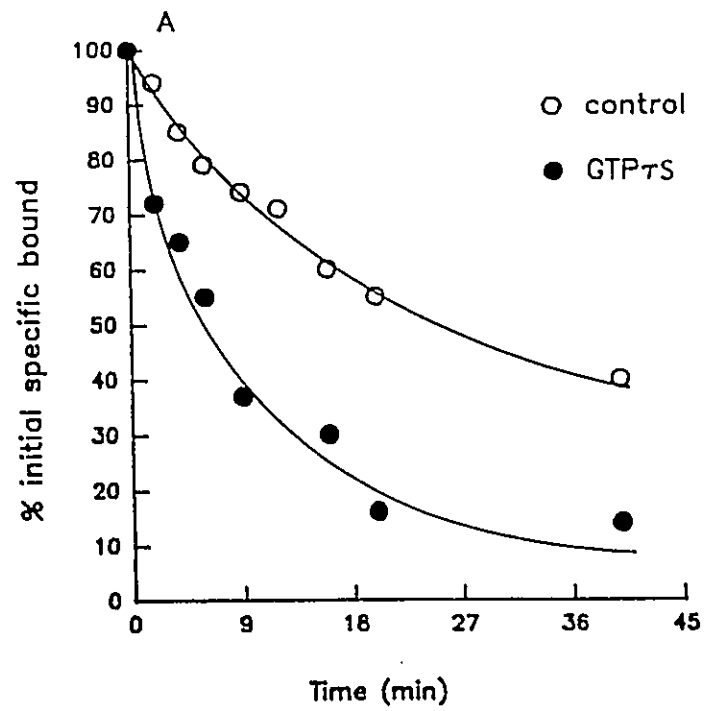
**Figure 13.** Displacement of  $^{125}\text{I}$ -galanin from plasma membrane by increasing concentration of galanin or galanin fragments 1-16 (Gal. 1-16), 1-15 (Gal. 1-15), and 15-29 (Gal. 15-29) under standard binding condition described in Methodology. Specific binding was expressed as a percentage of maximal specific binding. Results are means  $\pm$  SD of three similar experiments carried out in triplicate.



*6-3-5. Effects of guanine nucleotides:*

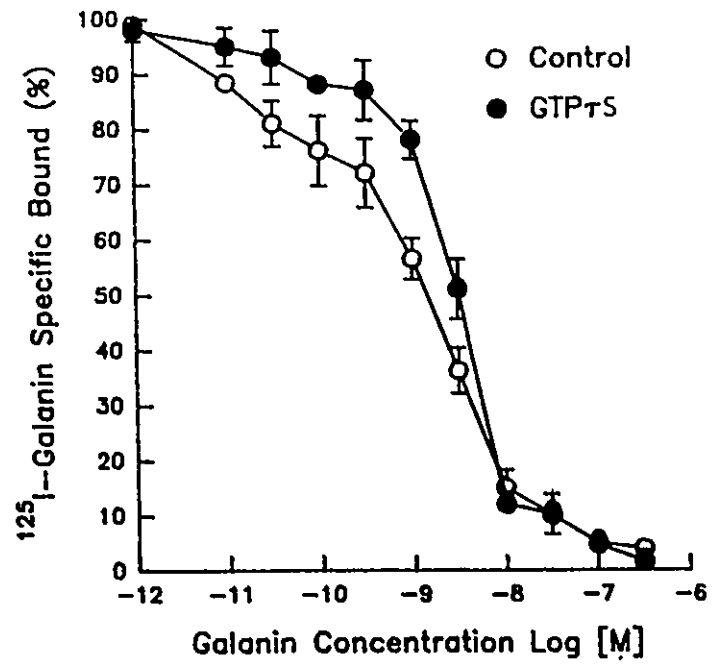
Addition of the nonhydrolyzable GTP analog, GTP $\gamma$ S caused an increase in the rate of dissociation of galanin binding (figure 13). Within the first 5 min after the addition of unlabelled galanin to the assay in the presence of GTP $\gamma$ S, 40% <sup>125</sup>I-galanin dissociated from the plasma membrane. The calculated rate constant of dissociation was  $0.064 \pm 0.004 \text{ min}^{-1}$ . GTP $\gamma$ S also decreased the high affinity binding of <sup>125</sup>I-galanin to the plasma membrane. As shown in figure 15, in the presence of the nucleotide, the upper part of the competition curve was shifted to the right in comparison with the control suggesting that the high affinity binding sites were abolished. Computer analysis confirmed that after GTP $\gamma$ S, the competition curve fitted a one-site model with  $K_i = 3.2 \pm 0.1 \text{ nM}$ , slope (nH) =  $0.9 \pm 0.1$ .

**Figure 14.** Effect of GTP $\gamma$ S on the time-course of dissociation of bound  $^{125}$ I-galanin from plasma membrane. Plasma membrane (20 ~ 25  $\mu$ g), in the presence of GTP $\gamma$ S ( $10^{-4}$  M) or absence of the nucleotide, was incubated with  $^{125}$ I-galanin (0.1 ~ 0.2 nM) under the standard binding condition. When equilibrium was reached, unlabelled galanin (1  $\mu$ M) was added. The radioligand remaining bound to the membrane was determined at different time intervals and expressed as a percentage of the initial (t=0) specific binding at equilibrium. The calculated rate constant of dissociation was  $0.064 \pm 0.004 \text{ min}^{-1}$ .





**Figure 15.** Effect of GTP $\gamma$ S on displacement of  $^{125}$ I-galanin from smooth muscle plasma membrane by increasing concentrations of unlabelled galanin. The nucleotide was added directly to the plasma membrane at a concentration of  $10^{-4}$  M. Values are means  $\pm$  SD of the experiments performed three times.

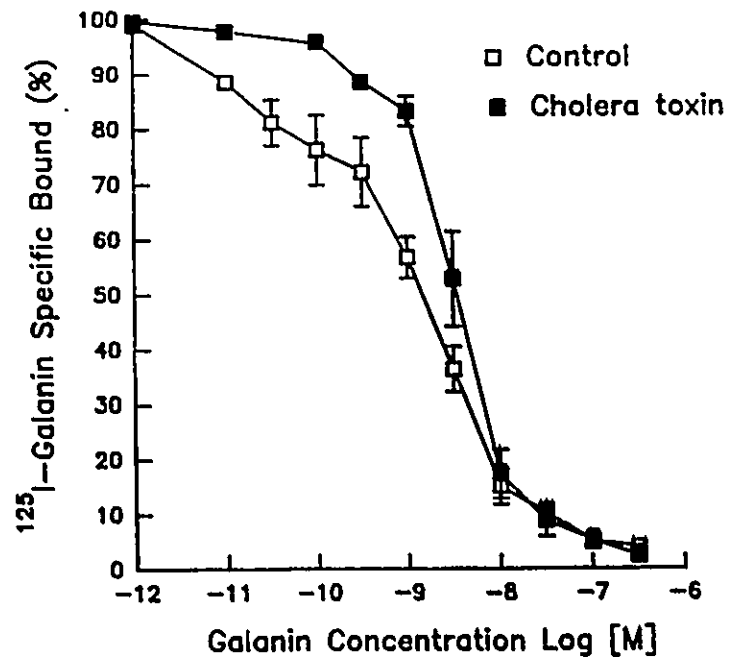


**6-3-6. Effect of cholera toxin:**

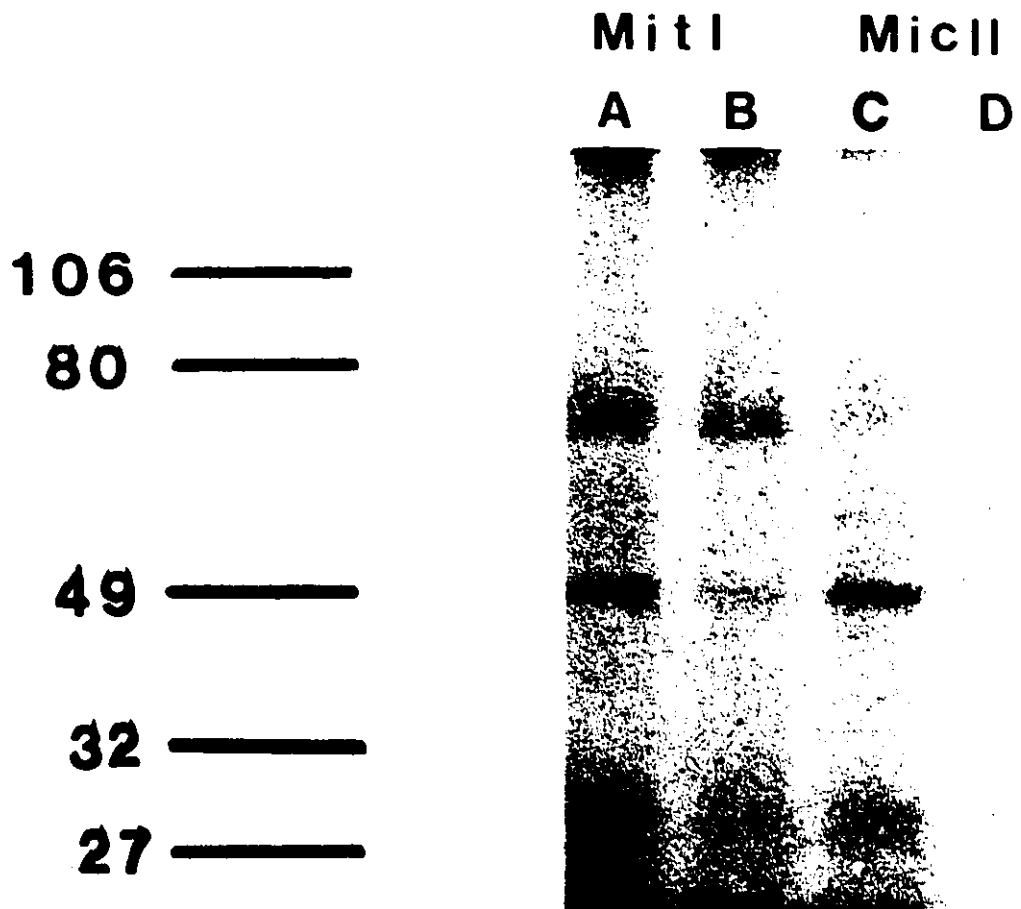
Pretreatment of canine small intestinal circular muscle plasma membrane with cholera toxin at a concentration of 2 mg/ml membrane abolished high affinity  $^{125}\text{I}$ -galanin binding (figure 16). The resultant competition curve was similar to that obtained after GTP $\gamma$ S addition. Computer analysis showed a one-site model with  $K_i=2.6 \pm 0.5$  nM,  $nH=1.0 \pm 0$ . PTX had no effect on competitive binding of  $^{125}\text{I}$ -galanin with unlabelled galanin to the plasma membrane (data not shown).

**6-3-7. Determination of the molecular weight of the galanin receptor:** SDS-PAGE analysis of galanin bound receptor in synaptosomes and smooth muscle plasma membrane revealed a similar band at an approximate molecular mass of 50 KDa (figure 17). In the presence of 1  $\mu\text{M}$  unlabelled galanin, the band disappeared indicating specific galanin binding sites in the neuronal and smooth muscle plasma membrane. In contrast, the bands at 70 KDa mass (for MitI fraction) was still visible in the presence of excess unlabelled galanin, indicating a nonspecific labelling.

**Figure 16.** Effect of cholera toxin (CTX) on the displacement of  $^{125}\text{I}$ -galanin from canine small intestinal circular muscle membrane by addition of increasing concentration of unlabelled galanin. The CTX was activated by DTT (50 mM) at  $30^{\circ}\text{C}$  for 2 hours. Then the plasma membrane was treated by activated CTX (2 mg/ml membrane) in the presence of ADP-ribosylating agents: 1 mM ATP, 0.2 mM NAD, and 20 mM arginine at  $30^{\circ}\text{C}$  for 30 min. Results are means  $\pm$  SD of four experiments performed in triplicates.



**Figure 17.** SDS-PAGE analysis of cross-linked  $^{125}\text{I}$ -galanin-receptor complex from plasma membranes of nerve (MitI) and smooth muscle (MicII) of canine small intestinal circular muscle and deep muscular plexus. The membranes (0.2 ~ 0.4 mg) were incubated with  $^{125}\text{I}$ -galanin (0.5 nM) in the presence (lane B and D) or absence (lane A and C) of 1  $\mu\text{M}$  unlabelled galanin at 25°C for 25 min. The galanin bound membrane fractions were then treated with 1 mM cross-linker disuccinimidyl tartarate (DST). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide slab gels to determine the molecular mass as described in **Methodology**. Autoradiograms of dried gels are shown. The numbers on the left of the photograph indicate the position of the standard molecular weight markers.



## **Chapter VII. Discussion**



### 7-1. Localization of Galanin Receptors in Small Intestine

An ultimate aim of our study was to determine the loci of the binding sites for galanin in canine small intestine. Earlier functional studies have shown that galanin has both myogenic and neuronal roles in regulation of small intestinal circular muscle motility in dog (Fox *et al*, 1986, 1988). Thus, the hypothesis of the existence of galanin receptors on both synaptosomal and smooth muscle plasma membrane of canine small intestinal circular muscle/DMP preparation seems reasonable. However, in order to localize the galanin receptor, either by radioligand binding assay, or by other assays, such as autoradiography, a separation of nerve and muscle plasma membranes from each other, and from the subcellular organelles is essential. By taking advantage of this membrane purification technique which was established in our laboratory (Ahmad, *et al* 1989), we were able to obtain relatively pure synaptosomal membranes and smooth muscle plasma membranes from circular muscle/DMP of canine small intestine. The purification of nerve and muscle membranes was judged by the presence of selective highly-specific and sensitive markers, i.e.  $^3\text{H}$ -saxitoxin binding and 5'-nucleotidase activity. It has been widely accepted that 5'-nucleotidase is a good marker for plasma membranes of smooth muscle cells from many tissue sources (Maeda *et al*, 1983, Forsman and Gustafsson, 1985, Kwan and Kostka, 1984), including the circular muscle of canine small intestine (Ahmad *et al*, 1989). In the present study, we showed that 5'-nucleotidase had highest activity in fraction MicII, as compared to other fractions obtained by differential centrifugation of the tissue homogenization of canine small intestinal circular muscle/DMP (figure 1). After discontinuous sucrose gradient

centrifugation of MicII, 5'-nucleotidase was enriched in M2 subfraction. An important result to indicate that MicII fraction was not contaminated by neuronal membranes was that MicII had much lower  $^3\text{H}$ -saxitoxin binding as compared to that in MitI fraction which had over 15 times higher  $^3\text{H}$ -saxitoxin binding than MicII. Since  $^3\text{H}$ -saxitoxin has been shown to be a good marker for neuronal membranes (see introduction, and Ahmad *et al*, 1988), it could be concluded that MitI and its discontinuous sucrose gradient subfractions F3, F4 are enriched in synaptosomal membranes, and MicII and M2 were mainly composed of smooth muscle plasma membranes.

However, as shown in figure 1 and 2, MitI, F3, F4, also contained considerable amounts of the marker for smooth muscle plasma membranes. The presence of low activity of 5'-nucleotidase in neuronal membranes has been shown in purified nerve membranes or myenteric plexus preparation from the intestine of dog (Kostka *et al*, 1987), monkey, rabbit (Wachstein and Meisel, 1957), and guinea-pig (Forsman and Gustafsson, 1985). In the present study, the lower activity of this enzyme in synaptosomes-rich fraction (MitI), as compared to that in muscle plasma membrane fraction (MicII), may reflect the role of 5'-nucleotidase in adenylate nucleotide metabolism in neuronal tissues (Phillis and Wu, 1981) or it may indicate a small contamination with smooth muscle membrane.

Figure 1 and 2 also showed that binding of  $^{125}\text{I}$ -galanin was much higher in MicII and MitI fractions and M2, F3, F4 subfraction. There was a good correlation between  $^{125}\text{I}$ -galanin binding,  $^3\text{H}$ -saxitoxin binding and 5'-nucleotidase activity. Therefore, we concluded that  $^{125}\text{I}$ -galanin binding sites are present on both synaptosomal and smooth

muscle plasma membranes of the canine small intestinal circular muscle preparations.

## 7-2. Binding Properties of the Galanin Receptor

The  $^{125}\text{I}$ -galanin binding sites on nerve and smooth muscle membranes showed high affinity and high capacity ( $K_d = 1.1 \text{ nM}$ ,  $B_{\text{max}} = 244 \text{ fmol/mg}$ , and  $K_d = 0.58 \text{ nM}$ ,  $B_{\text{max}} = 389 \text{ fmol/mg}$ , respectively). Table 2 summarizes the binding properties of galanin receptors in synaptosomes and smooth muscle plasma membranes of the canine small intestinal circular muscle. The  $^{125}\text{I}$ -galanin binding parameters in other tissues are summarized in Table 3. The galanin receptors in canine small intestinal circular muscle synaptosomes and muscle membranes have similar  $K_d$  values but higher capacity than those for rat brain, pancreatic  $\beta$  cells and intestinal membranes.

Although the saturation study gave one-binding site for  $^{125}\text{I}$ -galanin, both competition and kinetic studies suggested that the galanin receptor, on synaptosomes or smooth muscle plasma membranes, was a two-site model, one with high affinity ( $K_d = 0.01 \sim 0.02 \text{ nM}$ ) and the other with lower affinity ( $K_d = 1.1 \sim 2.8 \text{ nM}$ ). The evidence for the existence of two-binding sites rather than cooperation between binding sites are as follows: 1) over 40 ~ 50% of bound  $^{125}\text{I}$ -galanin could not be dissociated from the receptor within 40 ~ 45 minutes after adding excess unlabelled galanin ( $1 \mu\text{M}$ ); 2) the competitive binding curve fitted a two-site model, and 3) in the presence of GTP analog,  $\text{GTP}\gamma\text{S}$ , which is known to regulate the receptor by converting high-affinity sites to low-affinity ones, the dissociation of bound galanin was complete in 40 minutes after the addition of  $1 \mu\text{M}$  unlabelled galanin.

**Table 2:** Comparison of galanin receptors in synaptosomes and smooth muscle plasma membranes from canine small intestinal circular muscle/deep muscular plexus preparations:

| Parameters    | Nerve (MitI)          | Muscle (MicII)        |
|---------------|-----------------------|-----------------------|
| Kd            | 1.1 ± 0.13 nM         | 0.58 ± 0.085 nM       |
| Bmax          | 244 ± 2.1 fmol/mg     | 389 ± 29.9 fmol/mg    |
| Binding sites | high and low affinity | high and low affinity |
| Recognition   | N-terminal            | N-terminal            |
| G protein     | PTX-sensitive         | CTX-sensitive         |
| M.W           | 50 KDa                | 50 KDa                |

Values are means ± SD of 3 – 4 experiments performed in triplicate.

**Table 3:** Summary of characterization of galanin receptors in various tissue sources:

| Tissues         | Kd (nM)    | E <sub>max</sub> (fmol/mg) | Reference               |
|-----------------|------------|----------------------------|-------------------------|
| rat Brain       | 0.8 ± 0.03 | 26 ± 3                     | Chen, 1992              |
| rat spinal cord | 0.6 ± 0.2  | 55 ± 15                    | Bedecs, 1992            |
| rat hippocampus | 1.9 ± 0.4  | 107 ± 15                   | Fisone, 1989            |
| rat Rin m5F     | 0.3 ± 0.2  | 82 ± 22                    | Lagny-Pourmir,<br>1989b |
| Hamster β cells | 1.5 ± 0.2  | 43 ± 5.6                   | Amiranoff, 1986         |
| rat Rin 14B     | 1.6 ± 0.2  | 270 ± 14                   | Amiranoff, 1991         |
| rat stomach     | 2.7 ± 0.8  | 58 ± 11                    | Rossuwski, 1990         |
| rat jejunum     | 4.9 ± 1.7  | 32 ± 5.7                   | Rossuwski, 1990         |

In the case of synaptosomes, the dissociation appeared to be biphasic, thus two  $K_d$  values were obtained, one ( $K_d = 0.6$  nM) was similar to the low-affinity site given by saturation and competition studies, the other ( $K_d = 0.012$  nM) fits the high-affinity site given by the competition curve. In contrast, however, in plasma membrane of smooth muscle, we were unable to see the biphasic dissociation of bound  $^{125}\text{I}$ -galanin. This might be explained by two reasons: A) the rate of dissociation of bound galanin from low-affinity sites was too fast to catch up; B) the ligand concentration used in kinetic study was around 0.2 nM. This concentration did not lead to binding of galanin to a significant fraction of low affinity sites with  $K_d$  value of 2.8 nM, thus it was reasonable to assume that the low-affinity site were not effectively occupied at equilibrium. Combining reasons A and B, the dissociation of the low-affinity site-bound galanin may occur in the earliest stage of dissociation (may be just seconds after addition of the excess unlabelled galanin) which could not be assayed.

The reason that we could not obtain high-affinity binding sites from the saturation study in synaptosomes might be: 1) the population of the high affinity binding sites was much smaller than that of low affinity. Data from the competition curve showed that the high affinity binding sites were only 25 ~ 30% of the total number of the binding sites; 2) at very low concentration of the radioligand ( $< 0.01$  nM), most of the binding was found on filters thus low specific binding of galanin to the receptor was obtained when low concentration of  $^{125}\text{I}$ -galanin were used. In equilibrium studies of plasma membranes of smooth muscle, the low-affinity binding site was not detected because at high concentration of the radioligand ( $> 2$  nM), the non-specific binding increased and

occupied more than 50 ~ 75% of the total binding, thus it was hard to differentiate between total and non-specific binding when an high concentration of the radioligand was used.

The physiological, and/or pharmacological significance of high- and low-affinity sites of a receptor has not so far been revealed. The observations, from the present study and others (Provow and Velicelebi, 1987, Huang and Rorstad, 1988), that guanine nucleotides abolish only the high affinity sites or states of the receptor support the hypothesis that the high affinity site may play a greater functional role in receptor coupling to effect mechanisms, *e.g.* adenylate cyclase.

The previous functional studies (Fox *et al*, 1986, 1991) reported that galanin at a concentration of  $1.5 \times 10^{-10}$  moles inhibited phasic activity of canine small intestine *in vivo*, and galanin at  $10^{-8}$  M and  $10^{-7}$  M produced inhibition of VIP release. However, since galanin was delivered to the tissues differently in these two studies (intraarterial bolus and intraarterial infusion respectively), the final concentrations of galanin for eliciting the myogenic and neuronal effects were unknown. Considering the  $K_d$  values obtained from the present study, it is not known if only the high-affinity binding sites are responsible for galanin-receptor mediated biological effects, or both high- and low-affinity binding sites are involved.

It is interesting to note that our present study was the first, probably the only one, demonstrating that galanin binds to a two-class of receptors. Previous receptor binding studies, either in Rin m5F cell line or in rat hippocampus, showed a single class of binding sites. However, the newly synthesized high-affinity galanin antagonist, galantide,

recognizes a high- ( $K_1 = 0.001$  nM) and a low-affinity ( $K_2 = 1$  nM) binding site in the Rin m5F cell line (Lindskog *et al*, 1992). It was reported that galantide functions as a galanin-antagonist by completely blocking the galanin-induced inhibition of insulin secretion. No evidence of effect of galantide as an antagonist on small intestinal smooth muscle cells has been reported. If an highly-specific galanin antagonist is available, it will be an useful tool in the direct study of the biological action of galanin in GIT and subtyping galanin receptors between different tissues.

A 50 KDa band was revealed when bound  $^{125}\text{I}$ -galanin was cross-linked to either synaptosomal or smooth muscular membranes by DST. This labelling was specific for galanin as evidenced by displacement of the label with unlabelled galanin. If we assume that one molecule of galanin is bound and cross-linked per molecule of receptor, the size of galanin receptor will be 47 KDa.

### **7-3. Structure-Activity Relationship in Receptor Recognition**

The fact that the N-terminal 15-amino acid residues of galanin are identical in different animal species, while the C-terminal part of the molecule is quite varied, suggests that the active part of the galanin molecule may be in the N-terminal region. In dog, the N-terminal fragment, galanin 1-15, is equally effective on relaxation of canine intestinal circular muscle to galanin 1-29 *in vitro* and *in vivo* (Fox *et al*, 1988). The present study demonstrated that  $^{125}\text{I}$ -galanin binding, either on synaptosomes or smooth muscle plasma membrane, can be completely displaced by the unlabelled parent galanin molecule (1-29) and partially displaced by the N-terminal fragments, galanin 1-16, 1-15,



1-11. However, galanin 15-29 at a concentration of 1  $\mu$ M was totally inactive in competing for binding with radioiodinated galanin to the receptor on nerve or muscle membranes (Table 4). Our results are in contrast with the report that galanin 1-15 did not inhibit  $^{125}$ I-galanin binding to the rat gastric and jejunal smooth muscle membrane receptors while galanin 15-29 resulted in a loss of 90% of affinity (Rossowski *et al*, 1990). The reason for these differences in receptor recognition is not yet known, but species-related effect in recognition ability of galanin receptors, or the existence of subtypes of galanin receptors may be responsible.

**Table 4:** Inhibitory constants (Ki) of galanin, or galanin fragments for galanin receptors in synaptosomes and smooth muscle plasma membrane from canine small intestinal circular muscle/deep muscular plexus preparations:

| galanin<br>fragments | synaptosomes (MitI)        |             | plasma membrane (MicII)  |              |
|----------------------|----------------------------|-------------|--------------------------|--------------|
|                      | Ki (nM)                    | nH          | Ki (nM)                  | nH           |
| galanin 1-29         | 0.02 ± 0.005<br>1.05 ± 0.3 | 0.61 ± 0.04 | 0.01 ± 0.05<br>2.8 ± 0.2 | 0.59 ± 0.017 |
| galanin 1-16         | 5.5 ± 1.8                  | 0.86 ± 0.2  | 6.6 ± 0.8                | 0.41 ± 0.13  |
| galanin 1-15         | 12.4 ± 2.2                 | 0.46 ± 0.1  | 42 ± 2.7                 | 0.63 ± 0.2   |
| galanin 1-11         | 20.4 ± 5.1                 | 0.95 ± 0.21 | —                        |              |
| galanin 15-29        | inactive                   |             | inactive                 |              |

Values are means ± SD of 2 ~ 3 experiments performed in triplicate.

#### 7-4. Galanin-Receptor Interactions with G Proteins

The present study supports the hypothesis that GTP-binding proteins may be involved in the interaction of galanin with the receptor on both neuronal and smooth muscle plasma membranes in canine small intestinal circular muscle. This is based on several observations: 1) binding of galanin to the receptor was highly sensitive to the GTP analog, GTP $\gamma$ S. The addition of GTP $\gamma$ S apparently abolished the high affinity galanin binding site, leaving only a low affinity receptor site; 2) GTP $\gamma$ S increased the rate of dissociation of prebound iodinated galanin from the receptor; and 3) binding of galanin to the receptor was inhibited by the bacterial toxins, *i.e.* PTX or CTX, which are known to ADP-ribosylate the G $\alpha$  subunit of G proteins (see below).

The G proteins complexing with the galanin receptor on synaptosomes seems to be different from those on smooth muscle plasma membrane. In nerve membranes, galanin receptors are functionally coupled with a PTX-sensitive, CTX-insensitive G protein, while the G protein coupled to the galanin receptor on smooth muscle membrane was sensitive to CTX but not to PTX.

The negative heterotropic interaction between the binding of guanine nucleotide, *e.g.* GTP $\gamma$ S, and the binding of agonist to the receptor is criteria for identification of involvement of a G protein in ligand-receptor interaction. The decrease of the affinity of galanin for its receptor is the consequences of the binding of the nonhydrolysable GTP analog (GTP $\gamma$ S) to G protein which in turn generates a permanently active G $\alpha$ GTP complex.

A distinctive characteristic of G proteins is that the  $\alpha$  subunits of individual G

proteins are substrates for ADP-ribosylation catalyzed by the bacterial toxins, *Vibrio cholera* and *Bordetella pertussis*. The toxin induced ADP-ribosylation reactions have been of great experimental value in identification of various G proteins involved in ligand-receptor interaction. G proteins have been classified as  $G_s$ ,  $G_i$ ,  $G_o$ , and  $G_t$ , according to the different  $\alpha$  subunits which are linked to various effectors (Ui, 1990). Most of these G proteins are ADP-ribosylated by PTX at a site on the  $\alpha$  subunit. An exception is  $G_s$ , which is the substrate for CTX. In addition,  $G_t$ , which mediates light-induced activation of cGMP phosphodiesterase in retinal cells, can be ADP-ribosylated by both PTX and CTX. Table 5 lists the various G proteins and their effectors (Gilman, 1987, Ui, 1990).

The cysteine, located four residues from the carboxyl terminus of the  $\alpha$ -subunit of G proteins, is the site of ADP-ribosylation by PTX or CTX (Ui, 1990). PTX or CTX induced ADP-ribosylation of  $G\alpha$  uncouples G proteins from receptors, thus the signals can not be transmitted from the receptors to the effectors. Our data clearly showed that both GTP $\gamma$ S and PTX or CTX caused a decrease in galanin affinity for the receptors on synaptosomes and smooth muscle membranes, suggesting the involvement of G proteins in galanin-receptor interactions.

Table 5: Types of G proteins (Modified from Gilman, 1987, Ui, 1990).

| G protein       | Mwt (KDa) | Substrate for toxin | Effectors                 |
|-----------------|-----------|---------------------|---------------------------|
| G <sub>s</sub>  | 46        | CTX                 | (+) AC                    |
| G <sub>i1</sub> | 40        | PTX                 | (-) AC                    |
| G <sub>i2</sub> | 41        | PTX                 | (+) PLC                   |
| G <sub>o</sub>  | 39        | PTX                 | K <sup>+</sup> -channel   |
| G <sub>HL</sub> | 40        | PTX                 | Ca <sup>2+</sup> -channel |
| G <sub>t1</sub> | 40        | CTX & PTX           | cGMP PDE                  |
| G <sub>t2</sub> | 40.4      | CTX & PTX           | cGMP PDE (?)              |

Abbreviations: Mwt, molecular weight; KDa, killo dalton; CTX, cholera toxin; PTX, pertussis toxin; AC, adenylate cyclase; PLC, phospholipase C; cGMP PDE, cGMP phosphodiesterase.

### 7-5. Mechanisms of Neuronal Action of Galanin

Studies on isolated perfused canine ileum showed that galanin modulates circular muscle motility by neural inhibition of VIP release (Fox *et al*, 1988). The effector system for the neuronal effect of galanin on the regulation of the neuropeptide release in GIT has not been revealed. It is known that GI peptides are released from their cell of origin by the process of exocytosis. The mechanisms that causes exocytosis are not clear. Evidence has been shown that the cytoplasmic concentration of  $\text{Ca}^{2+}$  plays a critical role in the occurrence of fusion of vesicles with the cell membranes. Increase of membrane permeability to  $\text{Ca}^{2+}$  and thus enhancement of influx of  $\text{Ca}^{2+}$  facilitates neuropeptide release, whereas decrease of  $\text{Ca}^{2+}$  entry inhibits it. Recent, evidence shows that cAMP- and  $\text{Ca}^{2+}$ -dependent phosphorylation of a synaptosomal substrate in nerve terminals plays a key role in regulation of neurotransmitter release (Camilli, 1990). This substrate has been isolated, identified and named "synapsins" (Hunttner *et al*, 1983). Synapsins are membrane-bound proteins containing four homologous proteins, *i.e.* synapsin Ia, Ib, synapsin IIa, IIb. The synapsins are concentrated in nerve endings. Ultrastructural studies demonstrated that within the "silent" nerve terminals (*i.e.* no nerve firing), synapsins are associated with synaptic vesicles thus no exocytosis occurs (Camilli, 1990). During nerve stimulation, an increase in the intraterminal  $\text{Ca}^{2+}$  concentration, and/or cAMP level, activates  $\text{Ca}^{2+}$ /calmodulin and/or cAMP-dependent protein kinase which phosphorylates the synapsins. The phosphorylation of synapsins reduces their binding to synaptic vesicles, thus more vesicles are available for fusion with the membrane, leading to neuropeptide release (Camilli, 1990). Therefore, phosphorylation/dephosphorylation of

synapsins by effectors coupled by G proteins to the receptor on synaptosomal membrane may determine activation/inhibition of neuropeptides release.

It has been demonstrated that many hormones and neuropeptides suppress other neurohormonal release by virtue of their ability to inhibit adenylate cyclase activity and subsequently attenuate cAMP production via the Gi proteins (Limbird, 1988). In addition to the receptor linked inhibition of adenylate cyclase, other types of PTX-sensitive G proteins couple ion channel activity to suppress neuropeptide release, including: 1) K<sup>+</sup> channel opening thus hyperpolarising the membrane; and 2) inhibition of voltage-dependent Ca<sup>2+</sup> channel therefore decreasing Ca<sup>2+</sup> entry (Limbird, 1988). A two component inhibitory mechanism of galanin on myenteric neurons has been proposed: one component is the hyperpolarization of the membrane by opening of K<sup>+</sup> channels, the other is blockade of voltage-gated Ca<sup>2+</sup> channels (Tauramura, 1988). Studies on galanin induced inhibition of insulin release revealed the involvement of a PTX-sensitive, G protein regulating the inhibition of adenylate cyclase. Furthermore, Ullrich, et al (1989) demonstrated that in Rin m5F cells, galanin inhibits insulin release by directly affecting the PTX-sensitive exocytotic process. The observation of G protein coupled PTX-sensitive galanin receptor on synaptosomes from the present study suggests that galanin may regulate VIP, and probably the release of other GI neuropeptides by an interaction with a specific receptor coupled to one or more than one of the effectors, including: inhibition of adenylate cyclase, activation of potassium and/or inhibition of calcium channels which in turn inhibit the exocytotic process. Further experiments need to be done to define which effector(s) is responsible for the neuromodulatory effect of galanin

on GIT.

### **7-6. Possible Effectors of the Myogenic Action of Galanin**

The presence of galanin receptor on the plasma membrane of canine circular muscle supports the hypothesis that galanin may act as an neurotransmitter in GI small intestine. Fox, et al (1986) showed that galanin inhibits contraction of canine small intestinal circular muscle, both *in vivo* and *in vitro*, by a TTX-insensitive direct myogenic mechanism. Botella, *et al* (1992) recently reported that galanin induced a concentration-dependent contraction of isolated ileum circular muscle cells from pig, rat, and rabbit. They also showed that galanin inhibited isolated circular muscle contraction induced by cholecystokinin 8 (CCK 8) in the dog ileum. Our present study showing the high affinity binding sites for galanin on smooth muscle plasma membrane strongly suggests that the direct myogenic action of galanin on GI smooth muscle activity is mediated via the interaction of galanin with specific galanin receptors on the muscle membranes.

The observation of a CTX-sensitive, PTX-insensitive G protein coupled galanin receptor on smooth muscle plasma membrane suggested that adenylate cyclase activation might be the effector for the galanin-receptor interaction, since G<sub>s</sub>, which couples to activation of adenylate cyclase, is the only G protein reported to be the substrate for ADP-ribosylation by CTX, but not by PTX (Ui, 1990). The activation of G<sub>s</sub> by galanin-receptor interaction might stimulate intracellular adenylate cyclase which in turn increases the cyclic AMP production (Gilman, 1987). It has been demonstrated that elevation of intracellular cyclic AMP, as a result of adenylate cyclase stimulation,



functions as a second messenger to mediate smooth muscle relaxation by 1) inhibition of  $\text{Ca}^{2+}$  release from endoplasmic reticulum (ER) and increase of  $\text{Ca}^{2+}$  uptake by the  $\text{Ca}^{2+}$  pump on the ER (Severi *et al*, 1987), 2) hyperpolarization of plasma membrane by opening the membrane  $\text{K}^+$  channels (Standen *et al*, 1989), and 3) phosphorylation of myosin light chain kinase (MLCK), thus decreasing its sensitivity to  $\text{Ca}^{2+}$ -calmodulin (De Lanerolle *et al*, 1984). Obviously, further studies need to be done to investigate the relationship, if there is one, between intracellular cAMP levels and the galanin-receptor interaction.

### **7-7. Galanin: A Neurotransmitter and/or Neuromodulator ?**

The demonstration of the presence of galanin receptors on the circular smooth muscle membrane and the deep muscular plexus nerves provides an explanation for the direct (on muscle) and indirect (on nerve) action of galanin on GI motility observed in dogs (Fox *et al*, 1986, 1988), and also, suggests that galanin may join other GI peptides, e.g. VIP, substance P, and function as an neurotransmitter and/or neuromodulator (regulate release of other neuropeptides ).

There are several lines of evidence for galanin as neuromodulator: 1) exogenous galanin inhibits the tonic release of VIP from the isolated perfused canine ileum (Fox *et al*, 1988); 2) galanin prejunctionally inhibits cholinergic enteric nerves and acetylcholine and substance P release in canine small intestine (Gonda *et al*, 1988) and guinea-pig taenia coli (Ekblad *et al*, 1985); 3) in guinea-pig small intestine, results from intracellular microelectrode studies of myenteric neurons revealed that galanin had an inhibitory action

on these neurons by hyperpolarization of the membrane, decreasing input resistance, and suppression of excitability (Palmer *et al*, 1986, Tamura, 1987); moreover, galanin depressed the acetylcholine release evoked by electrical stimulation or caused by VIP and substance P (Yau *et al*, 1986). So far, the neuromodulatory effect of galanin has been demonstrated only on the release of VIP, acetylcholine and substance P, but, it is possible that other neuropeptide or non-peptide mediators are also affected. The loci of the action site, *i.e.* the location of galanin receptors, could be at the presynaptic and/or postsynaptic level. It is not known if galanin directly affects neuropeptide release or indirectly via an unknown mediator.

The hypothesis of galanin as an neurotransmitter is supported by the following observations: 1) galanin immunoreactive nerve fibres are widely distributed in GIT (Ekblad *et al*, 1988, Gonda *et al*, 1989); 2) release of galanin can be elicited by nerve stimulation or acetylcholine application in isolated perfused porcine ileum (Messell *et al*, 1992); and 3) direct action of galanin on GI smooth muscle, either *in vivo* or *in vitro*. A question raised from these observation is if galanin is a inhibitory, or excitatory neurotransmitter or both? Since galanin shows highly-species dependent myogenic effects on GI smooth muscle activity, here, we propose a hypothesis that inhibitory or excitatory effects of galanin on GIT motility may be dependent on the subtype of galanin receptor, which is coupled by distinct G proteins. This hypothesis is supported by the evidence from our present study which showed a CTX-sensitive G protein in canine small intestinal smooth muscle, while other studies have demonstrated that a PTX-sensitive G protein, which results in an influx of  $Ca^{2+}$  into the cell, was involved in galanin-induced

smooth muscle contraction in pig ileum (Botella *et al*, 1992). Further experiments to elucidate the effector systems of the CTX-sensitive G protein coupled galanin receptor will be of great value in support of this hypothesis.

## **Chapter VIII. Concluding Remarks**

## 8-1. Conclusions

The major contributions of the present study are the following:

- 1) The localization of galanin binding sites was revealed in synaptosomal and smooth muscle plasma membranes of canine small intestinal circular muscle/DMP preparations.
- 2) The galanin binding sites were characterized using radioligand binding assays with radioiodinated porcine galanin as the labelled ligand. The binding of  $^{125}\text{I}$ -galanin to the membranes was saturable, reversible and specific.
- 3)  $^{125}\text{I}$ -galanin binding to synaptosome rich fractions or fractions enriched in smooth muscle plasma membranes revealed two binding sites, representing a high- and a low-affinity galanin receptor. The functional significance of these two binding site remains unknown.
- 4) The galanin receptor is a protein with molecular weight of 50 KDa and recognises predominantly the N-terminal region of porcine galanin.
- 5) The galanin-receptor interaction on synaptosomes involved a PTX-sensitive G protein, whereas on smooth muscle membranes, a CTX-sensitive G proteins are coupled to the receptor. However, the effectors linked to these two different G proteins remain to be elucidated.
- 6) The present study provides further evidence in support of the role of galanin as neurotransmitter and/or neuromodulator in regulation of canine GI motility proposed by previous functional studies *in vivo* and *in vitro*.

## 8-2. Future directions

The mechanism of transmembrane signalling of galanin-receptor interaction need to be identified by studying the ability of galanin to activate or inhibit adenylate cyclase, and/or regulate ion channels, e.g. K<sup>+</sup> channels and Ca<sup>2+</sup> channels.

The signals, either physiological or pharmacological, for galanin release have not been studied in species other than the pig ileum *in vitro*. Research on finding the relationship of galanin with other neuropeptides, such as VIP, substance P, or the newly discovered nitric oxide will be of great significance in elucidating the mechanism of neuropeptide release and the correlation between GI peptides and their role in the regulation of GIT functions.

Effort on finding a highly-selective galanin antagonist will be extremely helpful on clear-cut delineation of galanin receptor subtype between the species, or between the various tissues, e.g. nerve vs smooth muscle.

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