MORPHOLOGICAL AND FUNCTIONAL STUDIES OF SUBSTANCE P AND SOMATOSTATIN IN THE SMALL INTESTINE

By

Eric Anthony Accili, B.Sc. (1982), M.Sc. (1987)

The University of British Columbia

A thesis submitted in partial fulfillment of the requirements for the degree of

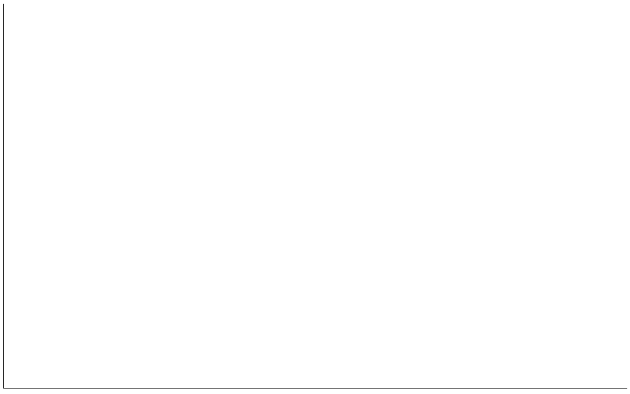
Doctor of Philosophy

in

The Faculty of Graduate Studies

Department of Physiology

We accept this thesis as conforming to the required standard



The University of British Columbia

June 1992

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of Physiology

The University of British Columbia
Vancouver, Canada

Date July 6 1992

ABSTRACT

The regulation of gastrointestinal function is partly dependent on the intrinsic neurones of the gut. Intrinsic neurones contain a large number of neurotransmitters, including neuropeptides, in different combinations. Neurones have been grouped according to the combination of neurotransmitters they contain and this practice is called chemical coding. The present studies were carried out to examine differences in chemical coding and enteric neuronal morphology in the human and canine small intestine, especially the submucosal plexus. The neuropeptides chosen for examination were substance P, somatostatin and vasoactive intestinal peptide because of their known involvement in both the physiology and pathophysiology of the small intestine. Further, primary cultures of submucosal neurones from human and canine submucosal plexus were utilized to determine whether differences in coding and morphology paralleled differences in somatostatin secretion.

Substance P immunoreactivity (SP-IR), somatostatin immunoreactivity (SS-IR) and vasoactive intestinal peptide immunoreactivity (VIP-IR) have been localized and their distributions have been compared in dog and human small intestine using immunocytochemistry (ICC). An antibody to protein gene product 9.5 (PGP) was used to localize all nerve cell bodies and fibres in the dog and human upper

jejunum. In addition, the proportions of peptide-containing neurones were determined by double staining.

Staining with PGP revealed neuronal cell bodies in the submucosal plexus (SMP) and the myenteric plexus (MYP) as well as extensive innervation by fibres throughout all regions of the small intestine. Canine submucosal ganglia contained 7.7 ± 0.6 neurones per submucosal ganglion (184 ganglia, n = 6 dogs), while the human ganglia contained 2.9 ± 0.3 (185 ganglia counted, n = 5 donors). Over 50 % of the ganglia in the human sections contained 3 or less neurones but over 10 % of the ganglia in canine sections contained 15 or more neurones. Finally, the canine circular muscle was shown to possess a distinct deep neural plexus, in contrast to that of human circular muscle.

The distribution of SP-IR, SS-IR and VIP-IR in canine and human jejunum was similar, confirming the results of previous studies. Double staining revealed that SP-IR and SS-IR were always co-localized in the human, but not canine, SMP and MYP. In both species VIP-IR was present in a population of neurones distinct from those containing SP-IR and SS-IR. In canine ganglia, 30% of neurones per ganglion in the SMP contained SP-IR, 35% contained SS-IR and 30% contained VIP-IR. In human ganglia, 42% of neurones per ganglion contained SP-IR and SS-IR while 40% contained VIP-

IR. These results sugest different functions for SP and SS in canine and human enteric ganglia.

The secretion of SS-IR, from intact preparations of small intestine, is difficult to interpret for two reasons. First, SS-IR has been demonstrated in vagal, submucosal and myenteric neurones as well as endocrine cells, of the small intestine. Second, enteric neurones have been shown to to contain the 14 amino acid form of SS-IR (SS-14) while endocrine cells have been shown to contain the 28 amino acid form (SS-28). A dispersed culture of submucosal cells from human small intestine was developed to examine the localization, release and molecular characteristics of SS-IR. After 72 h, the neurones were shown to be viable and to sprout neurites containing varicosities suggesting that they retained a morphologic phenotype similar to that observed *in situ*. Thirty percent of the submucosal neurones per ganglion in tissue sections and 35 % of cells per cluster in culture contained SS-IR. Acetic acid extracts of cultures contained 1990 ± 809 pg SS-IR/well. Incubation of cultures with phorbol 12ß-myristate 13-acetate (ß-PMA), an activator of protein kinase C (PKC), at concentrations up to 10^{-6} M for 120 min increased the release of SS-IR up to 23 times the basal level, and up to 27 times the basal level when extracellular K^+ was increased from 5 to 10 mM. Of the total SS-IR released in response to ß-PMA (10^{-6} M, 10 mM K^+), 59% was present in the medium after 30 min and 80%

after 60 min. Basal release of SS-IR could be reliably measured only after 120 min, therefore experiments which examined somatostatin secretion were carried out for this amount of time. The release of SS-IR by ß-PMA was not due to non-specific membrane effects since the inactive 4α-phorbol at the same concentrations did not alter basal release. Greater than 90 % of SS-IR present in acid extracts of cultures and released by ß-PMA eluted with the same retention time as synthetic SS-14 on reverse phase high performance liquid chromatography (HPLC).

In summary, the results presented in this thesis have shown that differences exist in the neuropeptide distribution and neuronal morphology between the canine and human small intestine. Moreover, SS-IR secretion from human submucosal neurones in response to SP and the phorbol ester were found to be different from the secretion of SS-IR from canine neurones. The results suggest that differences observed in the pattern of secretion in submucosal neurones probably reflect the differences noted in neuropeptide distribution and neuronal morphology. Furthermore, the present studies emphasize that the extrapolation of experimental data between species must be made with caution.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I extend my thanks to Dr. Alison Buchan for her time and committment to my work, and for her perseverance during the completion of this thesis. The assistance of Dr. Chris McIntosh in providing training and expertise for the HPLC, and imparting current information on everything known to man, was much appreciated. Advice on the design of pharmacological studies, especially the importance of industrial doses of skepticism in science, was graciously offered by Dr. Yin Nam Kwok and graciously accepted by myself. As the graduate advisor, Dr. Ray Pederson has always put the concerns of students first and I thank him for his enthusiastic interest in both my studies and my hobbies. I must thank Dr. John Brown because he would feel left out otherwise and more importantly because he was responsible for cultivating my interest in regulatory peptides and for encouraging me to pursue this interest within the MRC group. Finally, I am especially grateful to my friends and colleagues who have helped me with my curricular and extracurricular activities.

I. INTRODUCTION.

A. General Background.

Neuropeptides have been recognized as potential neurotransmitters since the discovery of the hypothalamic hormones. These include vasopressin and oxytocin from magnocellular neurones, sequenced by du Vigneaud (1953, 1954), as well as luteinizing hormone releasing hormone, thyrotropin releasing hormone and somatostatin (SS) from parvicellular neurones (Guillemin, 1978; Brazeau *et al*, 1973; Schally *et al*, 1973).

Information on their function is difficult to interpret, in part, because they are members of a larger group of regulatory peptides that also have hormonal and paracrine actions. Peptides have been demonstrated to exist in neurones and endocrine cells, which suggests that they may have endocrine, paracrine, neuroendocrine and neurotransmitter functions (Brown *et al*, 1971; Feyrter, 1953; Chang *et al*, 1971; Gullemin, 1978). For example, SS has been found in hypothalamic neurones and acts in a neuroendocrine manner to inhibit growth hormone release (Brazeau *et al*, 1973). Somatostatin has been localized to cerebral cortical neurones (Johansson and Hokfelt, 1980; Krisch, 1980) where it has been implicated as a neurotransmitter (Guillemin, 1978). Somatostatin has been

found in endocrine cells of the stomach, pancreas and small intestine, indicating a possible role as a hormone (Luft *et al*, 1974; Dubois, 1975; Polak *et al*, 1975). Finally, paracrine actions have been proposed for SS, in addition to its endocrine actions, based on its presence in endocrine cells particularly those which have long cytoplasmic processes (Larsson, 1979; Yamada, 1987). Other examples of peptides with multicellular origins include substance P, cholecystokinin and neurotensin.

Unlike classical neurotransmitters, the original consideration of neuropeptides as potential neurotransmitters was the result of their localization in neural tissue by immunocytochemistry or radioimmunoassay (Hokfelt, 1991) and in most cases this evidence remains the most compelling. The function of the putative neuropeptide neurotransmitters has been difficult to elucidate. The peptides must fulfil several criteria in order to be considered as neurotransmitters including the following (Dockray, 1987):

a. that the peptide is found in neurones.
b. that it is concentrated in nerve terminals from which it can be released by depolarizing stimuli with or without a calcium-dependent mechanism.
c. application of the purified peptide exerts an effect that is duplicated when the endogenous material is released.

d. a selective antagonist is able to block the actions of both endogenously released and exogenously applied peptide.
e. there are mechanisms for the breakdown, reuptake or the removal of the peptide.

There are difficulties in the identification of neuropeptides as neurotransmitters when compared to classical neurotransmitters such as acetylcholine and noradrenaline. Unlike classical neurotransmitters which are synthesized exclusively in nerve terminals, neuropeptides must be synthesized in cell bodies and transported to the nerve terminal where they are stored prior to release. There is presently no evidence for the uptake and reuse of neuropeptides by neurones.

The cell types which contain peptides belong to a family of cells with a common amine handling system present and hence were called APUD (Amine Precursor, Uptake and Decarboxylation) cells (Pearse, 1976). These cells include neuropeptide containing endocrine cells which share common characteristics with neuropeptide producing neurones.

Peptides have been shown to be members of families coded by the same or similar genes, but become processed in different ways in different cell types. The processing can occur at the level of mRNA, for example, by splicing such as in the case of the neuropeptide calcitonin gene-related

peptide (CGRP) and the hormone calcitonin. Peptides may undergo post-translational modifications such as enzymatic cleavage and C-terminal amidation, as in the case of the neuropeptide CCK-8 and the hormone CCK-33.

A further complication in the study of neuropeptides is that more than one neurotransmitter can be present in one neurone (Millhorn and Hokfelt, 1988). The transmitter substances may be neuropeptides, amino acids or a classical transmitter (noradrenalin or acetylcholine) which would allow the release of three types of neurotransmitters to convey fast, moderate or slow signalling from a single terminal (Iversen and Goodman, 1986).

B. Background on the Small Intestine.

The small intestine is innervated by both extrinsic and intrinsic nerves. The vagus and sacral nerves as well as the sympathetic ganglia supply the extrinsic parasympathetic and sympathetic innervation, respectively. However, the small intestine has an extensive intrinsic nervous component which, together with the processes of the sympathetic, parasympathetic and sensory neurones, was named the enteric nervous system. Langley (1921) considered this to be a portion of the autonomic nervous system separate from the sympathetic and parasympathetic branches.

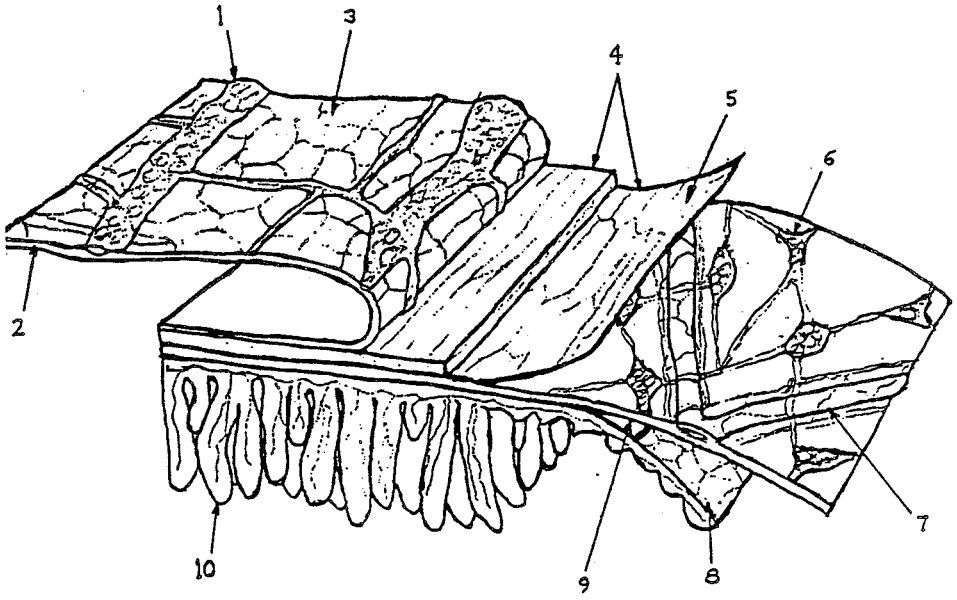
Enteric neuronal axons are unmyelinated and have been shown to have varicosities along their length, each of which represents a nerve ending from which a neurotransmitter can be released (Gabella, 1987).

The enteric nervous system is made up of two ganglionated plexuses called, respectively, the submucosal and myenteric. The submucosal plexus (SMP), described by Meissner (1857), is located in the connective tissue of the submucosa while the myenteric plexus (MYP), lies between the longitudinal and circular muscle of the muscularis externa (figure 1).

The ganglia of the SMP have been shown to be smaller than those of the MYP, and it has been suggested that they contain fewer neurones (Furness and Costa, 1980; Gabella, 1987; Gabella, 1990). The MYP has been shown to be structurally uniform along the length and circumference of the small intestine, and the myenteric ganglia were shown to have their long axis in the direction of the circular muscle (Gabella, 1987). The submucosal ganglia were shown to form smaller meshes, they did not demonstrate polarization with respect to the axes of the small intestine and they showed variation with respect to cell number along the length of the small intestine.

The enteric ganglia also show substantial variation between species (Gabella, 1987; 1990). The ganglia of the guinea pig small intestine have been shown to vary in size, but contain similar types of nerve cells and fibres (Wilson *et al*, 1981; Furness *et al*, 1984; Bornstein and Furness, 1988).

Figure 1. Diagram of a segment of intestine partly separated into layers showing the arrangement of enteric plexuses. (1) MYP (2) longitudinal muscle (3) tertiary plexus (4) circular muscle (5) deep muscular plexus (6) submucosal plexus (7) submucosal artery (8) mucosal plexus (9) muscularis mucosa (10) mucosa. (Adapted from Costa et al, 1987).


1
3
4
5
6
2
7
10
9
8

In many species including the dog and human, the ganglia of the submucosa have been shown to form outer and inner plexuses (Schabadasch, 1930), the latter which would become known as Schabadasch's plexus and the former which would be regarded as conforming to Meissner's original description of a plexus in the submucosa (Furness *et al*, 1989; Gunn, 1968; Scheuerman *et al*, 1989; Christensen and Rick, 1987; Stach, 1977). Submucosal ganglia of the outer plexus were shown to be more similar to the MYP, and the shapes, histochemical staining and patterns of innervation differ from the inner plexus (Gunn, 1968; Scheuerman *et al*, 1989; Stach, 1977).

The outer submucosal plexus does not exist in all species. For example, in the guinea pig, submucosal nerve cell bodies do not project to the circular muscle but exclusively to the mucosa where they have been shown to influence water and electrolyte absorption and secretion (Bornstein and Furness, 1988). The outer SMP is most evident in the colon of larger species such as the human, pig and sheep (Gunn, 1968; Stach, 1977; Crowe *et al*, 1992), whereas this plexus has not been found in the small intestine (Christensen and Rick, 1987), with the exception of the dog (Furness *et al*, 1989).

Interestingly, the interstitial cells of Cajal have also been found in the outer plexus of the mammalian

submucosa (Christensen and Rick, 1987). These cells were first described by Cajal (1893) who suggested they may be modified neurones, and have since been found in the muscle layers of the small intestine of several mammals (Gabella, 1987; Rumesson and Thuneberg, 1991) including the human. Their function has been the subject of considerable debate since they share characteristics of both neurones and non-neural cells such as fibroblasts (Rogers and Burnstock, 1966; Thuneberg, 1982). These cells have been implicated in generating the rhythmic activity of smooth muscle (Thuneberg, 1982; Huizinga, 1991). This has been supported by experiments in the dog which demonstrated that interstital cells at the muscle/submucosal interface possess electrical pacemaker activity (Barajas-Lopez *et al*, 1989).

The submucosal plexus has been suggested to be involved primarily in the control of mucosal secretion and absorption of electrolytes (Hubel, 1985; Keast *et al*, 1987; Cooke, 1988). The SMP is tonically active and suppresses ion transport (Cooke, 1988). The SMP of the small intestine and colon has been thought to help regulate motility by conveying sensory information to the MYP (Bulbring *et al*, 1958; Crema, 1970; Kottegoda, 1970). Its innervation of the inner circular muscle in larger animals such as the dog (Furness *et al*, 1989) would suggest direct involvement of the SMP in control of motility. A portion of circular muscle innervation of the rat also has been shown to come

from the SMP (Ekblad *et al*, 1987, 1988). To date, only the circular muscle of the guinea pig has been shown not to receive input from the SMP (Smith *et al*, 1988). Although implicated in the control of motility in the colon, the function of submucosal innervation of circular muscle has not been examined in detail in the small intestine. However, motoneurones from the submucosal ganglia have been shown to innervate and provide inhibitory and excitatory inputs in the circular muscle of the canine colon (Sanders and Smith, 1986).

The submucosal plexus receives inputs from the myenteric and extrinsic innervation in the guinea pig (Bornstein *et al*, 1988) but most mucosal fibres arise primarily from the SMP with the exception of some SP-containing fibres (Furness and Costa, 1987). The majority of fibres found in the mucosa of canine small intestine also appear to originate in the SMP while the submucosa receives little input from extrinsic sources (Furness *et al*, 1989). In the guinea pig, inputs from the MYP may be responsible for inhibitory synaptic inputs to the SMP (Bornstein *et al*, 1988), and may utilize SS as the neurotransmitter. The function of the MYP in control of ion transport has not been determined.

Substance P (SP), somatostatin (SS) and vasoactive intestinal peptide (VIP) were the peptides examined in this

thesis and are the focus the following discussion. The undecapeptide substance P was the first gut neuropeptide to be discovered (von Euler and Gaddum, 1931), on the basis of its ability to stimulate atropine resistant contractions of the rabbit ileum. Substance P was among the first known neuropeptides to be isolated and sequenced (Chang *et al*, 1971) and among the first whose presence in the gut was demonstrated by immunocytochemistry (Pearse and Polak, 1975).

Somatostatin is a tetradecapeptide originally isolated from bovine hypothalamus as a factor which inhibited growth hormone release (Brazeau *et al*, 1973) and has since been localized in many different regions including the endocrine cells (Polak *et al*, 1975) and neurones (Keast *et al*, 1985) of the gut.

Vasoactive intestinal peptide was isolated from porcine duodenum on the basis of its vasodilatory ability (Said and Mutt, 1970) and, unlike SP and SS, has been localized exclusively to neurones including those of the gut (Larsson, 1977).

Somatostatin occurs in two major molecular forms, SS-14 which was demonstrated to predominate in enteric nerves, and SS-28 which predominates in mucosal endocrine cells in the human small intestine (Penman *et al*, 1983; Keast *et al*,

1984; Baldissera *et al*, 1985). Exogenous application of either form has been shown to inhibit both small intestinal motility in the guinea pig (McIntosh *et al*, 1987a) as well as secretion of electrolytes in porcine small intestine (Brown *et al*, 1989). Inhibition of ion transport was shown to be partly mediated by enteric nerves in the guinea pig by SS-14 (Keast *et al*, 1987), or by both SS-14 and S-28 in the pig (Brown *et al*, 1989). Somatostatin has been shown to inhibit VIP-stimulated secretion in the dog but not the human, whereas basal secretion was not affected in either the dog or human. (Keast, 1987; Krejs and and Fordtran, 1980).

Substance P and VIP have been shown to increase secretion and decrease absorption of electrolytes in both canine and human small intestine *in vivo* (Krejs *et al*, 1980; Hubel *et al*, 1984; McFadden *et al*, 1986). Using human small intestine *in situ*, these effects were shown to be direct and not mediated by intrinsic nerves (Hubel *et al*, 1984). Similar studies have not been carried out in the dog, and therefore whether or not the secretory effects of VIP and SP were mediated by intrinsic nerves in this animal was not determined. The effect of SP has been shown to be partly mediated by intrinsic nerves in several species (Hubel *et al*, 1984; Keast *et al*, 1985; Perdue *et al*, 1987) but VIP has been demonstrated to act directly on the mucosa in all

species tested (Cassuto *et al*, 1983; Binder *et al*, 1984; Carey *et al*, 1985).

Substance P and VIP have opposite effects on gastrointestinal motility. Vasoactive intestinal peptide has been shown to inhibit motility in human colon (Couture *et al*, 1981; Furness and Costa, 1982) and the small intestine of the guinea pig (Furness and Costa, 1982). Substance P was shown to have excitatory effects on the motility of human gastrointestinal muscle (Zappia *et al*, 1978; Couture *et al*, 1981).

Intracellular recordings from enteric neurones have revealed fast and slow excitatory postsynaptic potentials (EPSPs) and fast and slow inhibitory postsynaptic potentials (IPSPs) (Wood, 1987). Since membrane potentials or ionic conductance can be measured over a period of hours, putative neurotransmitters can be applied and receptors can be characterized using pharmacological methods (North, 1986). Substance P has been shown to mimic the non-cholinergic slow EPSP evoked in enteric neurones by stimulation of axon bundles between enteric ganglia both *in situ* (Katayama *et al*, 1979; Bornstein *et al*, 1984; Surprenant, 1984) and in culture (Willard, 1990). The signal transduction mechanism of the SP-mediated EPSP did not involve cAMP (Palmer *et al*, 1987). Somatostatin has been shown to produce two types of slow IPSPs in both myenteric and submucosal neurones (Mihara

et al, 1987). Interestingly, SS-14 and SS-28 have been shown to exert opposite effects in rat neocortical neurones (Wang *et al*, 1989). Somatostatin-14 was shown to increase the delayed rectifier K^+ current while SS-28 was shown to decrease this current. Both effects were mediated by GTP-binding proteins and mediated by distinct receptors. Different effects of SS-14 and SS-28 have not been demonstrated in enteric neurones.

These neuropeptides have been shown to have effects on neurotransmitter release from enteric neurones. Substance P has been shown to stimulate the release of Ach from guinea pig myenteric neurones (Yau *et al*, 1986) and neurotensin release from canine submucosal neurones (Barber *et al*, 1989). Substance P has diverse effects on SS-IR release depending on the cell type. It has been shown to inhibit SS-IR release from canine submucosal neurones (Buchan *et al*, 1990) and gastric somatostatin cells (Kwok *et al*, 1985) but to stimulate SS-IR release from the hypothalamus and pancreas (Reichlin, 1981). Vasoactive intestinal peptide has also been shown to stimulate the release of SS-IR from enteric neurones (Grider, 1989). Somatostatin has been shown to inhibit the release of neurotransmitters, such as Ach, from enteric neurones (Wiley and Owang, 1987).

Receptors for neuropeptides have also been studied using receptor binding assays (Quirion and Gaudreau, 1985). The

distributions of SP and VIP binding sites in human (Gates *et al*, 1989; Korman *et al*, 1989) and canine small intestine (Mantyh *et al*, 1988; Zimmerman *et al*, 1989) have been shown to be similar. In both species, binding of VIP was found predominantly in the epithelial layer while binding of SP was found mostly in the smooth muscle layers but also in the submucosal arterioles and venules, and in the epithelium. Only few studies have detected receptors for any neuropeptides on canine enteric ganglia and these include receptors for opioids and bombesin (Allescher *et al*, 1989; Ahmad *et al*, 1989; Vigna *et al*, 1987). Substance P binding sites have been demonstrated in guinea pig enteric ganglia (Bornstein and Burcher, 1987). Although useful for the determination of high and low density binding sites, these techniques have been shown to be insensitive, probably because of a lack of specific ligands to date.

C. Experimental Rationale.

The actions and secretion of SS and SP in the submucosal plexus of the small intestine has been difficult to interpret for several reasons. Peptides such as SS are present in multiple locations and in multiple molecular forms. Therefore, studies which utilize *in vivo* experimental models to examine the release or actions of SS and SP become confusing. Also, the distribution and the pattern of co-localization of peptides varies between

species. Differences in peptide distribution between species may result in differences in their function.

The studies presented in this thesis compare the distribution of SP-IR, VIP-IR and SS-IR in the submucosal plexus of the human and canine small intestine. In particular, the possibility of co-localization of these neuropeptides is examined. Further, the studies describe the development of dispersed cultures of submucosal ganglia from the human small intestine and utilization of canine and human cultures to study the release of SS-IR and SP-IR. Finally, the studies compare the secretion of SS-IR and the effect of SP on SS-IR secretion between the dog and human, and relate these to differences in their distribution.

D. Hypotheses

1 That interspecies variations in neuropeptide localization and enteric neuronal morphology exist between the human and canine small intestine.

2. That differences in neuronal chemical coding and morphology will be reflected in neuronal function.

3. That short-term cultures of human and canine SMP will provide a model system in which to examine whether differences in neuronal chemical coding and morphology will be reflected in neuronal function.

E. Specific Objectives

1. To develop dispersed cultures of submucosal neurones from human small intestine and compare these to cultures of canine small intestine.

2. To compare the morphology of canine and human small intestinal innervation using the antibody to protein gene product 9.5.

3. To compare the distribution of SP, SS and VIP in canine and human submucosal neurones using single and double stains, and to compare the morphology of these neurones.

4. To compare the total number of neurones and total number of SP-IR, SS-IR and VIP-IR containing neurones per canine and human submucosal ganglion using PGP/peptide double stains.

5. To compare the distribution of SP, SS and VIP in canine and human submucosa in culture.

6. To examine and compare, the content and secretion of SS-IR from canine and human submucosal neurones in culture.

7. To examine the content and secretion of SP-IR from canine submucosal neurones in culture.

8. To compare the effects of SP on secretion of SS-IR from human and canine submucosal cultures.

II. IMMUNOCYTOCHEMICAL METHODS

A. Tissue Sections

1. Tissue Preparation

Cross-sections of intact intestine (1 cm thick), and stripped submucosa (6 cm^2) were fixed in Bouin's solution for morphological study. The tissues were fixed (in Bouin's solution) for 2 h, washed and stored in 70% alcohol prior to processing. The tissue was dehydrated through graded alcohols and xylene, and embedded in wax. Seven micron sections were air dried on glass slides at 37^{o}C and the wax removed with xylene followed by clearing through petroleum ether.

2. Protocol

All antibodies were diluted in PBS containing 10% bovine or swine serum while avidin layers were diluted in PBS alone. Primary antibodies were incubated at 4^{o}C, and all other procedures were carried out at room temperature.

a. Primary Antibodies (Table 1).

The tissue sections were incubated for 48-72 h in primary antibody diluted with PBS with 10% horse serum. The bound antibodies were localized using peroxidase or immunofluorescence techniques.

b. Secondary Layers

i. Peroxidase

Sections which were utilized for peroxidase staining were previously incubated with a 0.01% solution of hydrogen peroxide to block endogenous peroxidase activity before incubation with the primary antibody. The endogenous enzyme becomes blocked because the peroxide oxidizes peroxidase. Incubation with a biotinylated (biotin conjugated) secondary antibody (Table 2) was then carried out for 1 h (canine tissue) or 2 h (human tissue, canine and human cultures). A further incubation was carried out for 1-2 h with a solution containing avidin and biotin-peroxidase (ABC) previously incubated for 10-30 min. Two methods were used to localize the resulting complex. The first method utilized a solution of diaminobenzidene (DAB, 4 mg/ml) and hydrogen peroxide (0.03 %), in 0.1 M Tris buffer, which was filtered and added dropwise on slides and cultures to develop the peroxidase reaction. The second method utilized 100 ml of 0.1 M Tris

buffer containing 200 mg dextrose, glucose oxidase (0.3 mg), ammonium chloride (40 mg) and DAB (4 mg) in which the sections or cultures were incubated for 1-1.5 h. After counterstaining with hematoxylin, the staining was observed with a Zeiss Axiophot microscope equipped with phase contrast optics.

ii. Immunofluorescence.

Primary antibodies were visualized by immunofluorescence in two ways. An indirect method utilized a 1-2 h incubation with a fluorophore-conjugated second antibody (Table 3). A second method was carried out by incubation with a biotinylated second antibody for 1 h (canine tissue) or 2 h (human tissue, human and canine cultures) followed by a 45-60 min incubation with an avidin-fluorophore third layer (Table 4). The fluorophores used were fluoro-isothiocyanate (FITC) and tetramethyl-rhodamine-isothiocyanate (rhodamine). The FITC and rhodamine staining was observed with a Zeiss Axiophot microscope equipped with epifluorescence, using barrier filters of band width 465-490 nm (green) and 510-560 nm (red), respectively.

Table 1. Primary Antibodies.

Antigen	Source	Dilution	Species	Type
VIP	CURE	1:1000	mouse	serum
VIP	Peninsula	1:1000	rabbit	serum
Soma	CURE	1:10,000	mouse	ascites
Soma	RPG	1:1000	mouse	ascites
SP	SL	1:2000	rabbit	serum
SP	JP	1:2000	rabbit	serum
SP	RPG	1:1000	guinea pig	serum
PGP	Immunonuclear	1:2000	rabbit	serum

CURE- Professor J. Walsh, Centre for Ulcer Research and Education.

RPG- Regulatory Peptide Group.

SL- Professor S.A. Leeman.

JP- Professor J.M. Polak.

c. Double Stains.

Double staining was carried out using the techniques described for immunofluorescence. It was necessary to choose antibodies to peptides raised in species such that cross-reactivity between primary and secondary antibodies did not occur. Second layers have been affinity purified to remove potential cross-reacting globulins, and have been developed specifically for double staining. For example, primary monoclonal antibodies were localized with secondary antisera raised in goat or donkey rather than the more usual rabbit antimouse IgG to eliminate cross-reactivity with rabbit primary antibodies.

d. Controls.

Extensive characterization of the antisera/antibodies has been carried out previously to eliminate the possibility of cross-reactivity with other related peptides. In addition, incubations using PBS in 10 % serum layers were carried out to determine the extent of non-specific staining due to the second and third layers.

B. Tissue Culture.

The cultures were fixed in Bouin's solution for 15-30 min, washed with distilled water followed by phosphate

buffered saline. The localization of primary antibodies was carried out as for tissue sections except for the following changes. Some cultures were frozen with liquid nitrogen before washing and/or incubated in first, second and third layers containing Triton X-100 (0.1%), in order to lyse the plasma membrane and allow the optimal penetration of antibodies into the cell. The durations of first, second and third layer incubations were up to 50 % longer than those for tissue sections. The concentrations of primary and secondary antibodies, and of avidin third layers were double those used in tissue sections.

C. Quantification of Peptide-containing Neurones.

In tissue sections, the percentage of the submucosal plexus occupied by ganglia was determined by planimetry in sections stained with PGP 9.5. Neurones immunoreactive for each peptide were counted in six ganglia per section in 6 sections to a total of 180 ganglia and compared to the total number of neurones in those ganglia. The total number of neurones was determined using the PGP 9.5 antiserum. In the cultures, cells immunoreactive for somatostatin were counted and compared to the total number of neurones in that group or cluster.

Table 2. Biotinylated Antibodies

Antigen	Source	Dilution	Species
Rabbit IgG	Vector Co.	1:300	goat
Rabbit IgG	Jackson Co.	1:1000	donkey
Mouse IgG	Vector Co.	1:300	horse
Mouse IgG	Jackson Co.	1:1000	goat

Table 3. Fluorophore-conjugated Antibodies

Antigen	Source	Dilution	Species
Rhodamine			
Rabbit IgG	Jackson Co.	1:1000	donkey
Mouse IgG	Jackson Co.	1:1000	goat
FITC			
Rabbit IgG	Jackson Co.	1:1000	donkey
Mouse IgG	Jackson Co.	1:1000	donkey

Table 4. Avidin Layers

Layers.	Source	Dilution
Avidin FITC	Vector Co.	1:1000
Avidin rhodamine	Vector Co.	1:1000
Avidin peroxidase	Vector Co.	1:1000

III. CHAPTER ONE. IMMUNOCYTOCHEMICAL STUDIES

A. Introduction

Enteric neurones in several mammalian species have been shown to contain a plethora of neuropeptides (Schultzberg *et al*, 1980; Furness and Costa, 1982). The enteric nervous system of the guinea pig has been the most thoroughly characterized with respect to the pathways of individual peptide containing neurones (Costa *et al*, 1987; Furness *et al* 1990). Differences in the distribution of peptides within nerve cell bodies of the MYP and external musculature of the dog and guinea pig have been shown (Tange, 1983; Daniel *et al*, 1985; Daniel *et al*, 1987; Furness *et al*, 1989). In particular, differences in innervation of the circular muscle of the guinea pig and dog may lie in the submucosa (Furness *et al*, 1990). Canine circular muscle has been shown to contain SP-IR and VIP-IR fibres whose origin was the outer submucosal ganglia, or Schabadasch's plexus. Guinea pig circular muscle receives SP-IR and VIP-IR fibres solely from the MYP.

Similarities exist between the dog and human with respect to enteric submucosal/mucosal neuropeptide distribution. Single staining of VIP, SP and SS was comparable between canine and human submucosa/mucosa. Each peptide has been found in both submucosal and myenteric

neuronal cell bodies and fibres in canine and human mucosa/submucosa (Ferri *et al*; 1982; Tange, 1983; Keast *et al*, 1984; Daniel *et al*, 1985; Keast *et al*, 1985; Wattchow *et al*, 1988; Furness *et al*, 1990). These studies have shown that fibres containing VIP immunoreactivity (VIP-IR) were more abundant than those containing SP-IR, which in turn were more abundant than those containing SS-IR.

Comprehensive examinations of neurotransmitter combinations in enteric neurones have been carried out in the guinea pig and rat and have revealed distinct differences (Furness *et al*, 1989; Pataky *et al*, 1990). This is often referred to as chemical coding of neurones (Furness *et al*, 1989). In the SMP of the guinea pig, two major sub-groups of neurones were distinguished. These were dynorphin/galanin/VIP containing and CCK/CGRP/choline acetyl transferase (Chat)/galanin/neuropeptide Y (NPY)/SS containing neurones (Costa *et al*, 1987; Furness *et al*, 1989). In the rat, the major subgroups were VIP/NPY containing and SS/SP/CGRP containing neurones (Pataky *et al*, 1990). These studies used multiple staining techniques with highly specific antisera/antibodies to characterize the neuronal types. However, no information was obtained about whether functional differences could be correlated with differential peptide coding.

Earlier studies with canine intestine demonstrated the presence of large ganglia containing a high proportion of neurotensin immunoreactive (NT-IR) neurones (Buchan and Barber, 1987). These NT-IR positive neurones had not been demonstrated in either the rat or the guinea pig. The chemical coding of canine SMP neurones probably differs from both the guinea pig and the rat. The basis for such a difference may be the diet and size of the animal (Powell, 1987; Gabella, 1990).

Immunocytochemical studies of peptide co-localization and chemical coding of human and canine neurones of the small intestine are few (Wattchow *et al*, 1988; Furness *et al*, 1990). In the human small intestine SP and enkephalin have been shown to co-exist in fibres which were suggested to be excitatory to the external muscle (Wattchow *et al*, 1988). These authors also showed that NPY and VIP co-existed in a separate population of fibres and were suggested to be inhibitory to the external muscle.

In the present study, immunocytochemical staining was carried out to examine the hypothesis that there is interspecies variation in the localization of SS-IR, SP-IR and VIP-IR between the human and canine small intestine. The availability of highly specific antibodies/antisera raised in different species (mouse, rabbit and guinea pig)

combined with affinity-purified secondary antisera was essential in these experiments.

In order to quantify the number of neurones within a ganglion, a general neuronal marker was required. In these studies PGP 9.5 was used. Protein gene product 9.5 is a soluble cytoplasmic protein originally detected in protein extracts of human organs by high resolution two dimensional polyacrylamide electrophoresis (Doran *et al*, 1983). The distribution of PGP is similar to neurone-specific enolase, since both have been found in neurones and neuroendocrine cells (Doran *et al*, 1983). Polyclonal antibodies to PGP have demonstrated peripheral nerve cell bodies and nerve fibres with clarity and intensity (Gulbenkian *et al*, 1987; Lauweryns and Van Ranst, 1988; Wilson *et al*, 1988).

B. Results

1. Human Tissue Sections

a. Protein Gene Product 9.5.

The antibody to PGP strongly stained human neuronal cell bodies and fibres throughout the small intestine. This staining revealed the nerve cell bodies of the SMP and nerve fibres throughout the mucosa (figure 2). Submucosal ganglia were anatomically separated into those adjacent to the interface with the mucosa and those situated by the circular muscle. The myenteric ganglia were strongly stained and there was extensive innervation of the circular and longitudinal muscle (figure 3).

Quantification of the relative proportion of peptide containing neurones was carried out using double stains of PGP 9.5 in combination with the particular neuropeptide (for an example see figure 4). The intensity staining was much greater for PGP than for SP-IR, SS-IR or VIP-IR and revealed more abundant nerve fibres found throughout the ganglia. The volume of the myenteric ganglia was larger than that of the submucosal ganglia because of larger amounts of fibres and other neuronal processes (figure 4).

Figure 2. PGP 9.5 staining of human mucosa. Note cell bodies (large arrows) immediately below muscularis mucosae. Immunoreactive fibres were present throughout the mucosal layer (small arrows).

x 200.

Figure 3. PGP 9.5 staining of human muscularis (CM = circular muscle, LM = longitudinal muscle). Note fibres in both muscle layers (small arrows) and intense staining of the neurones in the MYP (large arrow).

x 100.

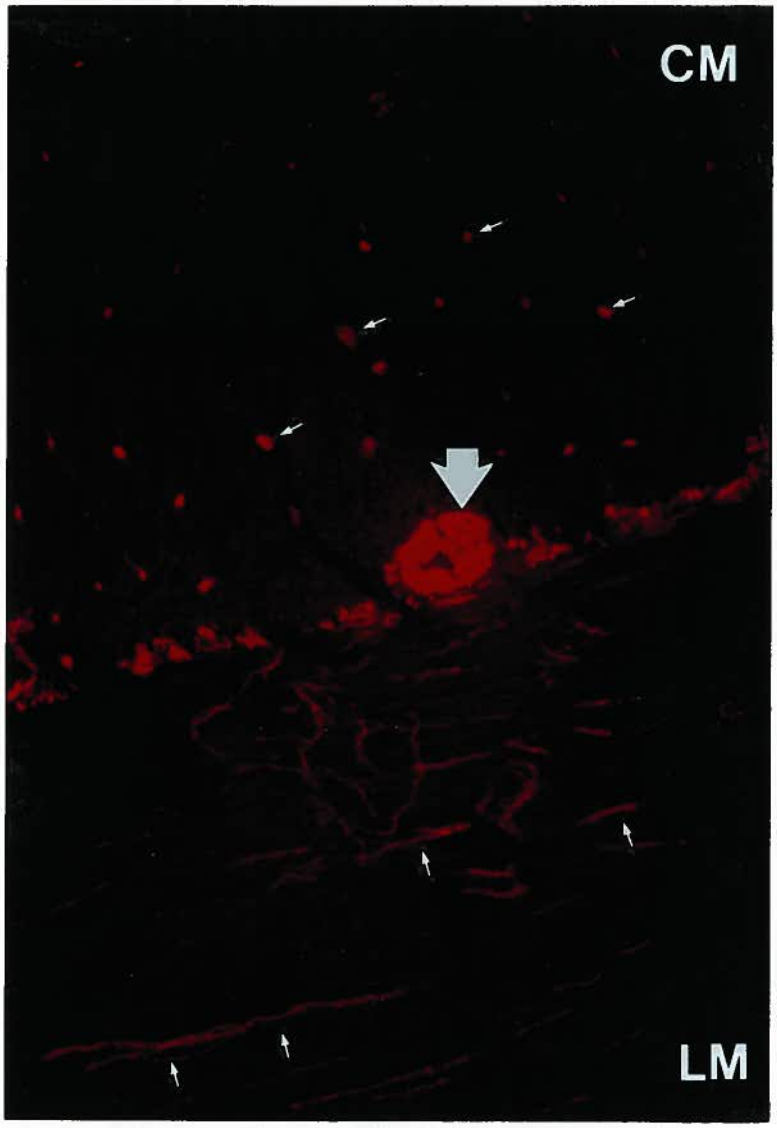

CM
LM

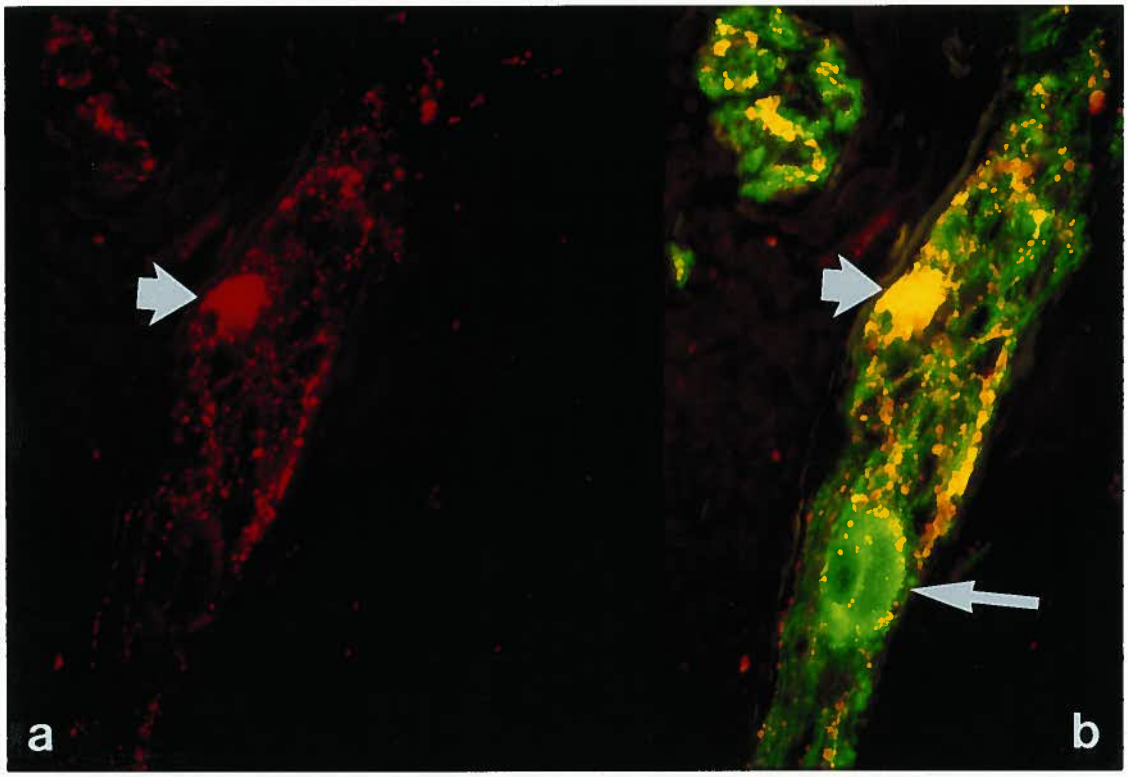
Figure 4.

a) VIP-IR neurone (large arrow) and fibres in the human MYP.

x 400.

b) The same ganglion in a double exposure showing VIP-IR neurone double stained by PGP 9.5 and a single neurone stained by PGP-IR 9.5 (small arrow)

x 400.


a
b

The average number of neurones per ganglion in the SMP was 2.9 ± 0.3 (184 ganglia, n = 5 donors) (figure 5) and made up only 5% of the submucosa while collagen made up over 85% of this layer.

b. Autofluorescence.

Certain neurones demonstrated autofluorescence when viewed under ultraviolet light which was often associated with neuropeptide containing neurones (figure 6). Autofluorescence was indicative of the high intrinsic amine content of these neurones and fibres. The autofluorescence was visible at 3 wavelengths (380, 480 and 570nm) but could be differentiated from Rhodamine (570 nm) and FITC (480 nm) because these were not observed at the 340 nm wavelength.

c. Single Staining of SS-IR, SP-IR and VIP-IR.

Single staining of human small intestine with antibodies to SS, SP and VIP revealed a pattern similar to that found previously in other laboratories. Somatostatin-IR was found in endocrine cells, submucosal ganglia and myenteric ganglia (figure 7 a, b, c). Endocrine cells containing SS-IR were abundant in the mucosal epithelium, stained more intensely than neurones containing SS-IR and were concentrated at the base of the crypts (see also figure 8 a).

Figure 5. The relative size distribution of human submucosal ganglia of the small intestine. Note that over 50 % of ganglia contained 3 or less neurones.

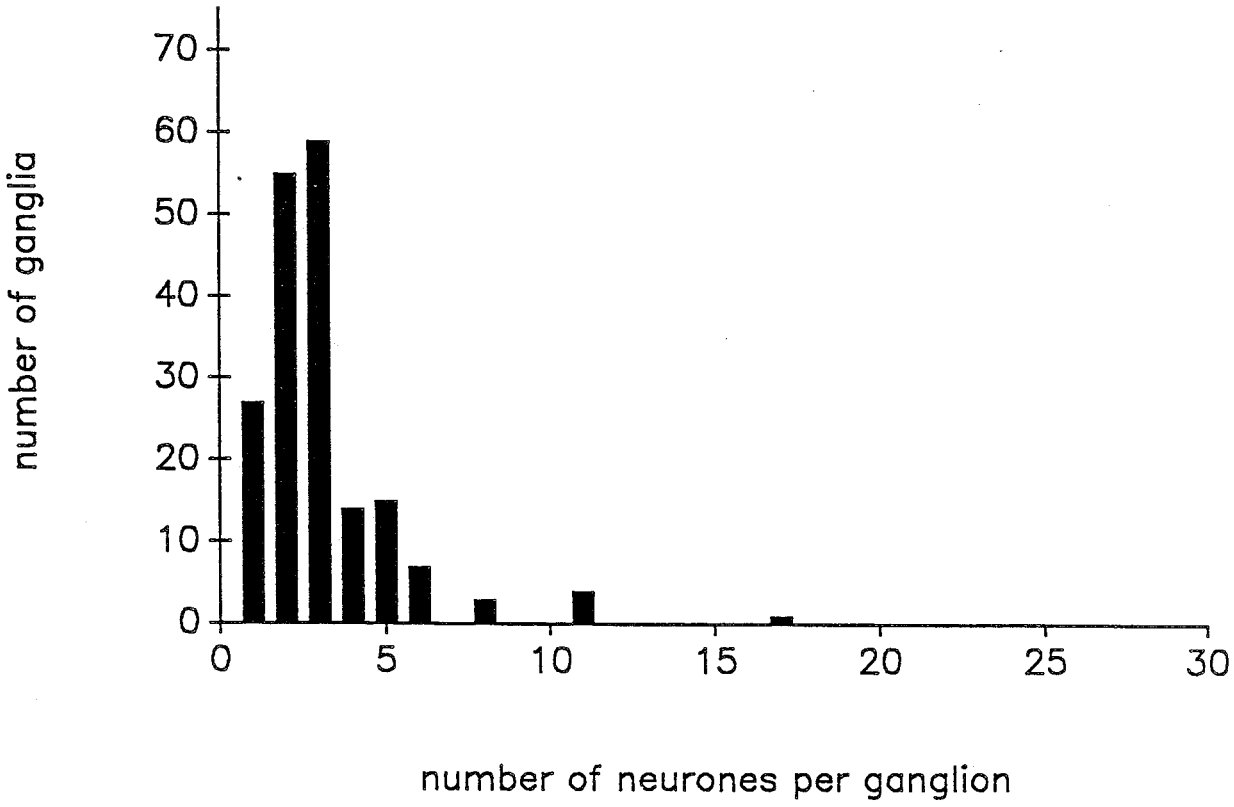

number of ganglia
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
number of neurones per ganglion

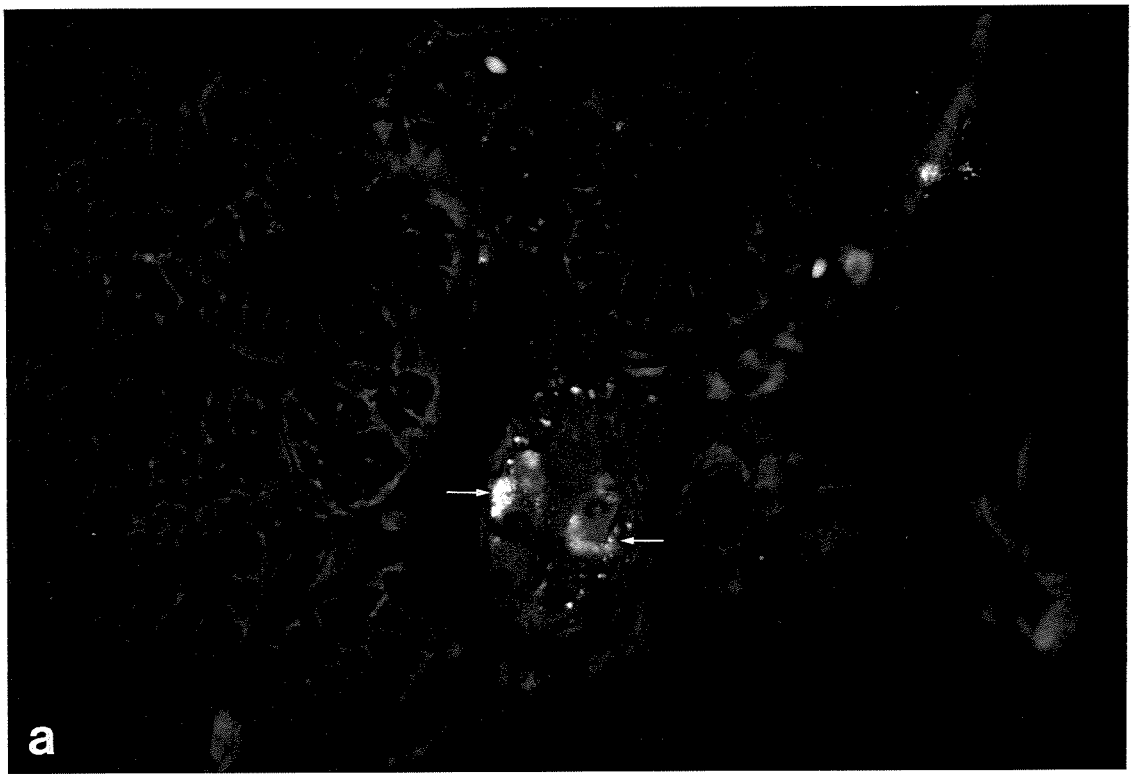
Figure 6.

a) a section of human duodenum photographed under ultraviolet light (370 nm). Note the presence of intense autofluorescence (arrows) in the SMP ganglion.

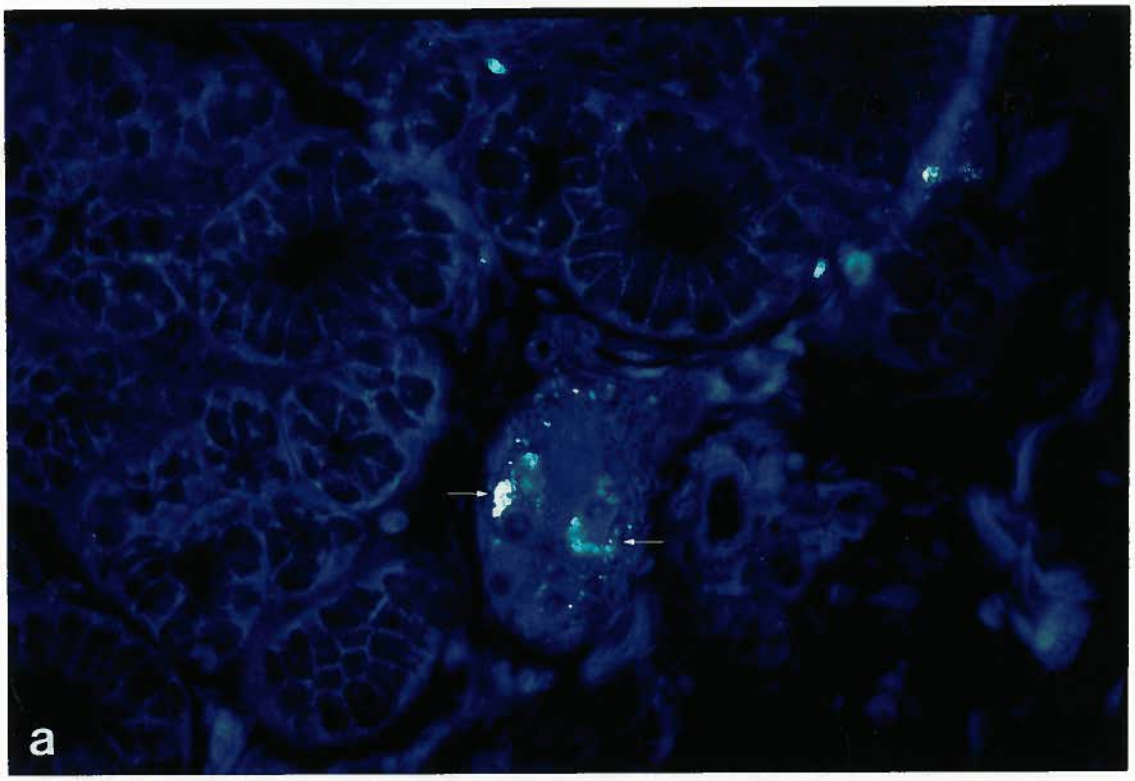
x 200.

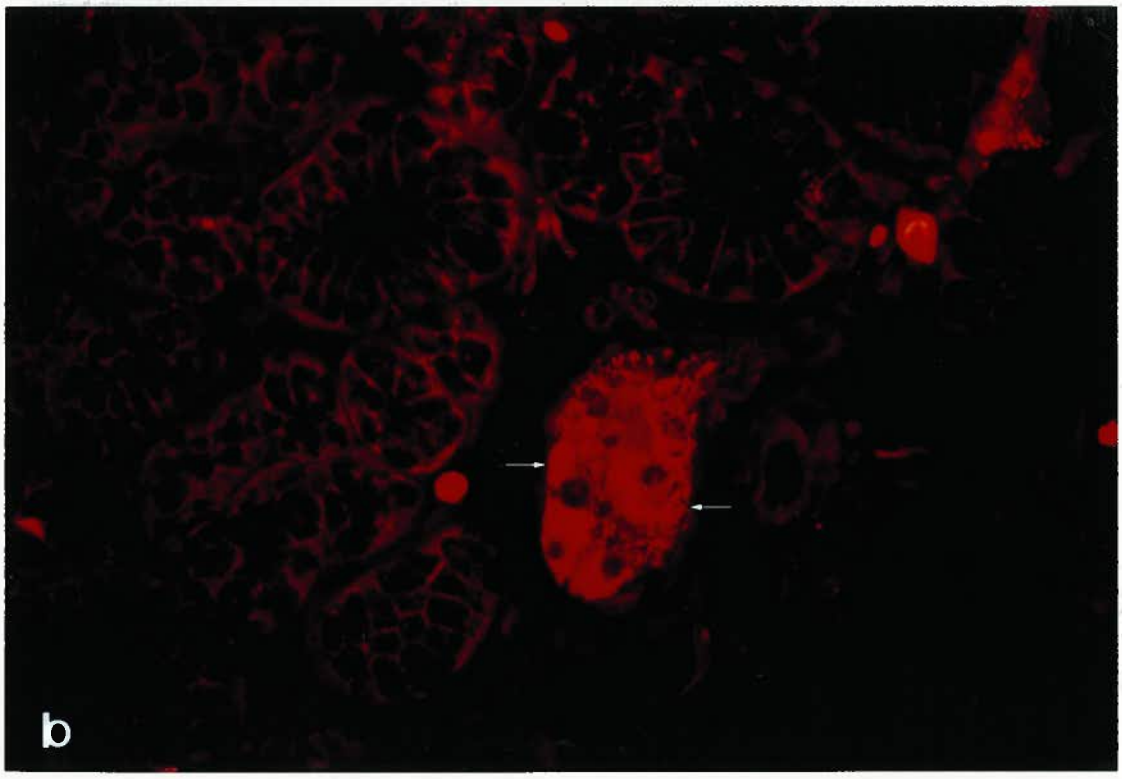
b) The same area photographed under long wavelength (570 nm) to show SP-IR neurones. Note the close association of the autofluorescent areas (arrows) with the positively stained neurones

x 200.


a


b


a


b

Submucosal and myenteric ganglia also contained SS-IR nerve fibres but these were less abundant than those containing SP-IR or VIP-IR. Forty two percent of neuronal cell bodies per human submucosal ganglion contained SS-IR.

Cell bodies containing VIP-IR were again found in myenteric (separate from neurones containing SS-IR) and submucosal ganglia (figure 7 d, e). Occasional nerve cell bodies were observed in the smooth muscle and submucosa. Fibres containing VIP-IR were found within the ganglia running between other neurones. Fibres were also found throughout the MYP, SMP and the smooth muscle layers. Fibres containing VIP-IR were also found in the mucosa (figure 7 f), and in general were found in similar amounts to SP-IR fibres, and greater than SS-IR fibres. Forty percent of neurones per human submucosal ganglion contained VIP-IR. Endocrine cells containing VIP-IR were not observed.

Cell bodies containing SP-IR were found in human myenteric (not shown) and submucosal ganglia (figure 7 g). Fibres containing SP-IR were found within the myenteric and submucosal ganglia and seemed to form pericellular baskets around other neurones. Fibres were also found in the muscle layers, the SMP, the mucosa and the mucosal villi. Substance P fibres were more plentiful than fibres containing SS-IR and were present in amounts similar to

those containing VIP-IR. Forty two percent of neuronal cell bodies per human ganglion contained SP-IR while endocrine cells containing SP-IR were not found.

d. Double Stains.

Substance P-IR and SS-IR were found to completely co-exist in the neurones of human submucosal (figure 8 a, b, c) and myenteric ganglia. Large numbers of fibres were demonstrated in all layers of the gut which contained only SP-IR while endocrine cells of the mucosa were found to contain only SS-IR.

Neurones containing VIP-IR were demonstrated to be a population completely distinct from those neurones containing SS-IR/SP-IR in both the myenteric and submucosal neurones (figure 9, and 10 a, b). Double stains revealed neuropeptide containing fibres and varicosities of each neuropeptide distributed around other neuropeptide containing neurones within the ganglia. Some fibres formed pericellular baskets around neuropeptide containing neurones. Certain small ganglia were made up of neurones which were exclusively of the VIP-IR type or SS-IR/SP-IR type.

Figure 7. Human intestine representative immunostains.

a) SS-IR endocrine cell (arrow) x 200.

b) SS-IR SMP neurones (arrow) x 200.

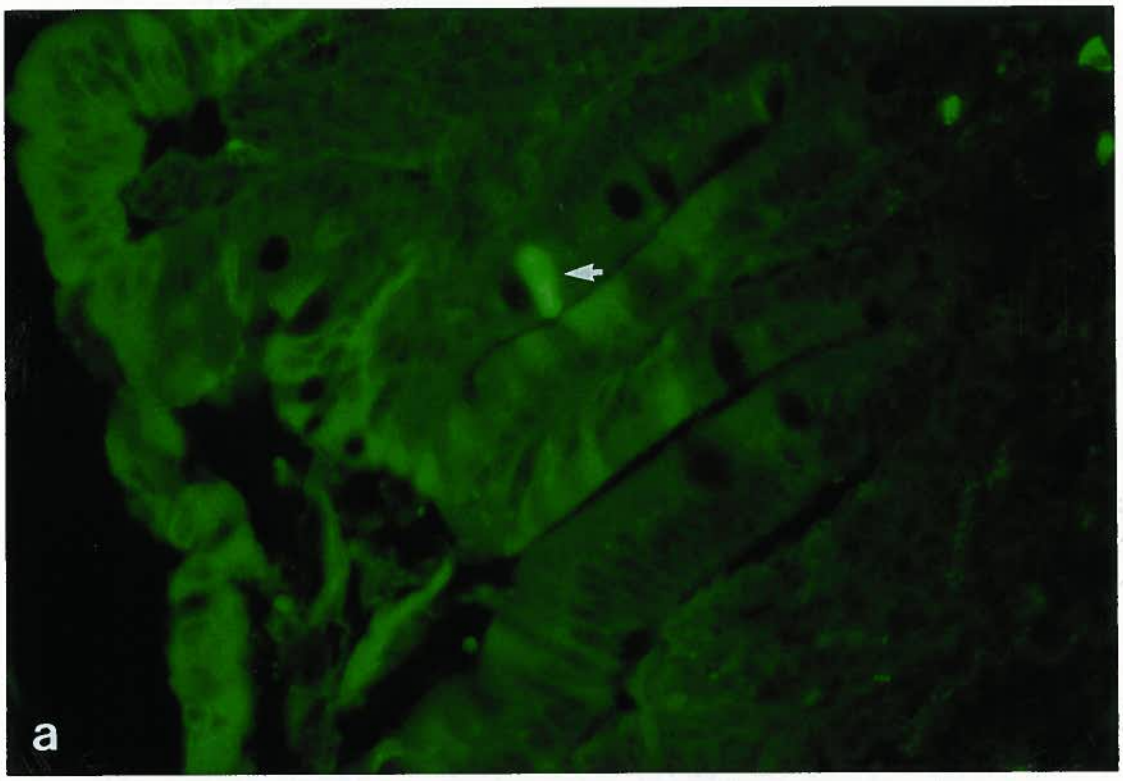
c) SS-IR neurones (arrows) and nerve fibres in the MYP x 200.

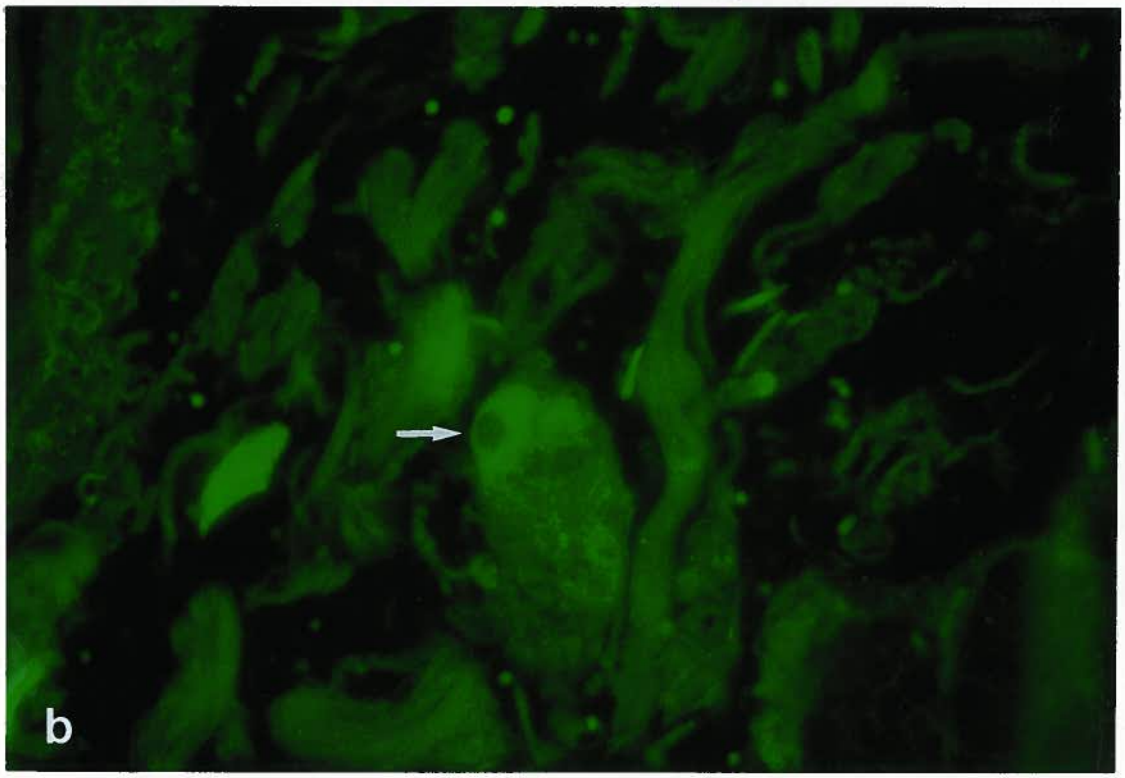
d) The same ganglion as in 'c' stained for VIP-IR. Note the separation of neurones. SS-IR (arrow heads), VIP-IR (long arrows) x 200.

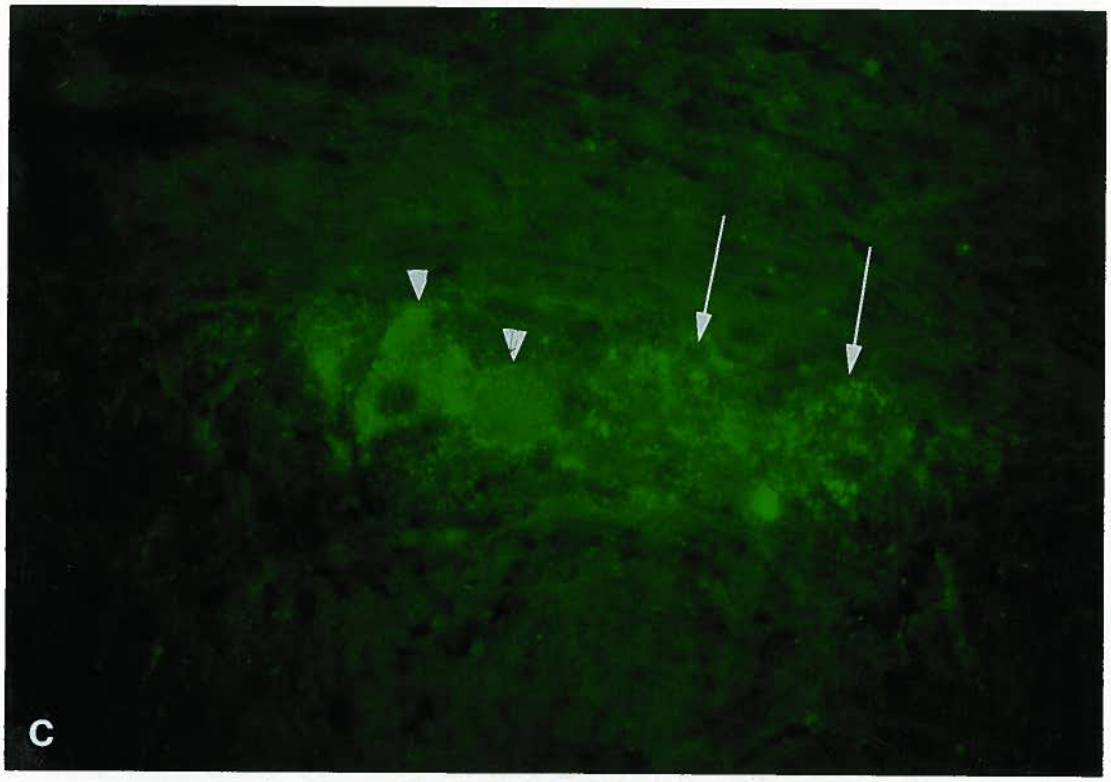
e) VIP-IR neurones (arrows) in a ganglion of the SMP x 200.

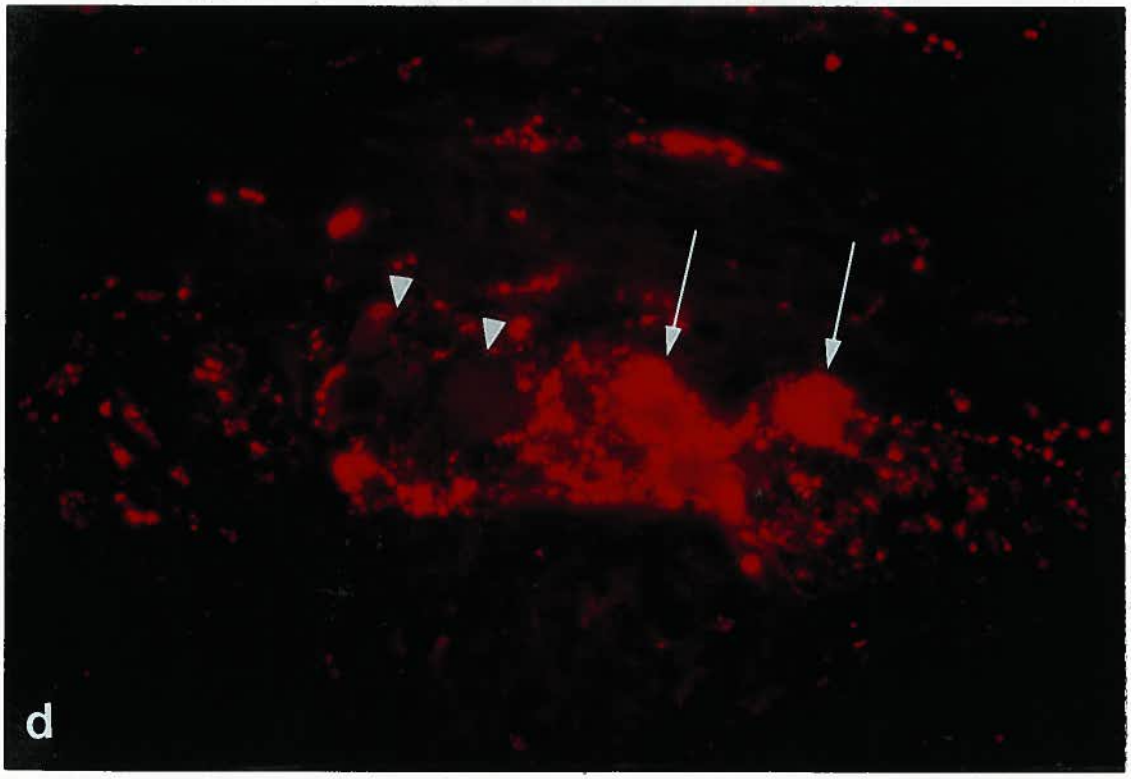
f) VIP-IR fibres (arrows) in the lamina propria of a villus in the duodenal mucosa x 200.

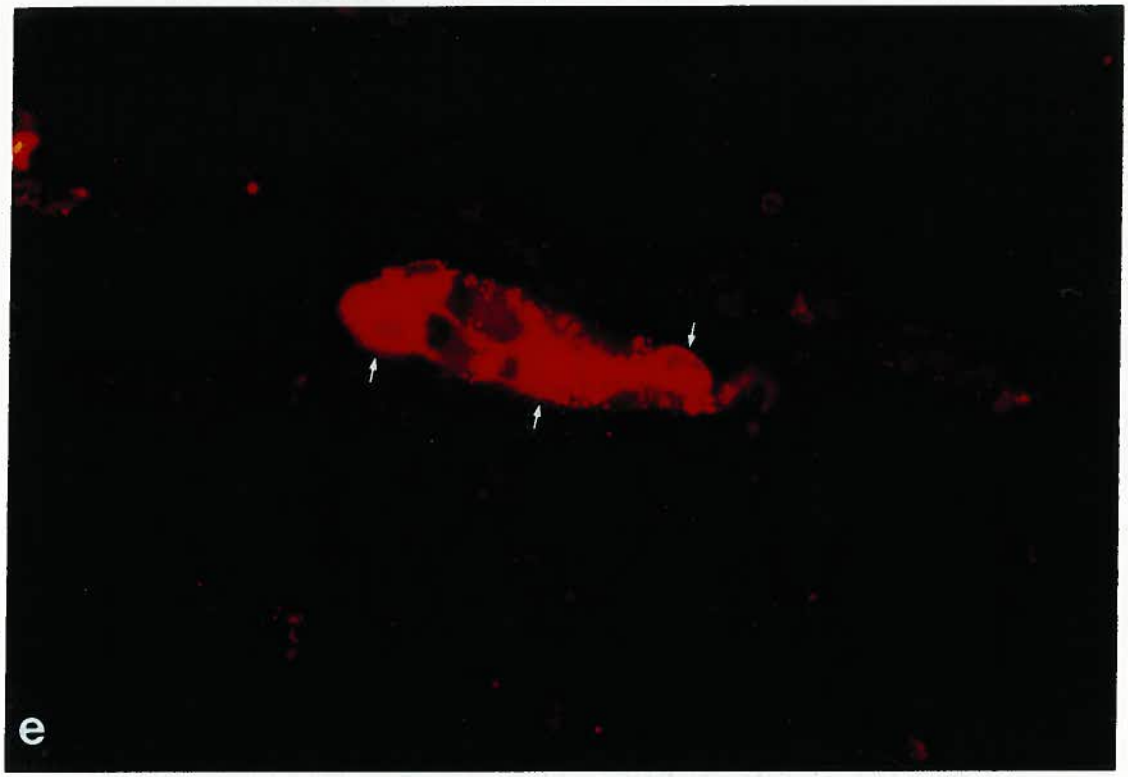
g) SP-IR neurones (arrows) and fibres in a ganglion in the duodenal SMP x 200.

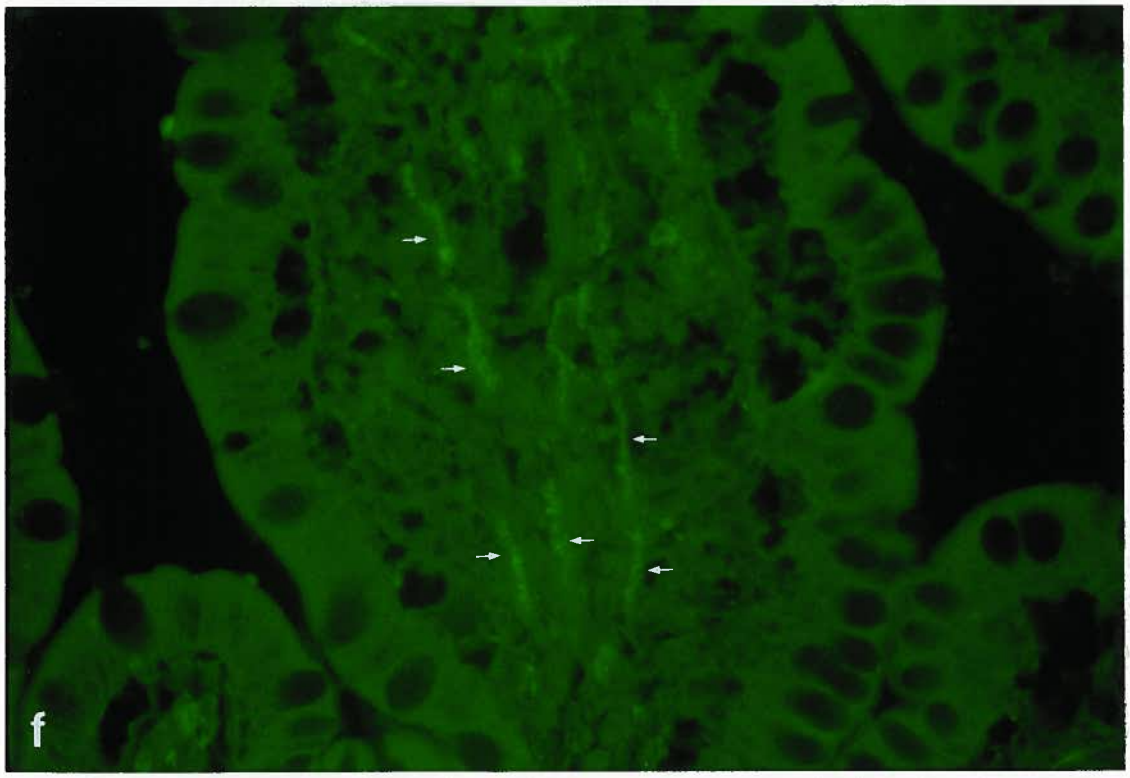

a


b


c


d


e


f


g

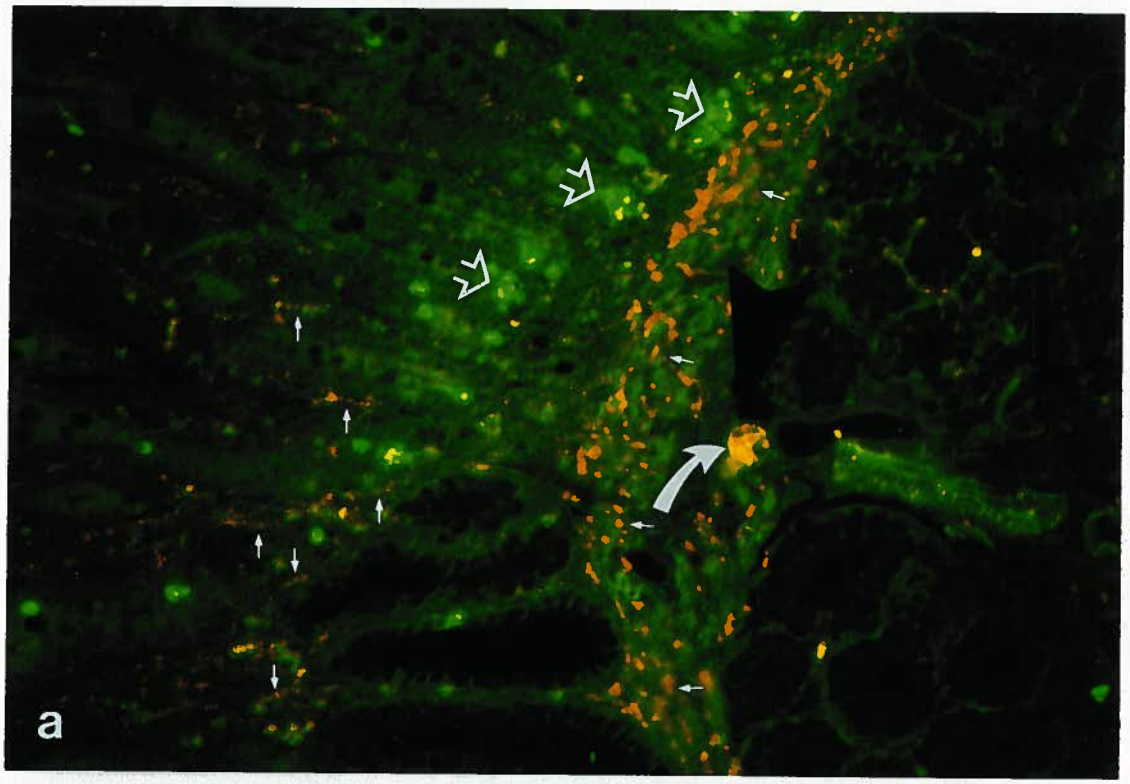
Figure 8. Human duodenal sections double stained for SS-IR (FITC) and SP-IR (Rhodamine).

a) A low magnification overview showing SS-IR epithelial endocrine cells (open arrows) which do not contain SP-IR; SP-IR fibres in the lamina propria and muscularis mucosae (small arrows) which do not contain SS-IR; a ganglion in the SMP (large black arrow) containing a neurone double stained for both SS-IR and SP-IR (large white arrow)

x 50.

b) A higher magnification micrograph of SP-IR in a SMP neurones co-localized with c) SS-IR

x 500


a

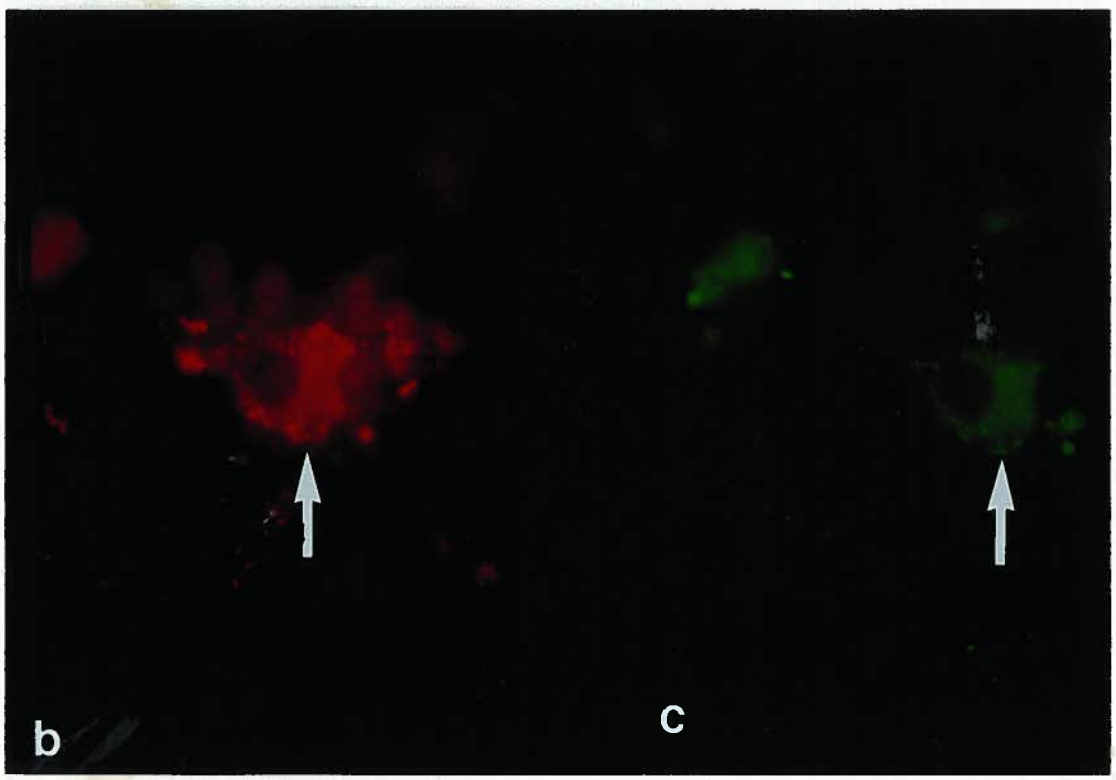

b
c

Figure 9. Human duodenal SMP stained for SS-IR (Rhodamine, large arrows) and VIP-IR (FITC, small arrows). Note the complete separation of the two neurone types and the presence of SS-IR varicosities around the VIP-IR neurone (small arrow heads) x 500.

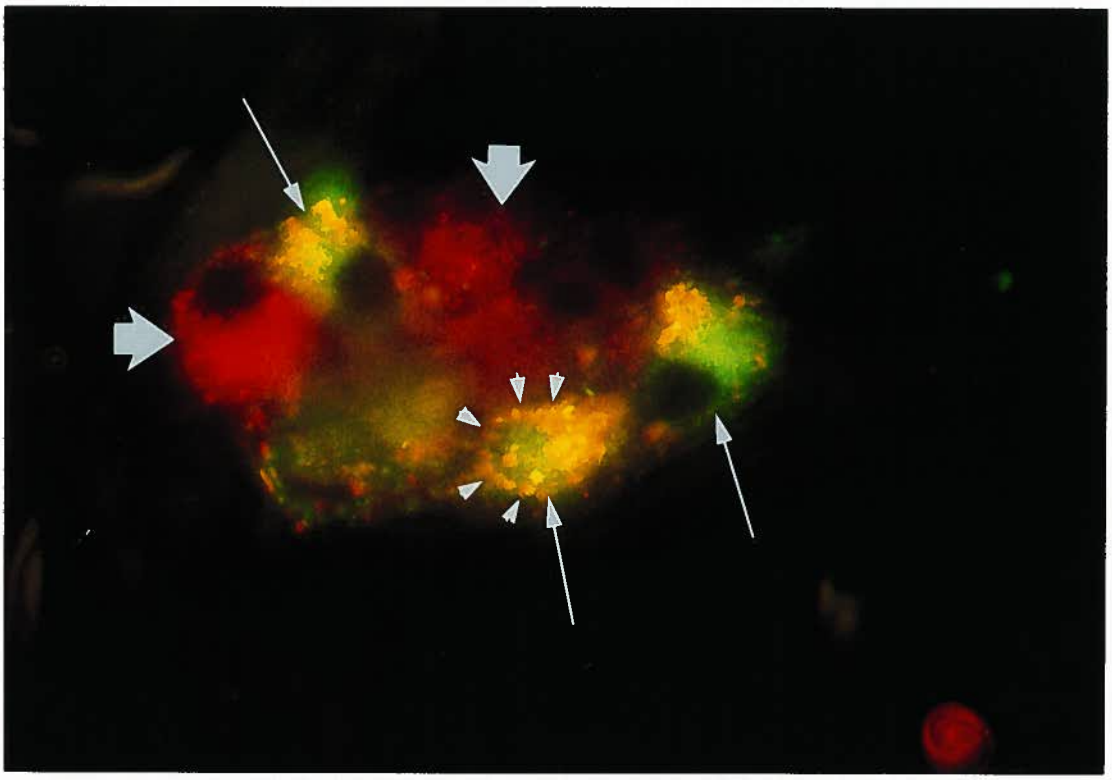
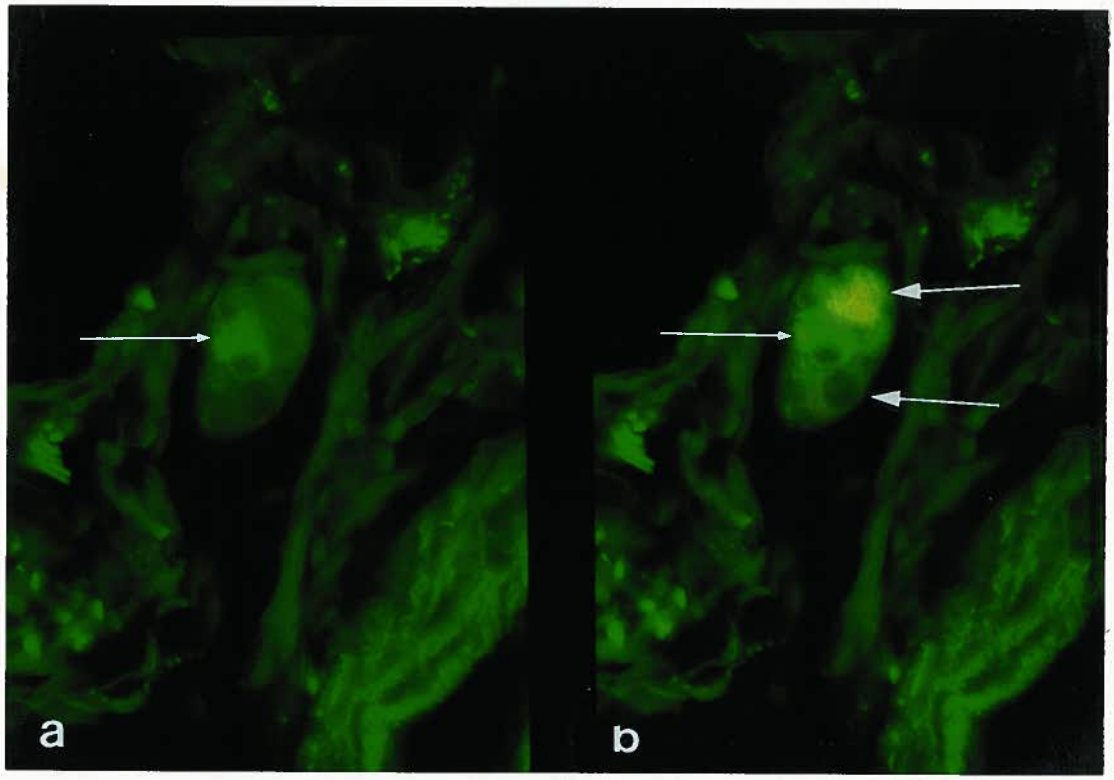


Figure 10. Human duodenum double stained for VIP-IR (FITC) and SP-IR (Rhodamine).

a) A ganglion in the SMP with a single VIP-IR neurone (small arrow) x 200.

b) A double exposure of the same ganglion showing the VIP-IR neurone (small arrow) and two SP-IR neurones (larger arrows) x 200.
Note the lack of co-localization.


a
b

2. Canine Tissue Sections

a. Protein Gene Product 9.5.

The antibody to PGP strongly stained canine neuronal cell bodies and fibres throughout the small intestine. This staining revealed the nerve cell bodies of the SMP and nerve fibres throughout the mucosa (figure 11). Canine submucosal ganglia were also present in two distinct groups, one closer to the mucosa and one near the interface with the circular muscle (figure 11, 12, 13). Unlike the human small intestine, PGP 9.5 staining revealed the presence of a discrete deep muscular plexus, situated near the outer layer of the submucosa (figure 13). Canine myenteric ganglia were strongly stained and there was extensive innervation of the circular and longitudinal muscle. The intensity of staining was much greater for PGP than for SP-IR, SS-IR or VIP-IR and more abundant nerve fibres were apparent throughout the ganglia. The volume of the myenteric ganglia was larger than that of the submucosal ganglia again due to large amounts of neuropil i.e. fibres and other neuronal processes, and glia. The average number of neurones per ganglion in the SMP was 7.7 ± 0.6 (185 ganglia counted, n=6 dog) and made up 10% of the submucosa while collagen made up over 80% of this layer (figure 14).

Figure 11. PGP 9.5 staining of canine duodenum (MUC = mucosa, CM = circular muscle). Note the presence of two distinct sets of ganglia in the SMP, one close to the interface with the mucosa (upper dotted line), the other apposed to the circular muscle (lower dotted line).

x 50.

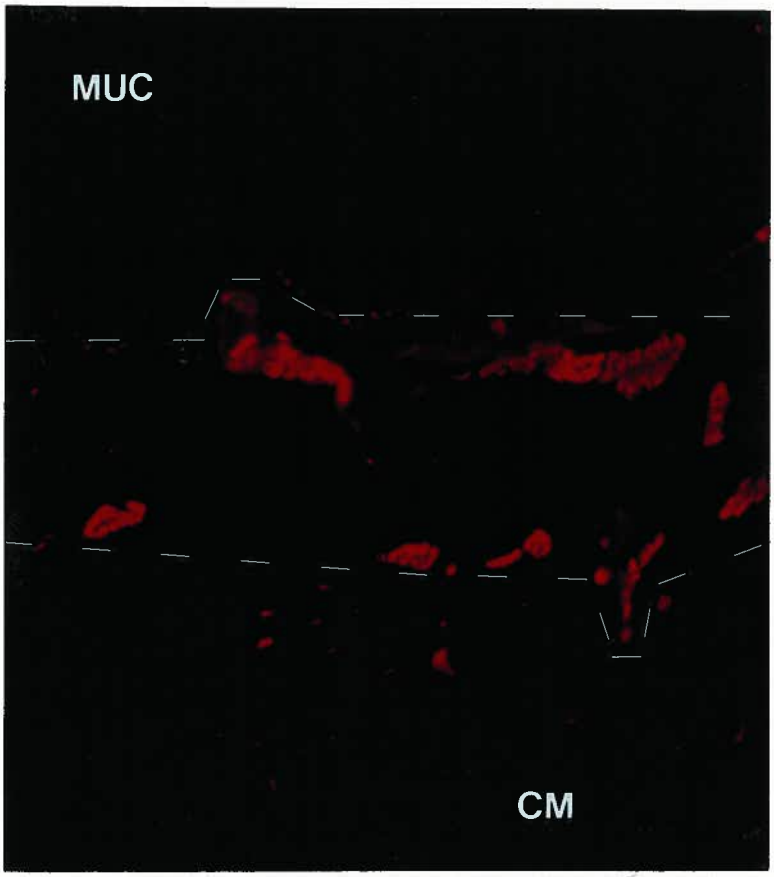

MUC
CM

Figure 12. Details of the PGP 9.5 staining in the canine duodenal SMP. Note the close association of the large ganglion with the muscularis mucosae (m = muscularis mucosae, CM = circular muscle, M = mucosa).

x 100.

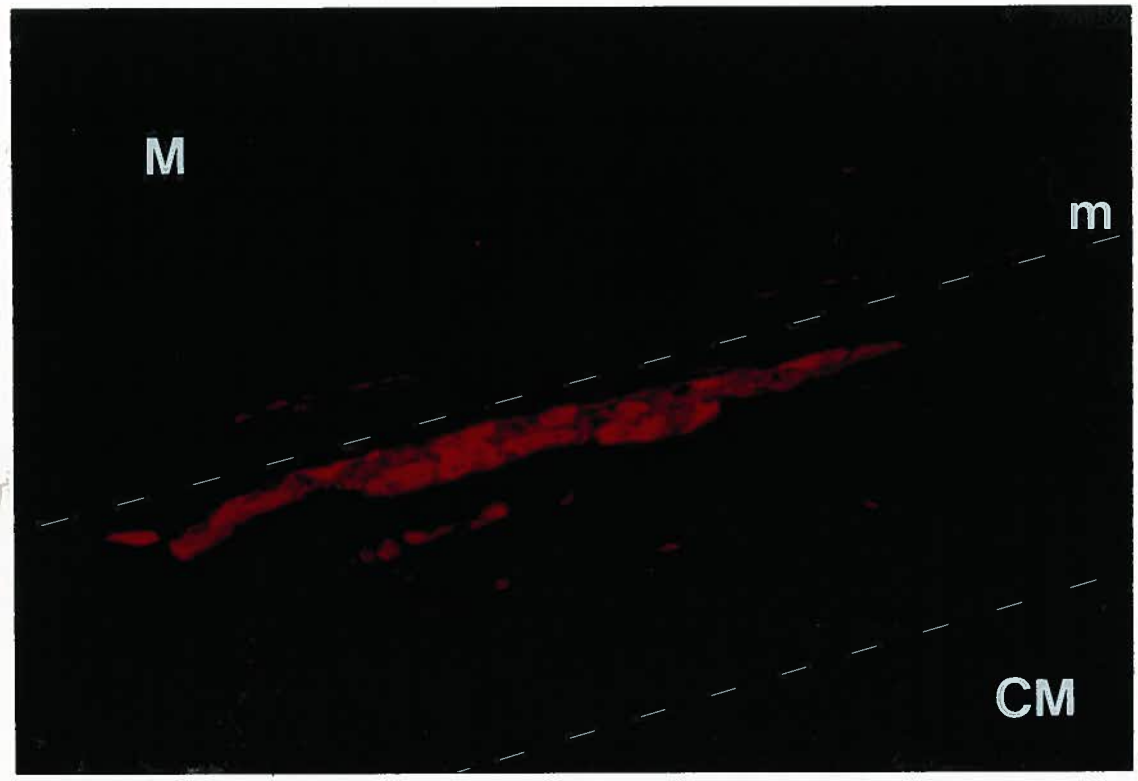

M
m
CM

Figure 13. Details of the PGP 9.5 staining in the canine duodenum. Note the band of immunoreactive fibres (small arrows) at the inner surface of the circular muscle forming the deep muscular plexus (dmp)

x 200.

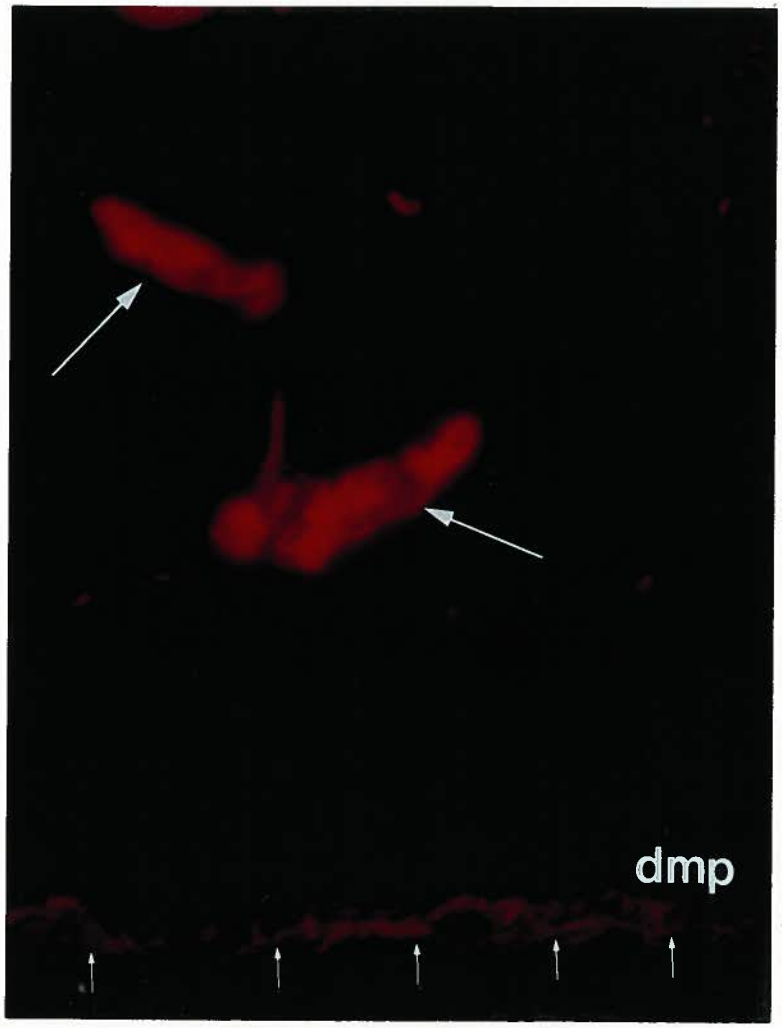
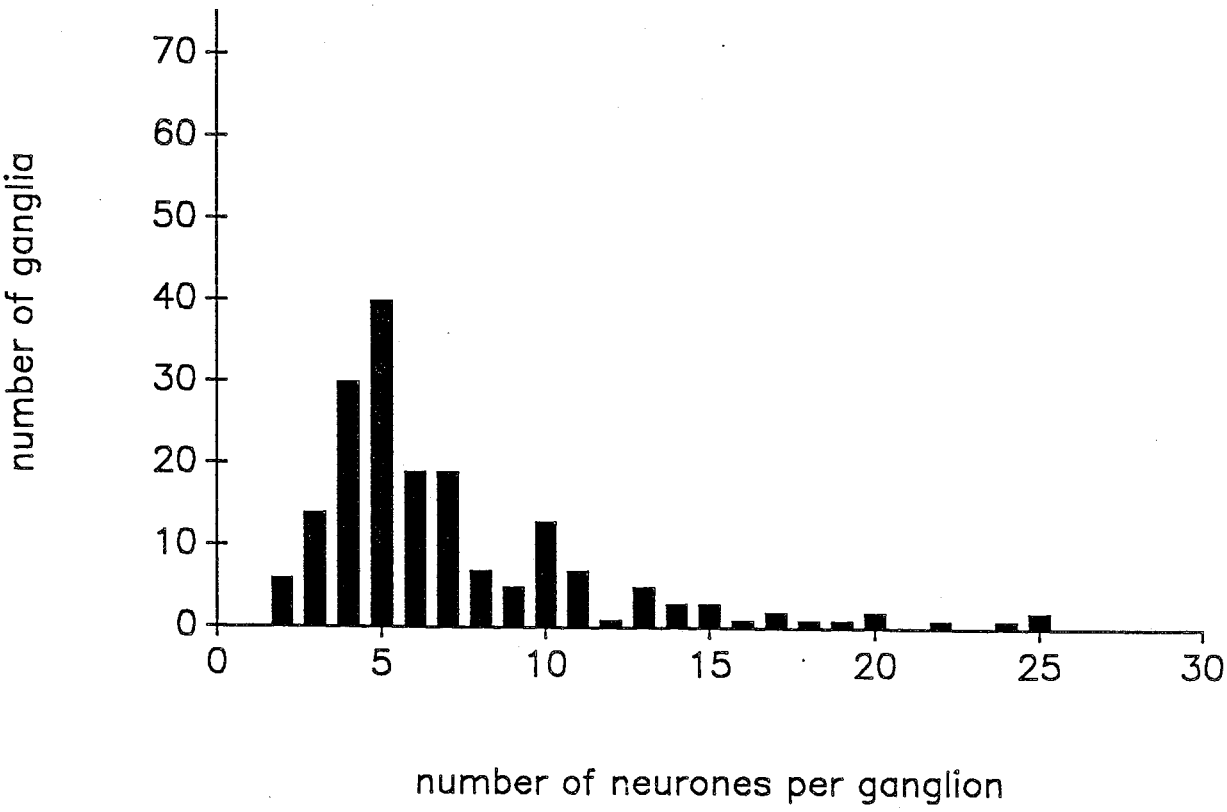

dmp

Figure 14. The relative size distribution of canine submucosal ganglia of the small intestine. Note that the majority of ganglia contained between 4 and 7 neurones. Large ganglia (those which contained > 15 neurones) made up 10 % of the population.


number of ganglia
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
number of neurones per ganglion

b. Single Stains of SS-IR, SP-IR and VIP-IR.

The pattern of single staining of canine small intestine with antibodies to SS, SP and VIP was again similar to that found in previous investigations. Cell bodies containing SS-IR were found in submucosal ganglia (figure 15 a), endocrine cells and myenteric ganglia. Submucosal and myenteric ganglia also contained SS-IR nerve fibres but were less abundant than those containing SP-IR or VIP-IR. The SS-IR neurones were often grouped into clusters within the ganglia whose fibres would exit the ganglia in the same direction. Thirty five percent of neurones per canine submucosal ganglion contained SS-IR. Endocrine cells containing SS-IR were predominant in the region of the crypts of the mucosal epithelium and were stained more intensely than neurones containing SS-IR.

Cell bodies containing VIP-IR were again found in myenteric and submucosal ganglia. Fibres were also found throughout the MYP, SMP and the smooth muscle layers. Fibres containing VIP-IR were also found in mucosa villi and, as in the human, were found in numbers similar to SP-IR fibres and greater than SS-IR fibres. Thirty one percent of neurones per canine submucosal ganglion contained VIP-IR. Endocrine cells containing VIP-IR were not observed.

Cell bodies containing SP-IR were found in canine submucosal (figure 15 b) and myenteric ganglia. Numerous SP-IR fibres containing varicosities were found within the myenteric and submucosal ganglia which were distributed amongst other neuronal cell bodies. As with the other peptide containing neurones, SP-IR neurones were segregated within the ganglia and their fibres exited the ganglia in the same direction. Fibres were also found in the mucosa, the SMP, MYP and muscle layers. Substance P fibres were more plentiful than fibres containing SS-IR and were present in amounts similar to those containing VIP-IR. Thirty percent of neurones per canine submucosal ganglion contained SP-IR while endocrine cells containing SP-IR were not found.

c. Double Stains.

The double stains revealed that, unlike the human, SS-IR and SP-IR were never present in the same neurone (figure 16 a, b). Neurones containing VIP-IR were demonstrated to be a population completely separate from those neurones containing SS-IR or SP-IR (figure 17 a, b). Double stains also demonstrated neuropeptide containing fibres and varicosities of each neuropeptide distributed around other neuropeptide containing neurones within the ganglia. Some fibres formed pericellular baskets around neuropeptide containing neurones. Finally, double stains provided

further evidence for the spatial segregation of different neuronal types within the ganglia.

The data obtained from the immunocytochemistry of sections of canine and human small intestine are summarized in tables 5, 6 and 7.

Figure 15. Representative immunostains of the canine SMP. Note that the neurons are grouped into small clusters within the larger ganglion (larger arrows) and that the fibres of a cluster appear to exit the ganglion as a unit (small arrows).

a) SS-IR x 200.

b) SP-IR x 200.


a

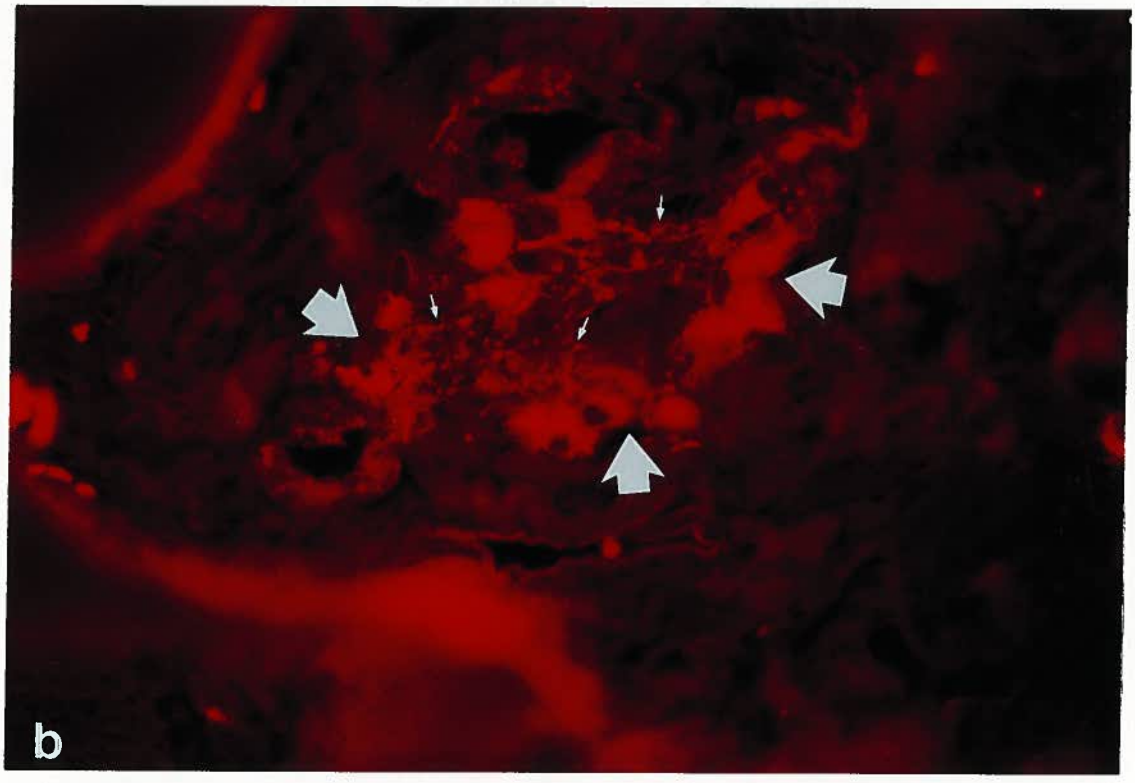
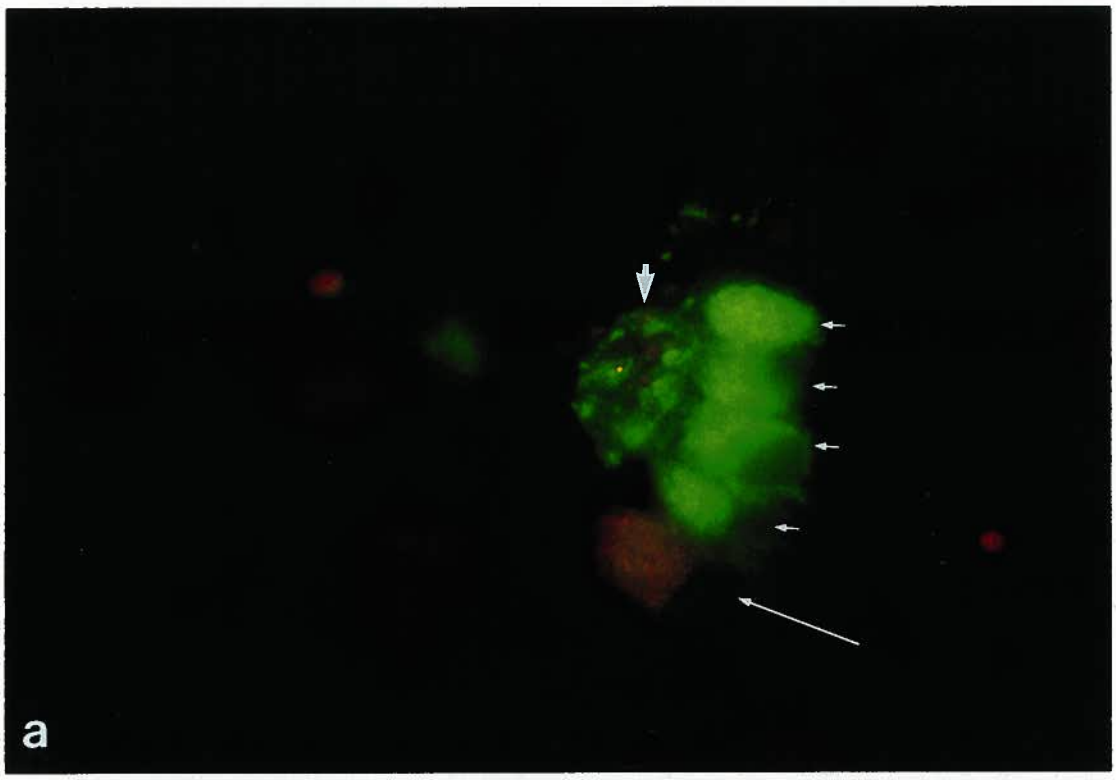

b

Figure 16, a and b. Double stains of canine SMP ganglia for SS-IR (FITC, small arrows) and SP-IR (Rhodamine, longer arrows). Once again note the grouping of the neurones into clusters of a single type and the merging of exiting fibres (see 'a', medium sized arrow)

x 500.


a

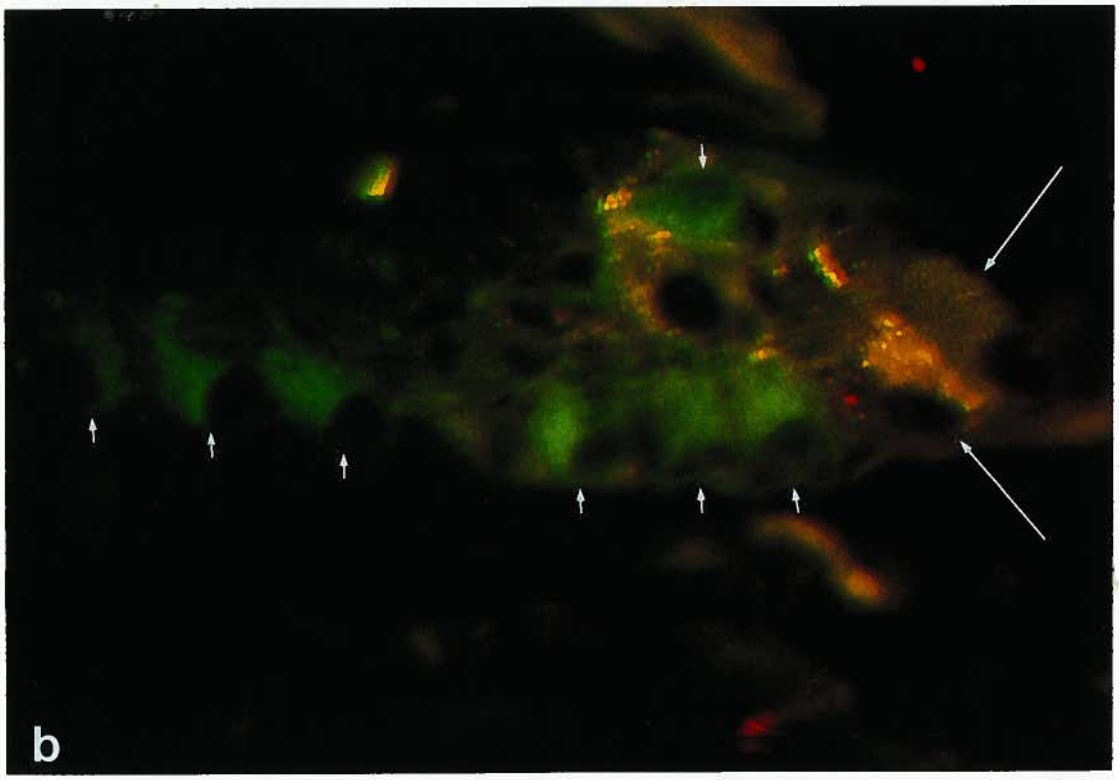
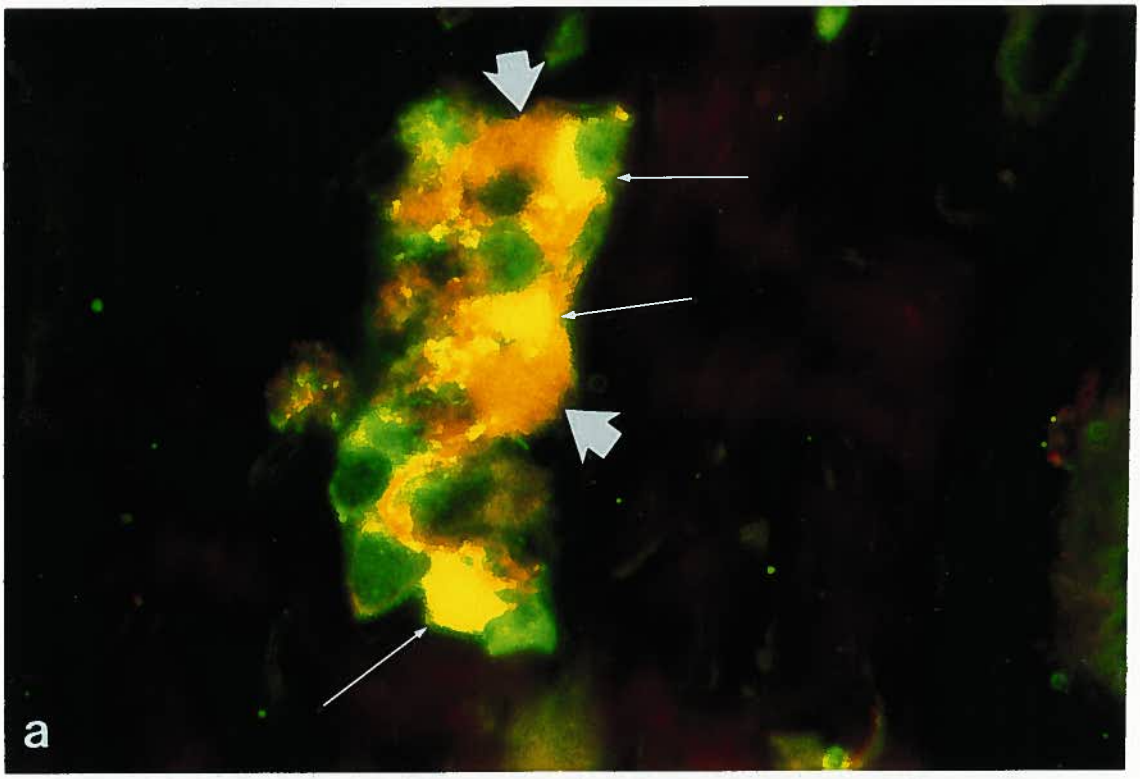

b

Figure 17. Double stains of canine SMP ganglia. a) VIP-IR neurones (Rhodamine, large arrows) and SS-IR (FITC, small arrows) x 500. Note the absence of co-localization. b) VIP-IR neurones (Rhodamine, large arrows) and SP-IR (FITC, small arrows) x 500. Note the absence of co-localization.


a

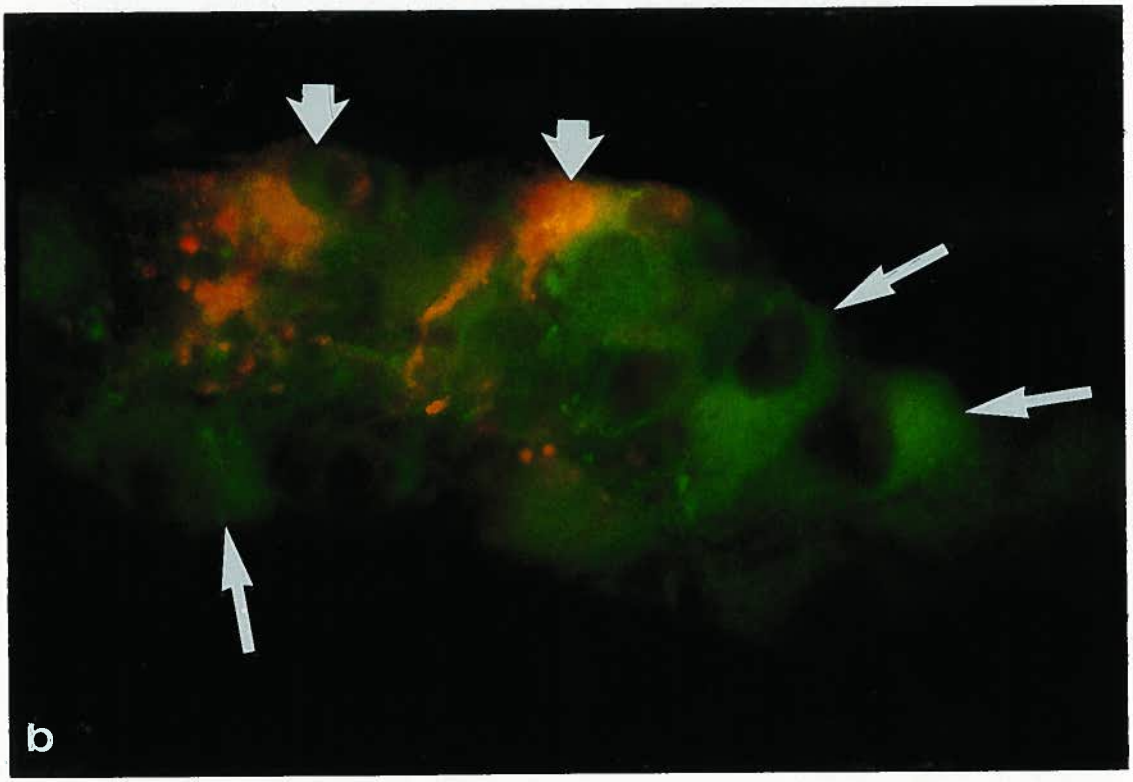

b

Table 5. Summary of Neuropeptide Distribution and Co-localization in the Human Small Intestine

	SS-IR	SP-IR	VIP-IR
Mucosa.			
endocrine cells	+++	-	-
nerve fibres	+	++	+++
Ganglia (SMP and MYP).			
neurones	+++	+++	+++
nerve fibres	+	++	+++
Co-localization.			
SS-IR		yes	no
SP-IR	yes		no
VIP-IR	no	no	

+++ most abundant.

++ less abundant.

+ least abundant.

Table 6. Summary of Neuropeptide Distribution and Co-localization in the Canine Small Intestine

	SS-IR	SP-IR	VIP-IR
Mucosa.			
endocrine cells	+++	-	-
nerve fibres	+	++	+++
Ganglia (SMP and MYP).			
neurones	+++	+++	+++
nerve fibres	+	++	+++
Co-localization.			
SS-IR		no	no
SP-IR	no		no
VIP-IR	no	no	
Deep Muscular Plexus	-	++	+++

+++ most abundant. ++ less abundant. + least abundant.

Table 7. Comparison of Quantification Data Between the Canine and Human SMP

	Dog	Human
Average number of neurones per ganglion.	7.7 ± 0.6	2.9 ± 0.3
% SS-IR neurones	35	42
% SP-IR neurones	30	42
% VIP-IR neurones	31	40

C. Discussion

Staining of tissue sections of canine and human small intestine with PGP 9.5, a general marker for peripheral nerves (Gulbenkian *et al*, 1987; Lauweryns and Van Ranst, 1988; Wilson *et al*, 1988), was used to localize all myenteric and submucosal neuronal cell bodies and revealed an extensive network of nerve fibres throughout the small intestine. The distribution of nerve fibres was similar between the dog and the human, but canine submucosal ganglia contained, on average, a greater number of neurones. Also, the density of neurones was greater in the canine SMP than human small intestine based on the smaller amount of collagen in the canine submucosa. It has been suggested that the size of the intestine determines the number of neurones per unit of serosal surface and the total number of neurones, with each of these values being parallel to the size (weight) of the animal (Gabella, 1987; 1990).

The average size of each submucosal ganglion was larger in canine small intestine than in the human small intestine. These data suggest that the size of submucosal ganglia does not parallel the size of the animal. The number of neurones per submucosal ganglion in the human was closer to that of the rat which has 3-5 per ganglion (Pataky *et al*, 1990), than to that of the dog. Sheep probably represent a third group of animals, based on the low spatial density of

neurones in the small intestine and the large size of the enteric ganglia. The small intestine of these ruminants has been shown to have a low spatial density of neurones but have neuronal ganglia which contain large numbers of neurones (Gabella, 1987).

It is generally accepted that mammalian myenteric ganglia contain a greater number of neurones than the submucosal ganglia (Gabella, 1987; Furness *et al*, 1987), although preliminary counts have indicated that this is not the case in the dog, while in the human the numbers of neurones in submucosal and myenteric ganglia seem to be equal (data not shown). A major portion of the volume of the myenteric ganglia in both the dog and human was made up of neuropil i.e. neuronal fibres and other neuronal processes, and glia. It could be argued that 2 dimensional renditions of 3-D structures do not accurately reflect neuronal number and volume, requiring the use of whole mount preparations. However, analysis of sections cut perpendicular and parallel to the direction of the circular muscle provided a reasonable construction of both plexi.

The myenteric ganglia were in an orderly array with their long axis travelling in the same direction as the circular muscle as noted in previous studies (Gabella, 1987). Whether the submucosal ganglia possessed a similar orientation could not be discerned from tissue sections but

most likely these ganglia were less structured, as has been demonstrated in previous studies (Gabella, 1990).

The presence of SS-IR in submucosal and myenteric neurones, and endocrine cells of the mucosa, and the small numbers of SS-IR containing nerve fibres in canine and human small intestine tissue sections was in agreement with the results of previous studies (Daniel *et al*, 1985; Keast *et al*, 1986). Nerve fibres throughout all layers of the small intestine and neurones of the myenteric and submucosal plexus contained SP-IR and VIP-IR, as demonstrated previously (Tange, 1983; Daniel *et al*, 1985). Mucosal endocrine cells did not contain SP-IR, although this has been reported in both the human and canine small intestine (Daniel *et al*, 1985; Keast *et al*, 1985). In studies carried out by these authors, endocrine cells containing SP-IR were few in number and stained less intensely than those endocrine cells containing SS-IR and SP-IR. Only the small intestine of the marmoset has been shown to contain large numbers of endocrine cells which stained brightly for SP-IR (Keast *et al*, 1985).

The double staining experiments revealed the co-existence of SP-IR and SS-IR in neurones of human, but not canine, small intestine which has not been previously demonstrated. Neurones containing VIP-IR were distinct from those containing SP-IR and SS-IR in both species, which is

in agreement with previous studies which have demonstrated no co-existence between SP-IR and VIP-IR fibres in the muscularis externa throughout the canine and human gastrointestinal tract (Wattchow *et al*, 1988; Furness *et al*, 1989).

Two separate populations of motoneurones have been proposed by these authors to innervate the external muscle of the human gastrointestinal tract, one containing VIP-IR and neuropeptide Y-IR and the other containing SP-IR and enkephalin-IR (Wattchow *et al*, 1988). Fibres containing VIP-IR and NPY-IR were called inhibitory by Wattchow *et al*, (1988), since both peptides have been shown to inhibit motility of human gastrointestinal muscle (Couture *et al*, 1981; Furness *et al*, 1982; Allen *et al*, 1987). The presence in the human small intestine of nerves which are excusively inhibitory is consistent with the results obtained in this study.

Fibres containing SP-IR and enk-IR were called excitatory by Wattchow *et al*, (1988), since these peptides have been shown to be excitatory on human gastrointestinal smooth muscle (Zappia *et al*, 1978; Couture *et al*, 1981). The presence of nerves which are exclusively excitatory in the human small intestine is not supported by the present study since neural SS-IR, which has been shown to inhibit motility in the small intestine (McIntosh *et al*, 1987a), was

found to co-exist in all SP-IR containing neurones in both the myenteric and submucosal ganglia. It may be argued that SS-IR may only act as an interneuronal neurotransmitter and thus not affect motility by acting directly on the muscle, in a physiological setting. An indirect action of SS on muscle, by inhibiting enteric neurones, has not been proven and furthermore each peptide may potentially affect adjacent neurones containing excitatory and inhibitory neurotransmitters making the terms "excitatory" and "inhibitory" neurone inappropriate (Cooke, 1989).

The interspecies alteration in peptide profiles demonstrated in this study parallels that seen between the guinea pig and the rat (Costa *et al*, 1987; Pataky *et al*, 1990). All neurones containing SS-IR were found to co-exist with SP-IR in the rat small intestine which was similar to the human small intestine (Pataky *et al*, 1990). The proportion of neurones containing SP-IR and VIP-IR in the rat (46 and 42 %, respectively) and human (42 and 39 %) was also similar. There were two major differences between the rat and the human in the distribution of peptides examined in this study. Not all rat SP-IR neurones contained SS-IR, and SS-IR neurones made up only 18 % of submucosal ganglia rather than 40 % as in the human. The similarities in peptide distribution between the rat and human small intestine were in addition to the similarity in total number of neurones per ganglion.

In the guinea pig, SP-IR and SS-IR have been shown to exist in separate neurones similar to canine submucosal ganglia (Costa *et al*, 1987). The proportion of neurones which contain SS-IR was similar between these species (29 % in the guinea pig and 32 % in the dog), whereas the proportion containing SP-IR and VIP-IR was different. The proportions of canine submucosal neurones containing SP-IR and VIP-IR were 32 % and 30 % respectively, but were 11 % and 45 % respectively in the guinea pig. Thus, even in those species in which there were similarities in chemical coding of the neurones, significant differences in ganglionic composition (i.e. the proportions of peptide-containing neurones) occurred. There is general agreement that interspecies variation in peptide localization of the enteric plexi will be present in almost all cases (Furness *et al*, 1989).

In human tissues, VIP-IR fibres innervated SP-IR/SS-IR containing neurones and SP-IR fibres innervated VIP-IR neurones. In addition, SP-IR containing fibres were associated with SP-IR containing neurones, providing anatomical evidence for interganglionic regulation.

In canine tissues, SP-IR fibres were distributed throughout the larger ganglia surrounding neurones containing both VIP-IR and SS-IR. The results provided

anatomical evidence for the action of SP on SS-IR secretion. It should be cautioned that anatomical evidence cannot be taken as conclusive, since data presented in this study do not support an action of SP on SS-IR secretion in the human, even though SP-IR fibres were found apposed to SS-IR containing neurones.

Fibres located in all regions were shown to contain only SP-IR in both canine and human tissue. In human tissue, the co-existence of SP-IR and SS-IR in neuronal cell bodies, but not fibres, could have been due to low levels of SS-IR. Another possibility for the lack of co-existence in fibres was that those containing SP-IR were extrinsic fibres, such as sensory afferents of the vagus. Substance P has been shown to commonly be present in vagal afferents in the small intestine and several other organs of many species where it does not co-exist with SS-IR (Costa *et al*, 1987).

The source of SP-IR and VIP-IR fibres in the outer layer of circular muscle in the canine small intestine has been shown to be in the MYP, whereas fibres in the inner layer and the innervation of the muscularis mucosae and mucosal layer originated in the submucosal plexus (Furness *et al*, 1989).

In both species, there was a spatial difference in the localization of neuronal types. In the canine sections, the

SS-IR, VIP-IR and SP-IR were clearly segregated within the ganglion. In the human sections, segregation was more extreme, with small ganglia (2-4 cells) being made up exclusively of VIP-IR or SS-IR/SP-IR neurones.

D. Summary

The data collected have supported the existence of major interspecies variations in chemical coding of enteric neurones between human and canine small intestine. They have confirmed the existence of two distinct plexuses in the submucosa of the canine small intestine. The presence of neuropeptide containing nerve fibres and varicosities within enteric ganglia has provided a morphological basis for neuropeptide actions on other neurones. Finally, it was demonstrated that different neuronal types are segregated within enteric ganglia, and send processes in the same direction. The factors which determine differences in gastrointestinal gross anatomy and function have been suggested to be related to diet (Powell, 1987) and size of the animal (Gabella, 1990). Thus, the gastrointestinal tract of omnivores such as rats and humans are more similar than the gastrointestinal tract of carnivores such as the dog, or ruminants such as sheep. In other words, the interspecies differences observed in this study may be due, in part, to differences in diet.

IV. CHAPTER TWO. ISOLATION OF CANINE AND HUMAN SUBMUCOSAL NEURONES

A. Introduction

The release of SS-IR and SP-IR from the mammalian small intestine using *in situ* and *in vivo* experimental models has been demonstrated (Andersson *et al*, 1982; Donnerer *et al*, 1984; Manaka *et al*, 1989) but the results have been difficult to interpret for two reasons. First, SS-IR and SP-IR have been found in three different groups of cells, namely neurones of the submucosal and myenteric plexi, and endocrine cells of the canine and human small intestine (Keast *et al*, 1986; Keast *et al*, 1985; Daniel *et al*, 1985). Thus, a peptide released from endocrine and/or neural cells could play a role in the physiological homeostasis of the small intestine. Second, SS-IR is present in the small intestine in two primary molecular forms, namely SS-14 and SS-28. Substance P is processed from the tachykinin gene which can express three different peptides, and it is not known which are expressed in different cell types (Dockray, 1987). Therefore, it has become necessary to establish models that circumvent difficulties associated with *in vivo* experiments in order to understand differential release of peptides from neurones or endocrine cells.

Organotypic cultures from small intestine have been developed using segments of the gut wall which contain the enteric plexuses in association with muscle layers and connective tissue (Gershon *et al*, 1980). The separation of the myenteric and submucosal plexuses from the gut wall using a combination of enzymatic treatment and microdissection, and the maintenance of explants in tissue culture was later carried out (Jessen *et al*, 1978; 1983). Dispersed cultures of rat small intestine have also been developed and have been extensively characterized with respect to their morphological, pharmacological and electrophysiological properties (Nishi and Willard, 1985; Willard and Nishi, 1985 a,b). These authors found that enteric neurones maintained in tissue culture possessed morphological, pharmacological and electrophysiological properties similar to those possessed by neurones *in situ*.

More recently, methods to examine the release of peptides from isolated cultured cells obtained from mucosal (Barber *et al*, 1986) and submucosal layers (Barber *et al*, 1989) of the small intestine have been developed and these offer certain advantages over *in vivo* studies. Acutely dissociated ganglia from myenteric neurones of guinea pigs (Grider, 1989) and a newly developed short-term culture of submucosal neurones from canine small intestine (Buchan *et al*, 1989) have been used specifically to study the release of SS-IR from enteric nerves. The use of acutely

dissociated neurones for secretion studies is limited because nerve fibres are not present after isolation. Thus, the release of neurotransmitter would have to be from the cell soma for which there is presently no evidence *in vivo*.

The control of peptide release *in vivo* is mediated via receptor regulation and/or a membrane voltage dependent mechanism. In culture studies, the role of receptors can be mimicked by the addition of specific pharmacological agents. These drugs activate second messenger pathways normally associated with receptor activation (Berridge, 1985). Three such drugs are commonly utilized to activate different intracellular pathways. The phorbol esters activate protein kinase C, the calcium ionophore (A23187) increases intracellular calcium and forskolin increases intracellular cAMP.

The effects of these drugs have been examined in cultures of canine SMP neurones. Of particular interest was the action of phorbol 12-myristate 13-acetate (ß-PMA) which, in the presence of the calcium ionophore A23187, stimulated the release of SS-IR from canine submucosal neurones in culture (Buchan *et al*, 1990). Phorbol esters, such as ß-PMA, have been shown to activate protein kinase C (PKC) (Blumberg, 1981). Protein kinase C is normally activated by diacylglycerol formed from phospholipase C-mediated cleavage of membrane phospholipids, which also produces

inositol triphosphate (Nishizuka, 1986). The activation of PKC has been shown to stimulate the release of hormones and neurotransmitters (Kaczmarek, 1987). The release of SS-IR by phorbol esters has also been demonstrated using dispersed cultures of fetal rat hypothalamus and cortex (Peterfreund and Vale, 1983).

The addition of the calcium ionophore or the phorbol ester alone was shown not to increase the secretion of SS-IR from canine submucosal neurones (Buchan *et al*, 1990). This indicated that increases in calcium per se or activation of PKC were not sufficient to stimulate SS-IR secretion.

Interestingly, the addition of forskolin did not stimulate the secretion of SS-IR from canine cultures, although it was able to stimulate the release of neurotensin from similar cultures (Barber *et al*, 1989; Buchan *et al*, 1990). Previous experiments carried out using glia-free cultures of rat cerebral neurones have shown that adenylate cyclase activation by forskolin was not sufficient to stimulate SS-IR secretion (Tapia-Arancibia *et al*, 1988).

Substance P has been shown to inhibit SS-IR release from canine submucosal neurones (Buchan *et al*, 1990) and gastric somatostatin cells (Kwok *et al*, 1988) and to stimulate SS-IR release from the hypothalamus and pancreas (Reichlin, 1981). The effect of SP on SS-IR secretion from canine neurones was

unexpected, since it has been shown to stimulate the release of NT from similar cultures (Barber et al, 1989). The inhibitory actions of SP on SS-IR release in canine cultures were likely indirect since this peptide has also been shown to release Ach from myenteric neurones (Wiley and Owang, 1987), increase intracellular calcium in dorsal horn neurones (Womack *et al*, 1988) and produce EPSPs in submucosal neurones (Surprenant, 1984). Its action on SS-IR secretion from human submucosal neurones was examined in the present study and compared to its effects on canine submucosal neurones.

The experiments outlined in the following chapter describe the development of a system to isolate human neurones from the upper small intestine and to maintain these neurones in tissue culture. These experiments test the hypothesis that the difference in chemical coding between canine and human neurones is reflected in their function. Specifically, the effects of the phorbol ester, the calcium ionophore and SP on the secretion of SS-IR from human submucosal neurones were examined. Preliminary experiments which examine the release of SP-IR from canine neurones are also described.

In addition to substance P, a variety of agents were tested for their ability to modify SS-IR secretion. Receptor independent secretagogues, the calcium ionophore

A23187 and the phorbol ester ß-PMA, were utilized to increase intracellular Ca^{2+} and PKC activity, respectively. It was expected that A23187 would require the concommitant addition of ß-PMA to stimulate secretion , since increasing intracellular calcium was insufficient in canine neurones. The phorbol, 4α-phorbol, is similar in structure to ß-PMA but does not stimulate PKC activity *in vitro* (Blumberg, 1981). Therefore this was used as a control for non-specific membrane effects, since phorbols are highly lipophilic and may cause non-specific destabilization of lipid membranes.

Experiments were carried out using 10 mM KCl, alone and concommitantly with the phorbol ester. This level of potassium would produce a depolarization of 20 mV, as determined by the Goldman equation using "normal" values for intracellular and extracellular ions. This was done for two reasons. First, this determined whether a small depolarization would stimulate the secretion of SS-IR from human submucosal neurones. Second, to determine whether a small depolarization would render the neurones more sensitive to stimulation by the phorbol ester.

It has been shown that cholinergic neurones are present in the submucosal plexus and may be involved in the release of other neurotransmitters (Barber *et al*, 1989). In order to determine the role of cholinergic neurones in the

secretion of SS-IR two agonists and two antagonists were used. Carbachol and methacholine are non-specific cholinergic agonists. Methacholine is more potent, has less nicotinic activity and has been shown to be more effective in stimulating the release of antral SS-IR (Buchan *et al*, 1991). Hexamethonium and atropine are nicotinic and muscarinic antagonists and were used to block both exogenous and endogenous cholinergic substances. Cholinergic agonists have been shown to produce both excitatory and inhibitory effects in enteric neurones (North *et al*, 1985) therefore the overall effects of the antagonists on SS-IR secretion were not predictable. These antagonists were also used to determine whether the effects of SP were direct or indirect via endogenous cholinergic action.

Calcitonin gene-related peptide (CGRP) has been shown to co-exist with SP in primary sensory neurones, and has been shown to stimulate the release of SS-IR in other systems (Dockray, 1987). Therefore, its effects on SS-IR secretion from human submucosal neurones were examined.

B. Methods

1. Human Donor Experiments

a. Procurement of Tissue

Twelve to fifteen inches of upper small intestine was obtained from multiple organ donors in association with the Pacific Organ Retrieval for Transplantation (PORT) program. Previous to surgical removal, the small intestine was perfused with Eurocollins (see appendix 2) at 4^{o}C to remove all red blood cells. The donors were pre-screened for transplantation and therefore had no known pathophysiological conditions.

b. Isolation of Submucosal Ganglia

The duodenal bulb was not taken nor was the initial portion of duodenum which contained Brunner's glands since the submucosa could not be properly separated in these areas. The mucosa and muscle layers were removed by blunt dissection, the remaining submucosa was washed in Hanks' Balanced Salt Solution (HBSS) containing 0.1% bovine serum albumin (BSA) and 20 mM N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES), and chopped finely. The Hank's buffer was used throughout the isolation for washing tissue between periods of incubation. The medium used for

incubation consisted of Basal Medium Eagle (BME) containing 0.1% BSA, 20 mM HEPES and collagenase, and was gassed with 5% CO_2 in O_2. The pH of the incubation and washing media was strictly maintained between 7.0 and 7.4, low enough to inhibit collagen reassembly yet remaining within physiological limits. Each 10 g of tissue was incubated with 50 ml of the incubation medium in 200 ml flasks, continually shaken in a water bath at 200 Hz and maintained at 37°C throughout each incubation period. The isolation was carried out in three stages. First, the tissue was incubated for 30 min in incubation medium containing 600 U/ml collagenase (type XI) and 4 mM $CaCl_2$. The partly digested tissue was washed and centrifuged for 5-10 min at 200 x g to remove collagen, fat and debris. The tissue was further digested for two periods of 60 min, each followed by washing and centrifugation. The suspension was then filtered through a 240 μ Nytex mesh and resuspended in HBSS.

c. Elutriation Centrifugation (figure 18)

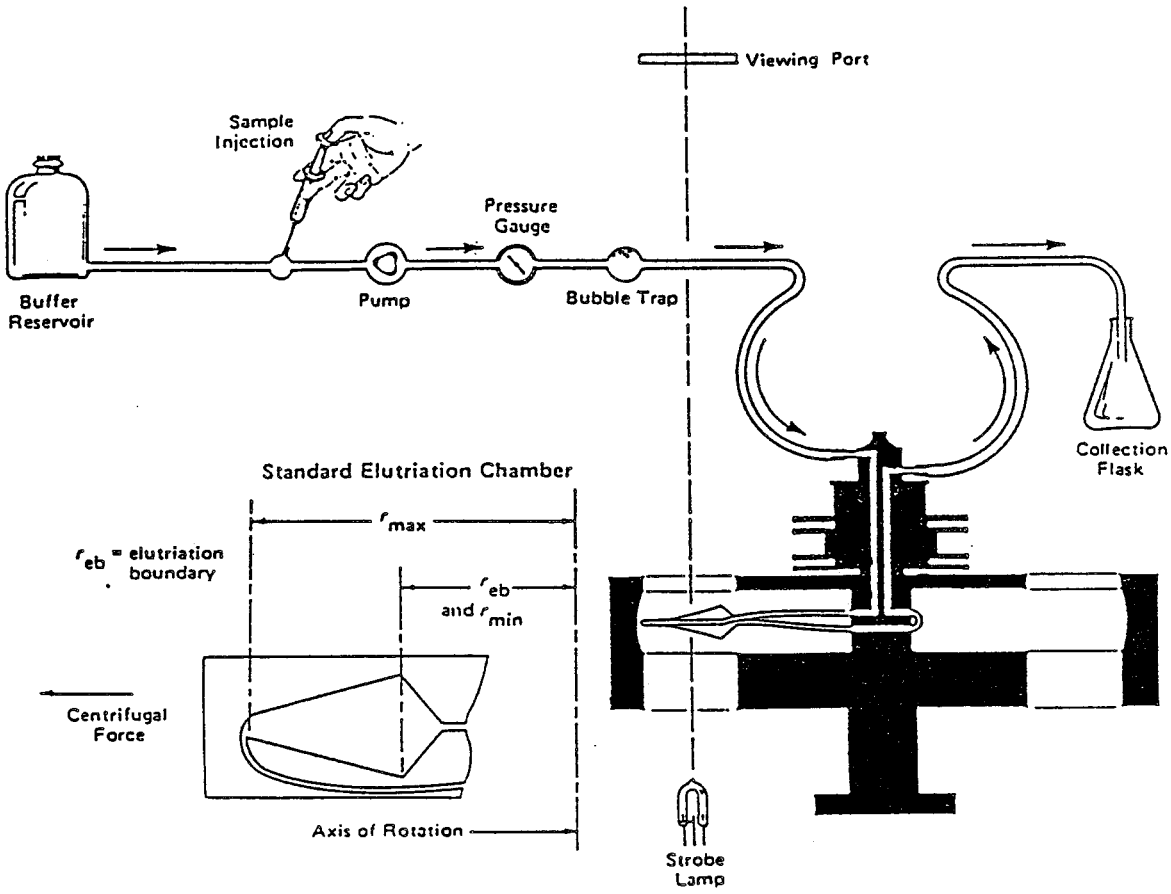
Elutriation centrifugation utilizes centrifugal force and flow, which act in opposing directions, to separate cells on the basis of their volume. Different fractions of cells can be removed by altering the flow rate of fluid passing through the elutriation chamber by altering the pump rate or by changing the speed of the centrifuge. Appropriate flow rates and centrifugation speeds were

determined empirically. The cell suspension was loaded into an elutriator rotor (Beckman) at 2500 rpm at a flow rate of 25 ml/min. Flow was supplied by a pump (Cole Parmer, Masterflex Model 7520-20) with a quick-loading head (Cole Parmer, model 7021-20) fitted with silicon tubing (Cole Parmer, type 6411-16), equipped with a pressure gauge. The pump flow rates were calibrated before each experiment at 2500 rpm. A fraction was collected at 2200 rpm at a flow rate of 35 ml/min which contained fibroblasts, red blood cells and cell fragments. A second fraction was collected at 800 rpm and a flow rate of 100 ml/min which contained single ganglia and clusters of two or three cells as well as undigested collagen fibres and fragments of blood vessels. Approximately 90% of the neurones were viable, as demonstrated by trypan blue exclusion.

d. Tissue Culture

The final fraction was again centrifuged at 200 x g for 5-10 min to concentrate the cells and resuspended in growth medium at a density of approximately $1x10^6$ cells/ml and plated on 12 well plates coated with rat tail collagen at 1ml/well. Single cells were counted and numbers were estimated for clusters of cells in order to obtain total counts. The growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) containing 5.5 mM glucose and supplemented with 20 mM HEPES, 2 mM glutamine, 200 mM

Figure 18. Diagram of elutriator rotor and chamber. Variations in flow rates and rpm allow cells to be separated on the basis of their volume.


Viewing Port
Sample Injection
Pressure Gauge
Buffer Reservoir
Pump
Bubble Trap
Collection Flask
Standard Elutriation Chamber
r_{max}
r_{eb} = elutriation boundary
r_{eb} and r_{min}
Centrifugal Force
Axis of Rotation
Strobe Lamp

cytosine ß-D-arabinofuranoside, 8 µg/ml insulin, 20 ng/ml nerve growth factor CS-7S, 100 µg/ml gentamicin, 1 µg/ml hydrocortisone, 4 µg/ml fungizone and 5% fetal calf serum. The cells were maintained in culture for 72 h at 37°C after which immunocytochemistry (ICC), release experiments, extraction and HPLC were carried out.

e. Somatostatin Secretion

i. General Protocol.

The cells were washed with 1 ml of release medium which consisted of DMEM containing 5.5 mM glucose, 1.0% aprotinin and 0.1% BSA. A 585 µl aliquot of release medium and 15 µl of drug or peptide solution was added to each well (total volume = 600 µl). The drug or peptide solutions were prepared at 40 times the desired final concentration in dimethyl sulfoxide (DMSO) and release medium. The highest ratio of DMSO to release medium was 1:400 and at this concentration the release of SS-IR was not affected. The cells were incubated for 120 min, after which the release medium was removed, centrifuged to remove any particulate matter, and stored at -70°C for radioimmunoassay (RIA). This procedure was used for ß-PMA, 4α-phorbol and all peptides. Comparisons were carried out using the same preparation i.e. in paired experiments.

ii. High potassium.

Experiments were carried out using release medium augmented with potassium chloride (KCl) in order to obtain a potassium concentration of 10 mM. This resulted in a depolarization of approximately 20 mV, as determined by the Goldman equation. The osmolality of the release medium was 346 mOsm/kg and 337 mOsm/kg, with and without the additional KCl, respectively, and therefore no adjustment to the composition of the medium was made.

iii. Time-course

Experiments were carried out to determine the time course of SS-IR secretion in response to ß-PMA (10-6 M, 10 mM KCL). ß-PMA was incubated in separate sets of wells for 15, 30, 60 and 120 min.

f. Somatostatin Extraction.

Each well was extracted to determine the content of SS-IR per well and the variability of SS-IR between wells (n=8). The isolated ganglia were dislodged from the bottom of each well using a rubber spatula after the addition of 600 μl of 2N acetic acid and boiled for 15 min. The extracts were centrifuged and the supernatant was frozen at -70^{o}C for RIA and HPLC analysis.

g. Somatostatin Recovery

The recovery of SS-IR was determined in two ways. Firstly, SS (500 pg) was added to release medium and incubated with submucosal cultures for 2 h. Secondly, SS (500 pg) was dissolved in 2 N acetic acid, boiled and lyophilized. SS-IR was then measured by RIA.

h. Characterization of Primary Molecular Forms.

i. Sample Preparation.

Extracts and release samples were added to a Waters Sep-pak C_{18} column that had been primed by the addition of 5 mls of 100% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) followed by 5 ml of 100% dH_2O with 0.1% TFA and dried with 5 ml of air. The sample and extracts were added to the column, washed with 1.5 ml of dH_2O and eluted with 1.5 ml of 70% ACN containing 0.1% TFA. The samples were gassed with 100% nitrogen to remove the ACN, frozen at -70^{o}C and lyophilized.

ii. Reverse Phase HPLC.

The samples were reconstituted in distilled water for subsequent reverse phase HPLC. Reverse phase HPLC was carried out on Waters equipment consisting of a 3.9 x 30 mm

μ-Bondapak C_{18} column, a model 512 WISP, two model 510 pumps and a model 441 absorbance detector. A gradient of ACN (28-35%) in 0.1% TFA run over a 10 minute period was used at a flow rate of 1 ml/min and 0.5 ml fractions were collected. The fractions were lyophilized, reconstituted in assay buffer and SS-IR was measured by RIA. Synthetic SS-14 and SS-28 were used for calibration of the column.

2. Dog Experiments

a. Procurement of Tissue

Mature mongrel dogs were sedated with phentanol triperidol (0.1 ml/kg) given with atropine (0.05 mg/kg), anaesthetized with sodium pentobarbital (30 mg/kg) i.v. and prepared for abdominal surgery. The animal was bled from the abdominal vena cava, the upper small intestine was removed and immediately placed in a container of ice-cold Hank's buffer.

b. Isolation of Submucosal Ganglia

The initial preparation of the tissue was similar to that which was carried out with the human small intestine. The isolation was carried out in two 1 h stages. First, the tissue was incubated for 60 min in incubation medium containing 300 U/ml collagenase (type XI, Sigma). The

partly digested tissue was washed and centrifuged for 5-10 min at 200 x g to remove collagen, fat and debris. The tissue was further digested for 60 min followed by washing and centrifugation. The suspension was then filtered through a 240 μ Nytex mesh and resuspended in HBSS.

c. Elutriation Centrifugation

The cell suspension was loaded into an elutriator rotor (Beckman) at 2500 rpm at a flow rate of 25 ml/min. A fraction was collected at 2200 rpm at a flow rate of 35 ml/min which contained fibroblasts, red blood cells and cell fragments. A second fraction was collected at 1600 rpm and a flow rate of 100 ml/min which contained single ganglia and clusters of two or three cells as well as undigested collagen fibres and fragments of blood vessels. Approximately 90% of the neurones were viable as demonstrated by trypan blue exclusion. In several experiments, a third fraction which contained large groups of cells, fragments of blood vessels and collagen fibres was collected at 800 rpm and a flow rate of > 100 ml/min.

d. Tissue Culture

The procedure for tissue culture of canine submucosal neurones was identical to that for human neurones.

e. SS-IR and SP-IR Secretion

i. General Protocol

The cells were washed with 1 ml of release medium which consisted of DMEM containing 5.5 mM glucose, 1.0% aprotinin and 0.1% BSA. A 1 ml aliquot of release medium and 10 μl of drug or peptide were added to each well. Drugs or peptides were prepared at 100 times the desired final concentration in distilled water. The cells were incubated for 45 min after which the release medium was removed, pipetted into Eppendorf tubes containing 110 μl concentrated HCl, centrifuged to remove any particulate matter and stored at -70^{o}C for RIA. Comparisons were carried out using the same preparation i.e. in paired experiments.

ii. Acetic Acid Extraction

Canine submucosal cells were extracted to determine the total cell content of SS-IR and SP-IR in the same manner as SS-IR from human submucosa.

iii. Sep-Pak Extraction and Concentration

Samples were extracted as previously described (Kwok and McIntosh, 1990). Release and acetic acid extract samples were applied to Sep Pak C_{18} cartridges which had been primed

with 10 ml of acetonitrile containing 0.1% trifluoroacetic acid (TFA), followed by 10 ml dH_2O and 10 ml 1% BSA. The cartridge was then washed with 10 ml dH_2O and 1 ml 20% acetonitrile containing 0.1% TFA. Two ml of 50% acetonitrile containing 0.1% TFA were added to the column to elute the peptides. The samples were lyophilized using a speed vac, and stored at -70°C for RIA. Each Sep Pak cartridge was used no more than twice, a procedure which has been shown not to alter the recovery of substance P and somatostatin.

iv. SP-IR Recovery

Known amounts of SP-IR (500 or 1000 pg) were added to release medium and incubated with canine submucosal cells for 1 h in wells which either did or did not contain the enzyme inhibitors captopril (20 μM) and bacitracin (20 μM).

3. Radioimmunoassay

Radioimmunoassay (RIA) was used to measure SS-IR and SP-IR in release medium and cell extracts of submucosal cultures, and the techniques for each have been previously described (Kwok and McIntosh, 1990; McIntosh *et al*, 1987b).

a. Somatostatin

i. Assay buffer

Sodium barbital (4.90 g), sodium acetate (0.32 g) and ethylmercurithiosalicyclic acid sodium salt (merthiolate; 0.10 g) were dissolved in 700 ml dH_2O. The pH was adjusted to 7.4 with HCl, and this stock buffer was stored at 4^oC. Aprotinin (trasylol; 100 K.I.U.) and BSA (Pentex; 5.0 g/l) were added to the stock buffer for preparation of assay buffer.

ii. Antibody

The SS-IR was assayed in duplicate using a monoclonal antibody (SOMA 3) to somatostatin (Buchan *et al*, 1985) which detects both SS-14 and SS-28 (McIntosh, 1987). The antibody was prepared from crude mouse ascites as previously described and kept at 4^oC until use. This stock solution was diluted with assay buffer to obtain a final titre of $4x10^6$ for the assay. The antibody has been shown to not cross-react with GIP, motilin, gastrin or substance P.

iii. Standards

Synthetic cyclic somatostatin was dissolved in 0.1 M acetic acid, diluted to 100 μg/ml using dH_2O containing

0.05% BSA (Pentex) and aliquots of 50 µl (5 µg) were lyophilized and stored at -20°C. An aliquot was dissolved in 100 µl of dH_2O, and serially diluted in assay buffer on the day of the assay to obtain standards ranging from 3.9 to 500 pg/ml.

iv. Preparation of ^{125}I-Somatostatin.

Synthetic Tyr^1-somatostatin was iodinated using the chloramine-T method, purified initially by adsorption to silica and lyophilized in aliquots of $1x10^6$ cpm. Aliquots were further purified on the day of the assay using a CM-52 Sephadex column previously equilibrated with 0.002 M ammonium acetate. An aliquot of ^{125}I-somatostatin was dissolved in 0.002 M ammonium acetate, applied to the column and eluted using 0.2 M ammonium acetate at a flow rate of 1 ml/min. One or two peak fractions were counted, neutralized with 2 M NaOH and diluted to 3000-3500 cpm/100 µl in assay buffer.

v. Separation

Activated charcoal (1.25%) and dextran (0.25%) were dissolved in 0.05 M phosphate buffer and this mixture was stirred for at least one h after the addition of 0.1% charcoal-extracted plasma (CEP).

vi. Assay Protocol (Table 8)

Assays were carried out on a refrigerated Table, maintained at approximately 4°C, using 12 x 75 borosilicate glass tubes. Total count, non-specific binding (NSB), zero binding and standard tubes were assayed in triplicate, while samples were assayed in duplicate. If necessary, samples were diluted with assay buffer so that concentrations fell within the most sensitive portion of the standard curve. For release experiments, 100 µl of release medium was added to NSB, zero and standard tubes in place of assay buffer.

After a 72 h period of equilibration at 4°C, separation of bound and free peptide was carried out by adding 1 ml of charcoal slurry to all except "total count" tubes. Tubes were vortexed and centrifuged at 3000 rpm for 30 min at 4°C. Tubes were decanted, dried over absorbant paper and counted on a gamma counter. The remaining pellet contained free iodinated and cold peptide.

Table 8. Somatostatin Assay Protocol

TUBES	NSB	zero	sample or standard	total count
buffer	300 µl	200 µl	100 µl	---
standard or sample	---	---	100 µl	---
antibody	---	100 µl	100 µl	---
label	100 µl	100 µl	100 µl	100 µl
total volume	400 µl	400 µl	400 µl	100 µl

vii. Calculation and Presentation of RIA Data

$$\% \text{ bound} = 100 \times \frac{(C_{NSB} - C_{sample})}{C_{total}}$$

where C = cpm

$$\% \text{ NSB} = 100 \times \frac{(C_{total} - C_{NSB})}{C_{total}}$$

A standard curve was plotted of % bound versus [somatostatin] on semilogarithmic paper and/or was transformed into a logit-log plot using a RIA software program (RIA Analysis v 1.0). Sample concentrations were determined from the original standard curve or by the RIA program using the mean of duplicate counts.

viii. Inter- and Intra-Assay Variation

Samples containing known amounts of somatostatin could not be kept for long periods of time because of a loss in immunoreactivity and therefore were not used as controls. Inter-assay variation was determined by comparing multiple standard curves while intra-assay variation was determined by comparing SS-IR standards randomly placed within the assay. Both inter-assay and intra-assay were less than 10%.

ix. Testing pH Effects.

Acetic acid (2N) was normally used to extract SS-IR from tissue and cultures and samples were diluted 1:20 and 1:40 for measurement in RIA. Therefore, the effects of adding acetic acid alone (2N, diluted 1:20 and 1:40) to the SS-IR standard curve were examined. The plots of % bound versus [somatostatin] showed that the "zero binding" was reduced from 50% to 36%, and the linear portion of the curve was reduced when the 1:40 dilution was used in the standard curve (figure 19). In addition, the non-specific binding of the label was increased when the 1:40 dilution was used. The standard curve could be corrected when the pH of the 1:20 and 1:40 dilutions of 2N acetic acid was adjusted to 7.4 with sodium hydroxide. It should be noted that the standard curve produced using the 1:40 dilution without the pH adjustment could be utilized to determine sample SS-IR concentrations. Interestingly, linear logit-log plots of the 1:40 undiluted, and the 'normal' standard curves were more similar than the plots of % bound vs. concentration (figure 20). This illustrates the inability of linear transformations of assay data to assess the usefulness of a standard curve.

Figure 19. Somatostatin standard curves (% bound vs concentration of somatostatin), without acetic acid, with acetic acid (1:40 dilution) and with acetic acid (1:40 and 1:20 dilution) where pH was adjusted with NaOH.

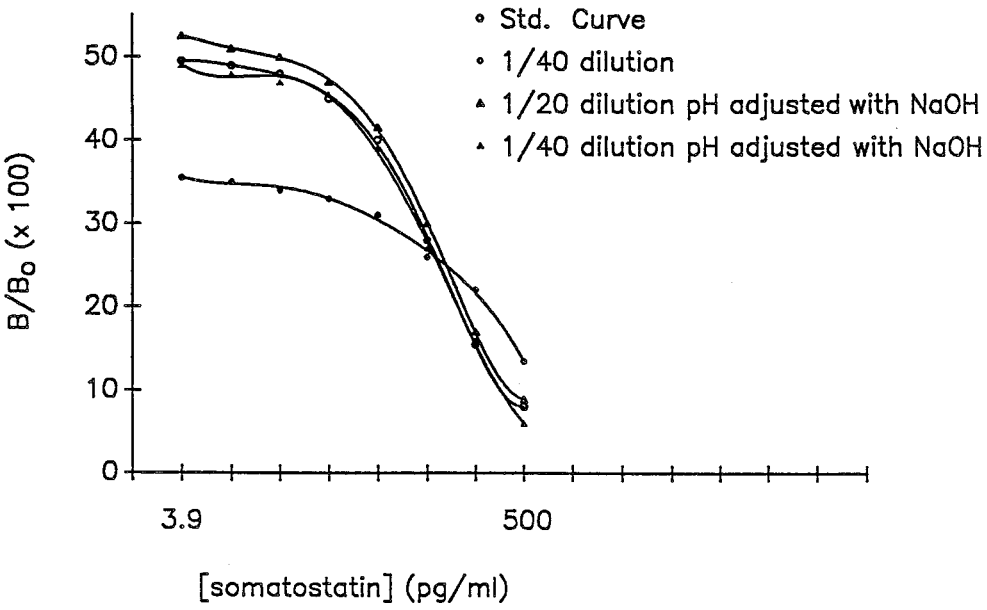
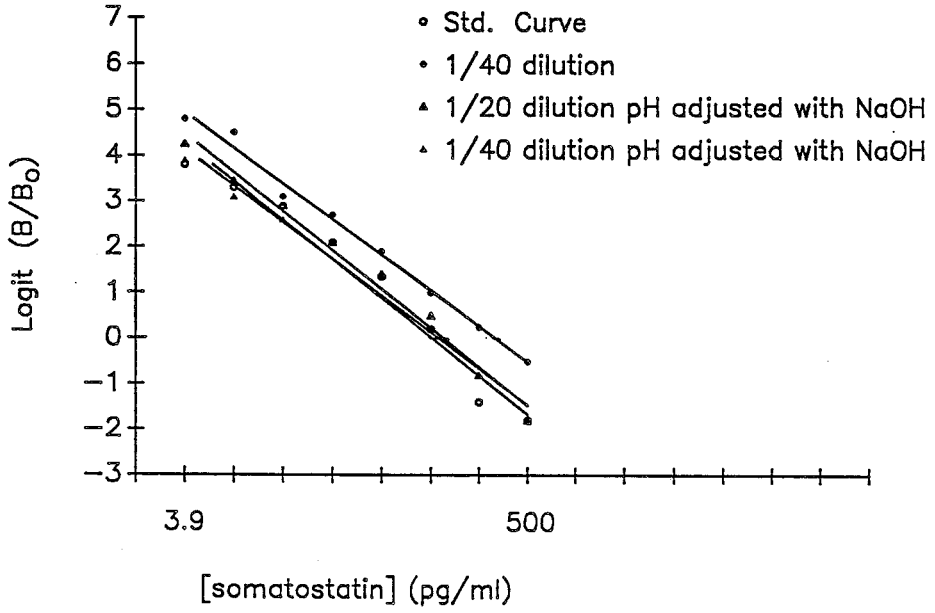

Std. Curve
1/40 dilution
1/20 dilution pH adjusted with NaOH
1/40 dilution pH adjusted with NaOH
B/Bo (x 100)
50
40
30
20
10
0
3.9
500
[somatostatin] (pg/ml)

Figure 20. Somatostatin standard curves (logit % bound vs concentration of somatostatin), without acetic acid, with acetic acid (1:40 dilution) and with acetic acid (1:40 and 1:20 dilution) pH adjusted with NaOH.


Std. Curve
1/40 dilution
1/20 dilution pH adjusted with NaOH
1/40 dilution pH adjusted with NaOH
Logit (B/Bo)
7
6
5
4
3
2
1
0
−1
−2
−3
3.9
500
[somatostatin] (pg/ml)

b. Substance P

The substance P RIA has been described previously (Kwok and McIntosh, 1990) and was identical to that for somatostatin with the following differences.

i. Assay buffer

Gelatin was used in place of BSA in the assay buffer.

ii. Antiserum

The antiserum KGP05 was raised in guinea pig using haemocyanin-conjugated SP and was used at a final dilution of 1:180,000. The antiserum has been shown to cross-react 100% with SP, SP-(3-11) and physalaemin, < 0.3% with kassinin, < 0.07% with SP methyl ester, < 0.04% with SP-(5-11), SP-(7-11) and eledoisin, < 0.009% with SP-(1-4), SP-(1-7), SP-free acid, neurokinin A, neurokinin B, bombesin and somatostatin.

iii. Assay protocol

^{125}I-Substance P was added to each RIA tube 2-3 h after the addition of the antiserum. Also, polypropylene (plastic) tubes were used rather than borosilicate tubes

since the latter were shown to result in a loss of measurable SP-IR due to the adsorption of peptides to glass.

iv. Calculations

The RIA data were analyzed in the same way as for somatostatin.

4. Data Analysis

The total amount of SS-IR or SP-IR per well was determined by adding the amounts extracted and released. Peptide release was calculated as a percent of total cell content (%TCC) in the following way:

$$\% \text{ TCC} = [r/(e + r])\ \times\ 100$$

where r = amount of SS-IR released
e = amount of SS-IR extracted
(e+r) = total SS-IR per well

All values are given as means ± SE and n always refers to the number of donors/dogs. The Mann Whitney U analysis was used for statistical comparisons of secretion data and differences were considered significant at the $p<0.05$ level.

To test human extract data, a one way ANOVA was utilized.

C. Results

1. Isolation and Characterization of Submucosal Cultures

a. Human

The isolation protocol described produced the highest yield of viable cells. Using less collagenase (300 U/ml) and increasing the time of digestion always resulted in more undigested tissue and a smaller yield of viable cells. Increasing the collagenase (1000 U/ml) and decreasing the time also resulted in a lower yield of viable cells. Maintaining the pH below 7.4 reduced the reaggregation and gelation of collagen. Likewise, several washing and centrifugation steps were required to remove digested collagen and reduce gelation. The post-collagenase digest consisted of single cells, clusters of cells, fragments of blood vessels, red blood cells (RBCs) and satellite cells. Following filtration and elutriation, the suspension contained no RBCs, fewer fragments, fewer single cells and less debris but individual ganglia could be seen clearly under the microscope.

Cells adhered to the collagen substrate overnight and their viability in culture remained > 90% for up to 5 days as shown by trypan blue exclusion conducted throughout this period. After 5 days, the viability decreased and the cells

became detached from the collagen. Cells aggregated around individual ganglia and there was abundant neurite outgrowth after 72 h in culture. The individual clusters of cells were linked by neurite extensions and resembled the submucosal plexus *in situ* (see canine isolation). Therefore, ICC, release experiments and HPLC extractions were carried out at this time point. An initial plating density of $1\text{-}2x10^6$ cells/ml/plate was chosen and was found to be optimal for the survival of the neurones for 72 h. At densities of $5\text{-}8 \times 10^5$ cells/ml, the majority of cells would not adhere while at densities of $3\text{-}5 \times 10^5$ cells/ml the cells would detach from the collagen substrate after 1-3 days.

The mitotic inhibitor cytosine arabinoside was included in the growth medium and effectively prevented the overgrowth of fibroblasts. A sheet of fibroblasts to support the attachment of neurones was not required with the use of plates coated with rat tail collagen.

Hematoxylin staining of sections of stripped submucosa confirmed that all mucosal and muscle tissue had been removed (not shown). Human neurones were phase bright, sprouted neurites which contained varicosities along their length and made anatomical connections to other cells after 72 h.

Human cultures contained neurones which stained for SS-IR (figure 21 a, b). In human cultures, 35 % of all cells per cluster contained SS-IR in culture, which was similar to the amount found in tissue sections. Positively stained fibres were abundant and varicosities present along the length of the fibres also contained SS-IR (figure 22 a, b, c). Submucosal neurones and fibres also contained SP-IR and VIP-IR (figure 23 a, b).

Myenteric cultures also contained neurones which stained for SS-IR (figure 24), as well as SP-IR and VIP-IR neurones. These neurones sprouted extensive neurites with varicosities which contained SS-IR, SP-IR and VIP-IR.

b. Dog

The isolation and culture of canine SMP differed from that of human tissue in several ways. The amount of collagenase and overall incubation time required to disperse the submucosa were less than for the human submucosa. This was due to a lesser amount of collagen contained in the canine submucosa.

Figure 21 a and b. High magnification micrograph of cultures of human duodenal neurones, grown on coverslips, stained for SS-IR.

x 500.

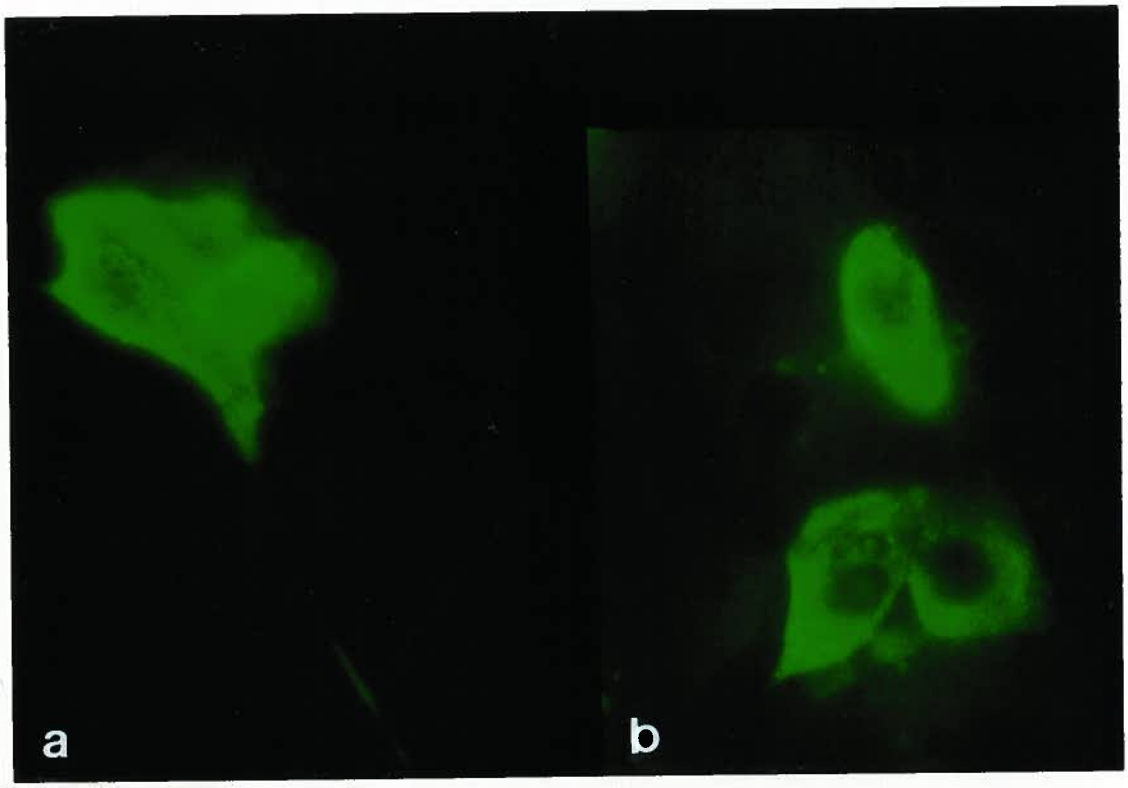
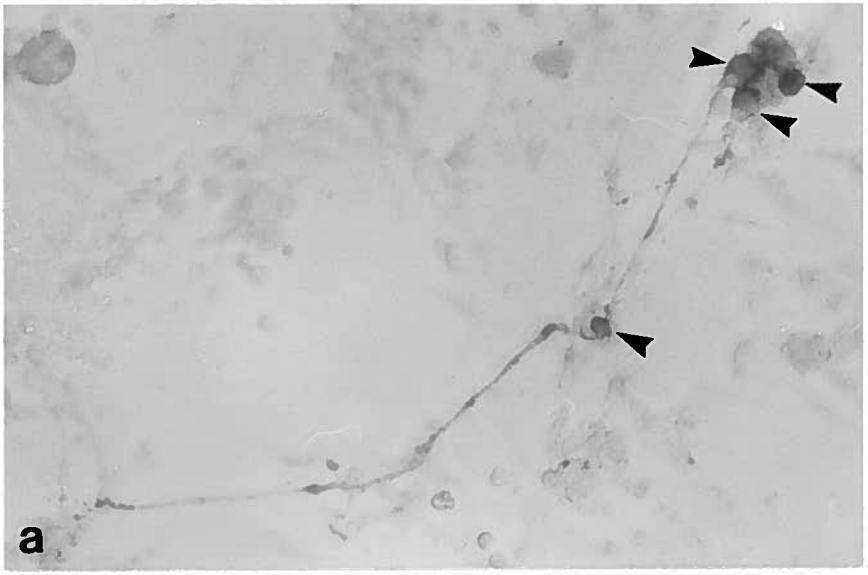

a
b

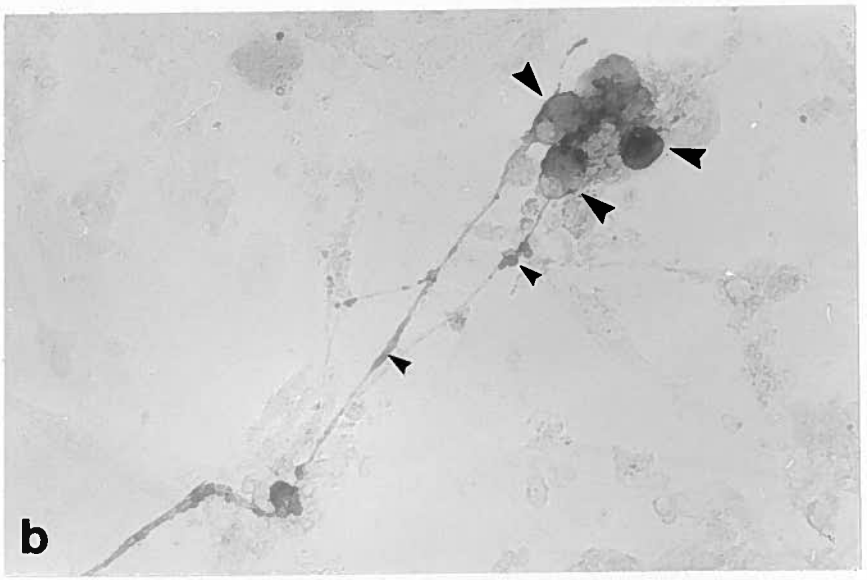
Figure 22. Cultures of human duodenal SMP stained for SS-IR. Note the presence of IR neurones (large arrows) and varicosities (small arrows).

a) x 100

b) x 200

c) x 200.


a


b

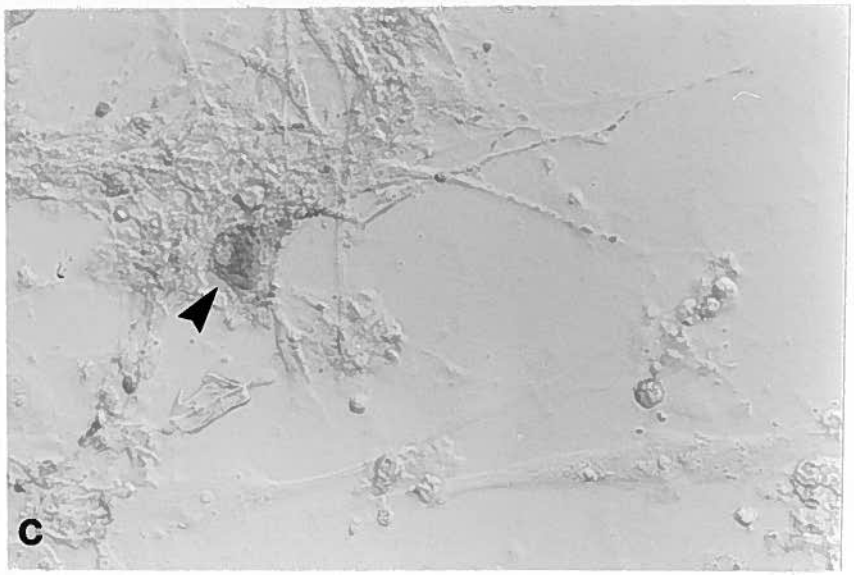

c

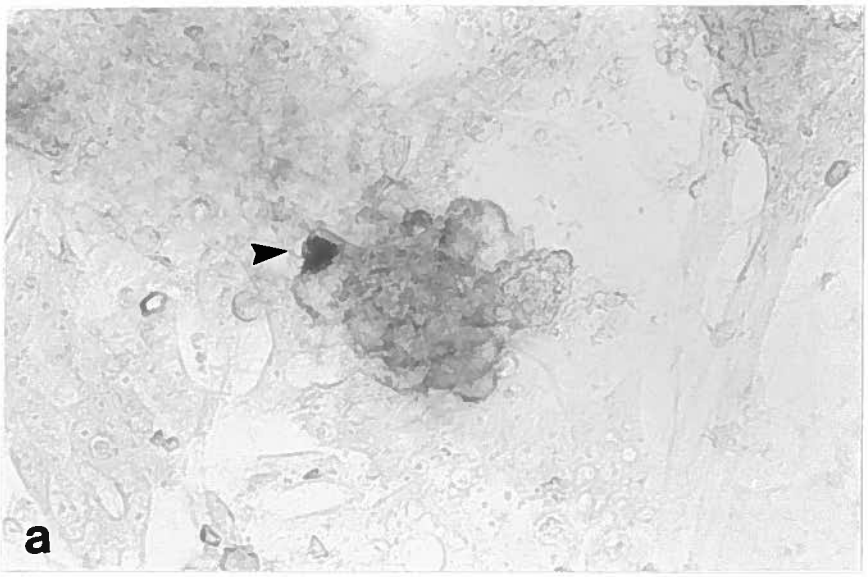
Figure 23. Immunostained cultures of human duodenal SMP.

a) SP-IR neurone (arrow).

x 200

b) VIP-IR neurones (arrow) and varicosities (small arrows)

x 200.


a

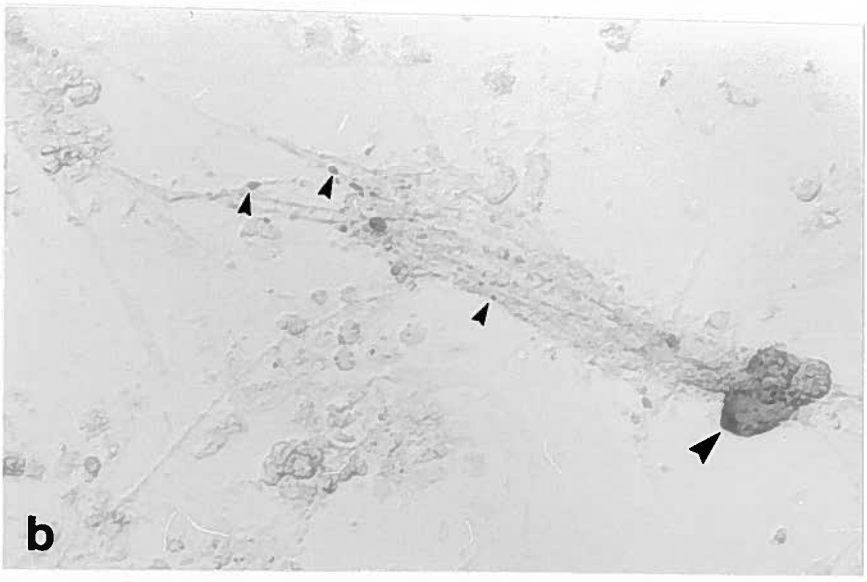
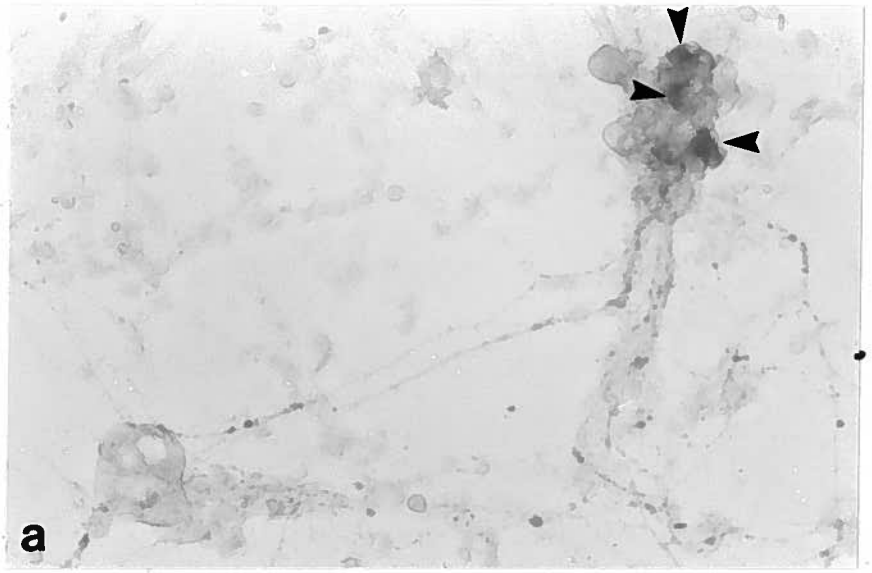
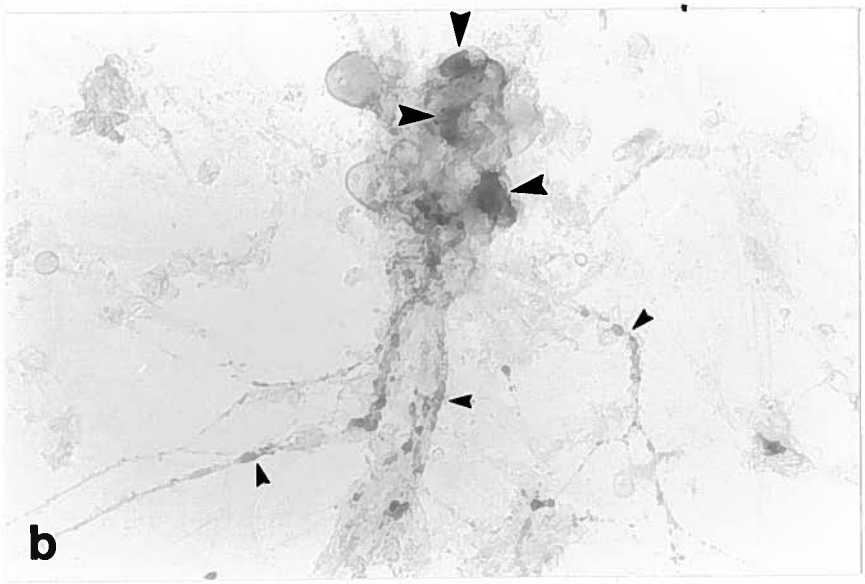

b

Figure 24. Cultures of human duodenal MYP stained for SS-IR. Note the presence of IR-neurones (large arrows) and varicosities (small arrows).

a) x 100

b) x 200.

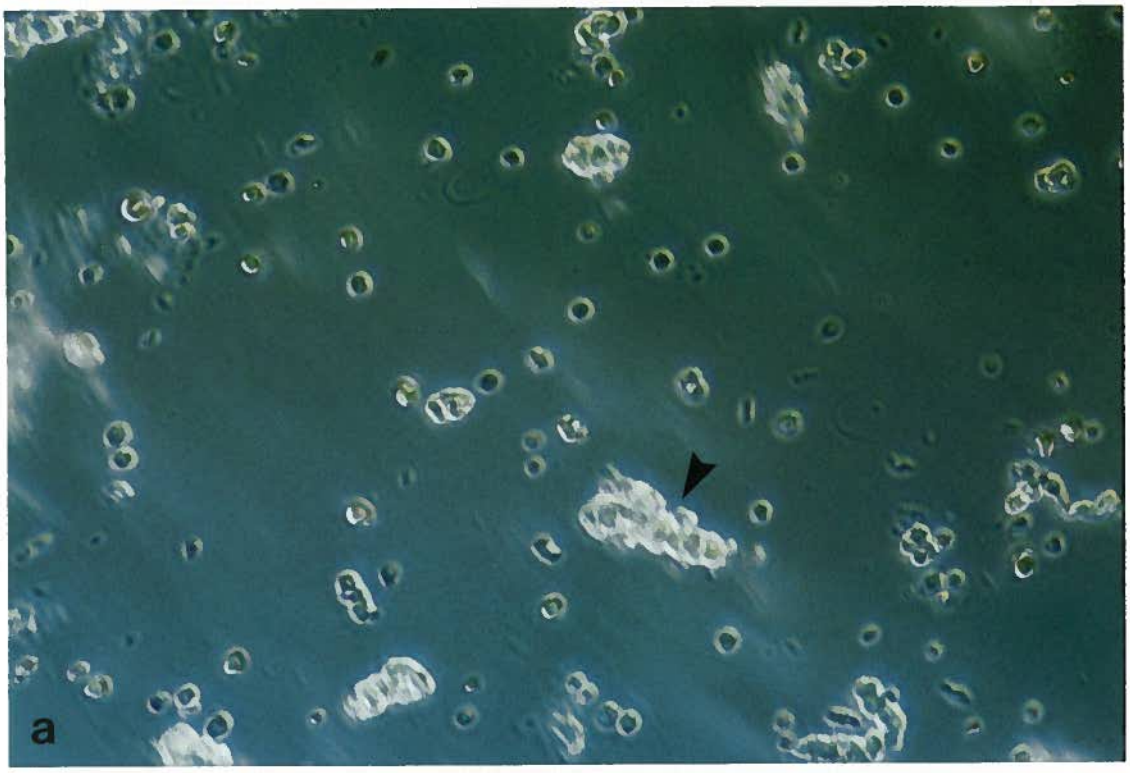

a


b

Three different protocols were tested for elutriation of canine tissue. Fraction 1 contained primarily single cells, including neurones and red blood cells. Fraction 2 contained mainly ganglia and groups of 2-5 cells. Fraction 3 was eluted according to the method used for human submucosa, and contained large fragments and large groups of cells. The viability of all fractions was > 95% immediately after elutriation, but fractions 2 and 3 contained the cells of interest. The elutriation procedure for fraction 2 was chosen for two reasons. First, only single ganglia were present in fraction 2, similar to the situation in the human cultures. Second, the cells in fraction 3 would flatten and sprout neurites but became detached from the collagen substrate after 2-3 days.

An examination of the time-course and progression of short-term cultures is shown in figure 25. Clusters were phase bright, and attached to the plates after 24 h in culture but no sprouting was observed (figure 25 a). After 72 h in culture, ganglionic structures formed and made interconnections (figure 25 b). After 120 h, many of the cells in the ganglionic structures were dead (figure 25 c). Therefore, 72 h was selected as the time when ICC and secretion experiments were carried out.

Figure 25. A time-course of the attachment and progression in short-term cultures of canine SMP neurones.
a) Day 1, 24 h after plating neurones clusters attached (arrow) but no sprouting of neurones was observed x 200.
b) Day 3, 72 h after plating, neuronal clusters formed ganglionic type structures (arrow) with interconnections x 200. c) Day 5, 120 h after plating, the majority of the cells in the ganglion-like structures were dead x 200.


a

b

c

Phase contrast microscopy revealed that the canine neurones were phase bright, sprouted neurites which contained varicosities along their length and made anatomical connections to other cells after 72 h (figure 26). Canine cultures contained neurones which stained for SS-IR (figure 27 a, b), SP-IR (figure 27 c) and VIP-IR (figure 27 d, figure 28). Positively stained fibres were observed and varicosities present along the length of the fibres also contained SS-IR and SP-IR.

Double stains of human cultures demonstrated co-localization of SS-IR and SP-IR (figure 29 a,b). Double stains of the canine cultures revealed separate populations of SS-IR and SP-IR neurones (figure 29 c,d). Thus, the neurones in culture displayed the same phenotype as neurones *in situ* with respect to SP-IR and SS-IR. Interestingly, the canine neurones also demonstrated the segregation of neuronal types normally observed in neuronal ganglia *in situ*.

Figure 26. Phase contrast micrograph of canine submucosal neurones. Note the presence of phase bright neuronal cell bodies (large arrows) and extensive sprouting of neurites containing varicosities along their length (small arrows) x 200.

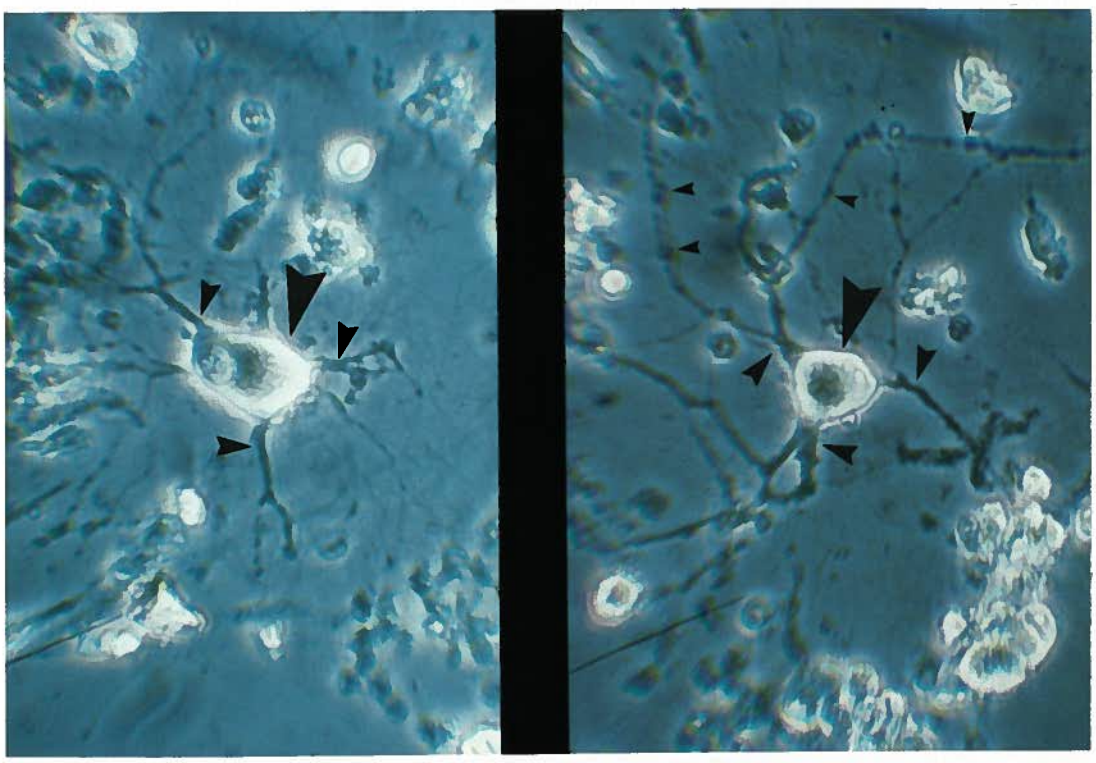


Figure 27. Immunostained cultures of canine SMP.

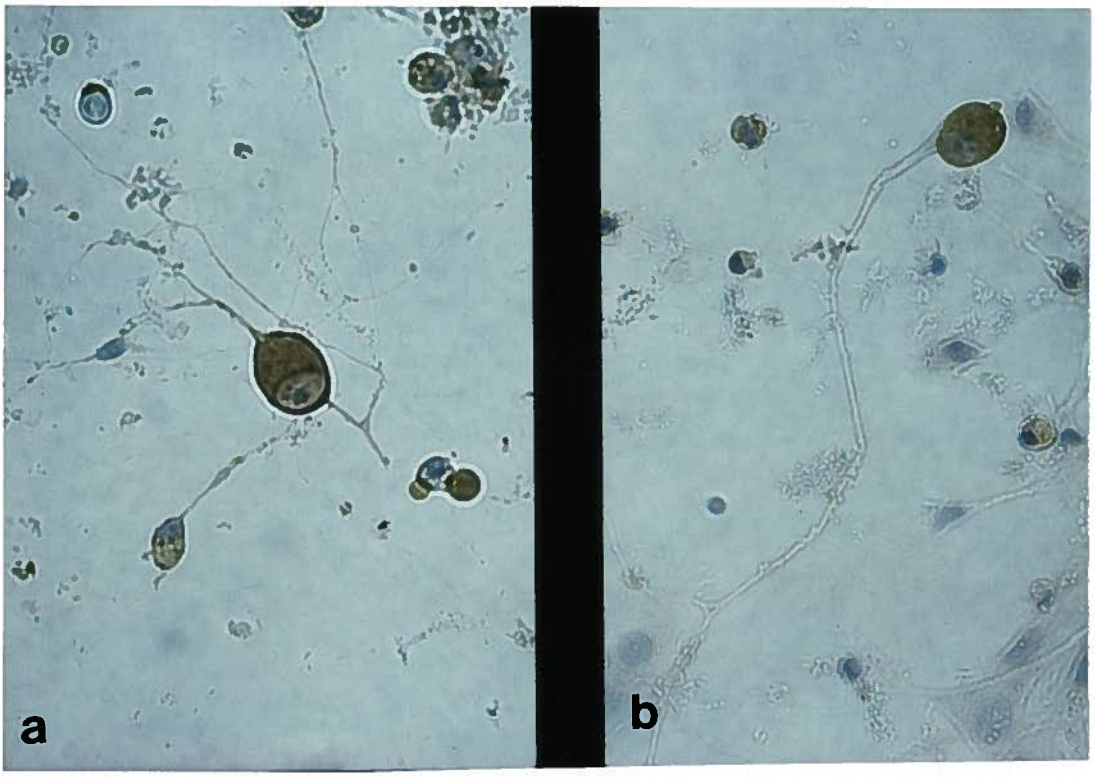
a) SS-IR neurones x 200

b) SS-IR neurones x 200

c) SP-IR neurones x 200

d) VIP-IR neurones x 200.

Note the extensive sprouting of neurites.


a
b

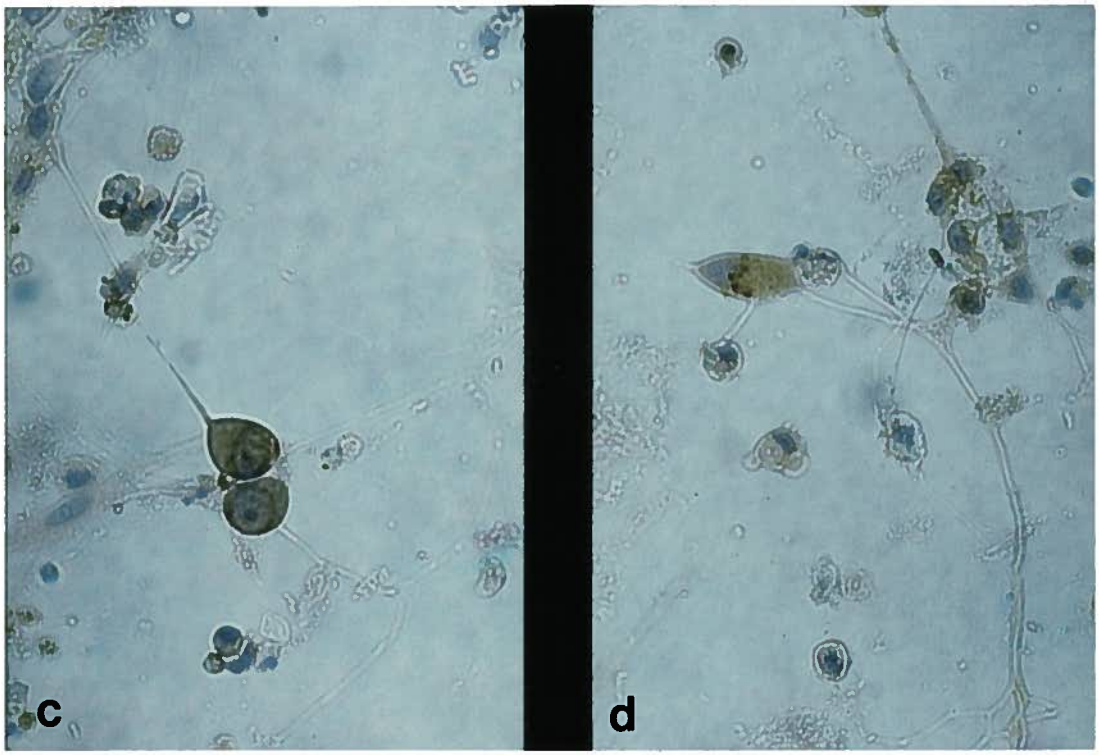

c
d

Figure 28. Cultures of canine SMP neurones stained for VIP-IR (arrows), which are located within ganglion-like clusters of cells

x 100

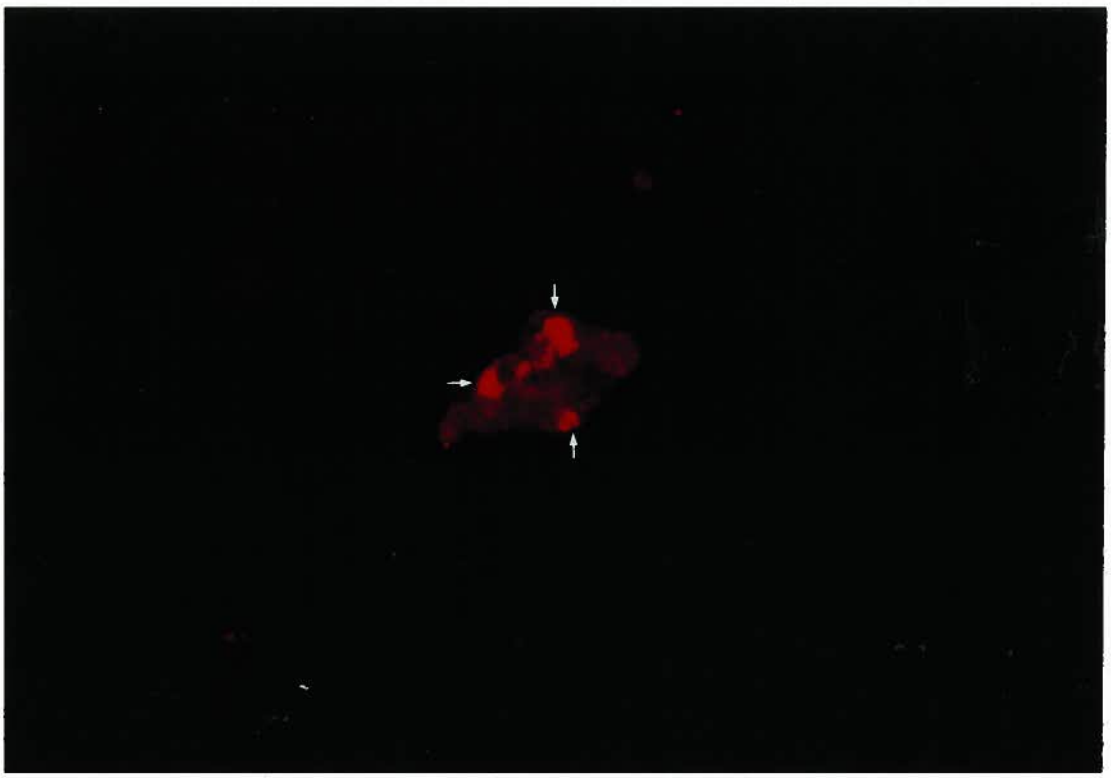
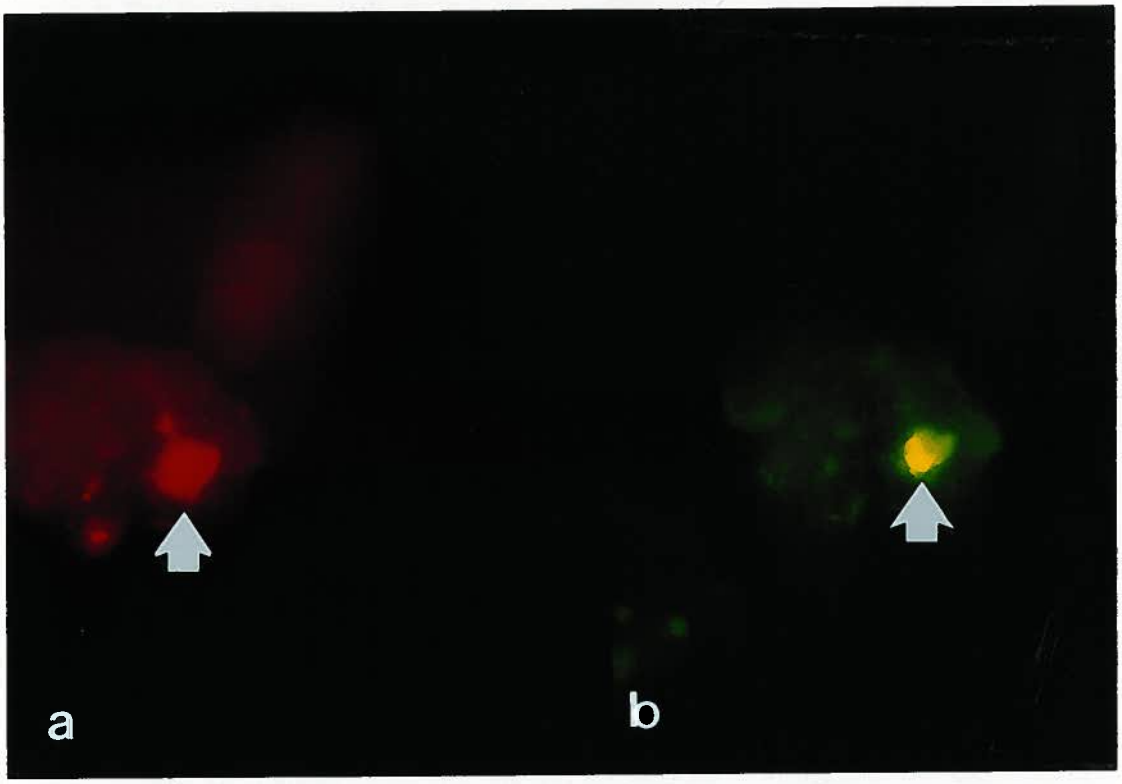
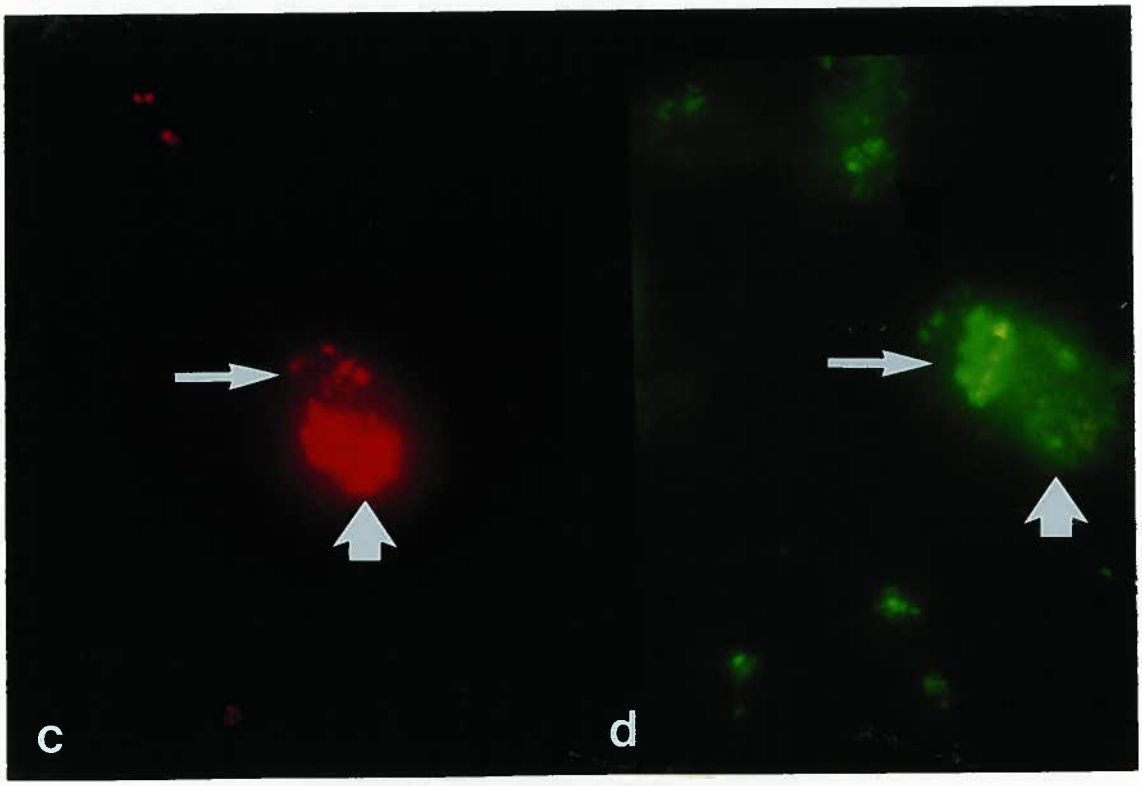


Figure 29. Double stains of human and canine cultures of SMP for SP-IR (Rhodamine) and SS-IR (FITC).

a) Human SP-IR neurones and b) SS-IR neurones (large arrows) x 200. Note the co-localization of SS-IR and SP-IR

c) Canine SP-IR neurones (large arrows) and d) SS-IR neurones (small arrows) x 100. Note the lack of co-localization. Also, note the grouping of each type of neurone reminiscent of what is observed *in situ*.


a
b


c
d

2. Somatostatin Secretion from Human Submucosal Neurones.

a. Effects of Secretagogues

The addition of ß-PMA at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M caused significant increases in the release of SS-IR (figure 30). Increasing the potassium concentration from 5 to 10 mM resulted in a further increase in the mean value of SS-IR released in response to ß-PMA, but this was not statistically significant (figure 31). The basal level of SS-IR over the two hour time period was 16 ± 6 (n=6) and 24 ± 4 (n=11) pg/600μl using release medium with and without added KCl, respectively. Basal release of SS-IR could be measured only after 120 minutes. The variability of SS-IR release in response to ß-PMA and 10 mM KCl was determined in 3 wells and was found to be < 5% (n=3). Variation in basal release levels was found to be < 3% between wells (n=6).

Of the total SS-IR released in response to ß-PMA (10^{-6} M, 10 mM KCl) after 120 min, 59% was present in the medium after 30 min, and 80% was present after 60 min (figure 32) (n=3).

Figure 30. Release of somatostatin immunoreactivity (SS-IR) as a percent of total cell content (% TCC) from submucosal neurones in response to incubation with ß-PMA for 120 min (n = 6 donors). "C" is the basal level of SS-IR after 120 min. Values are means ± S.E.

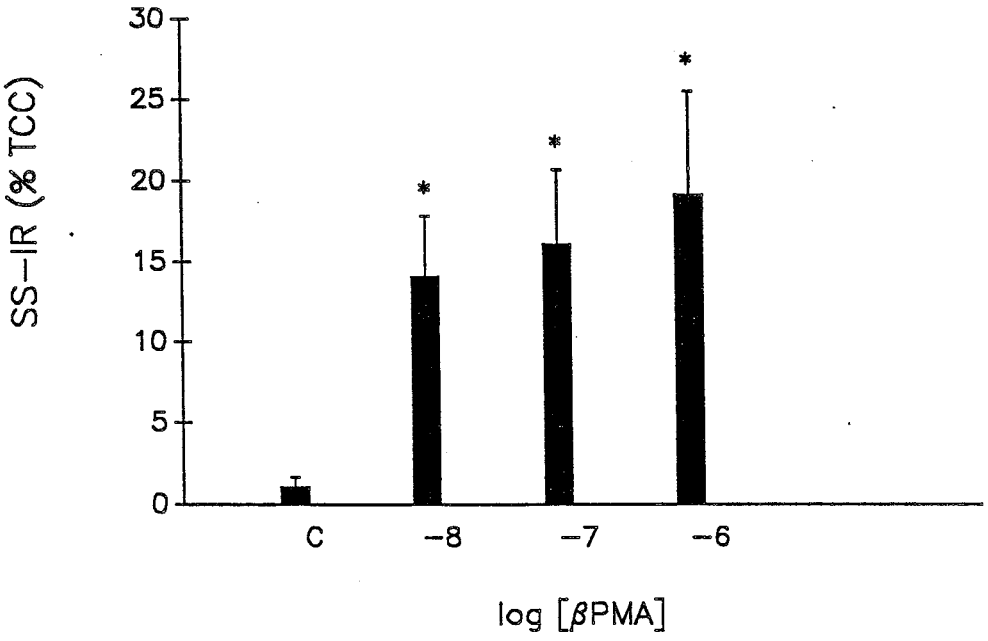

30
25
20
15
10
5
0
SS-IR (% TCC)
*
*
*
C
-8
-7
-6
log [βPMA]

Figure 31. Release of SS-IR (% TCC) from submucosal neurones in response to incubation with ß-PMA for 120 min in 5 mM and 10 mM KCl (n=6 donors). Values are means ± S.E. *Significantly different from basal release ($p \leq 0.05$).

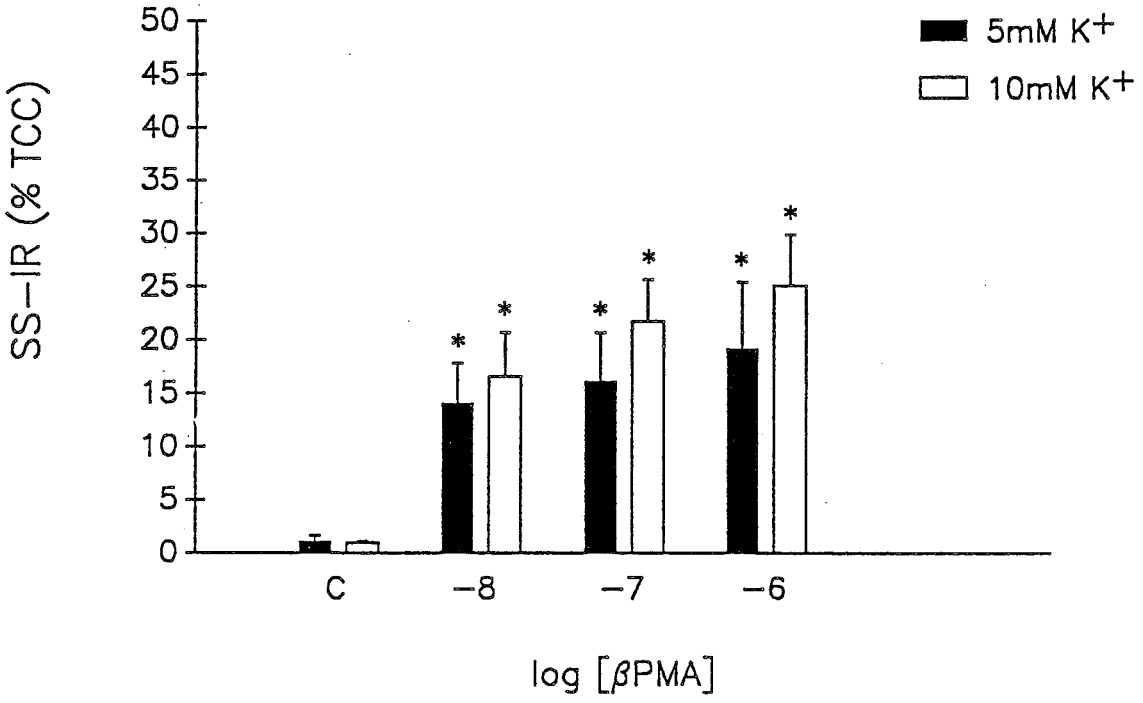
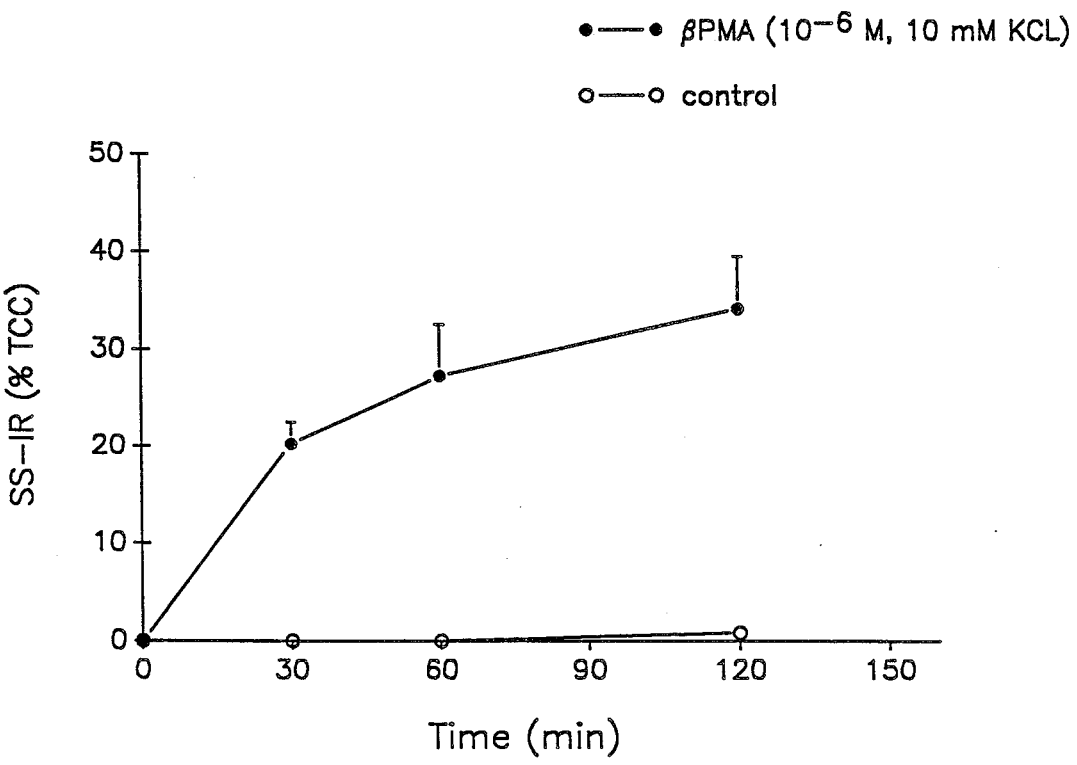

SS-IR (% TCC)
50
45
40
35
30
25
20
15
10
5
0
5mM K+
10mM K+
*
C
−8
−7
−6
log [βPMA]

Figure 32. Release of SS-IR (% TCC) from submucosal neurones in response to ß-PMA (10^{-6} M, 10 mM KCl) after 30, 60 and 120 min (n = 3 donors). Control values represent the amounts of SS-IR released in the absence of ß-PMA over 120 min. Values are ± means S.E.


βPMA (10^{-6} M, 10 mM KCL)
control
SS-IR (% TCC)
50
40
30
20
10
0
0
30
60
90
120
150
Time (min)

The addition of the inactive 4α-phorbol at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M did not cause significant increases in the release of SS-IR (figure 33) (n=3).

The addition of substance P did not cause any increase in SS-IR release in comparison to basal (figure 34). The addition of SP in the presence of hexamethonium (10^{-6} M) and atropine (10^{-6} M) did not cause any change in the SS-IR secretion, but did result in more variation of the basal levels (figure 35). The addition of SP (10^{-7} M) did not affect ß-PMA stimulated SS-IR release (figure 36).

The effects of tetrodotoxin (TTX, 10^{-6} M) on ß-PMA (10^{-6} M) -stimulated SS-IR release were examined in one experiment (figure 3 (figure 37). The secretion of SS-IR in response was partly attenuated by TTX.

The effects of CGRP 10^{-6} M and methacholine 10^{-6} M on SS-IR release were examined in one experiment (figure 38). The secretion of SS-IR was stimulated by CGRP, but not affected by methacholine. Methacholine attenuated CGRP-induced secretion of SS-IR.

The calcium ionophore (10-6 M, 5 x 10-6 M) was tested in two donors and it was found to not have any effect on SS-IR secretion (Table 9).

Figure 33. Release of SS-IR (% TCC) from submucosal neurones in response to incubation with ß-PMA and the inactive 4α-phorbol for 120 min (n = 3 donors). Values are means ± S.E.

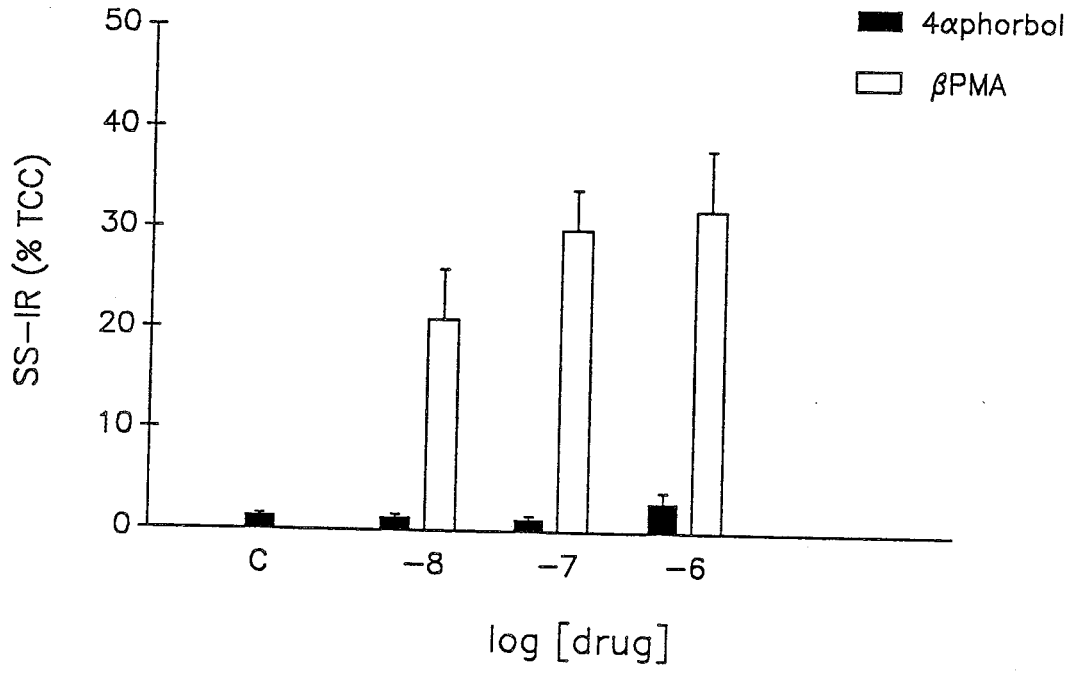

4αphorbol
βPMA
SS-IR (% TCC)
50
40
30
20
10
0
C
−8
−7
−6
log [drug]

Figure 34. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with substance P for 120 min (n = 4 donors). Values are means ± S.E.

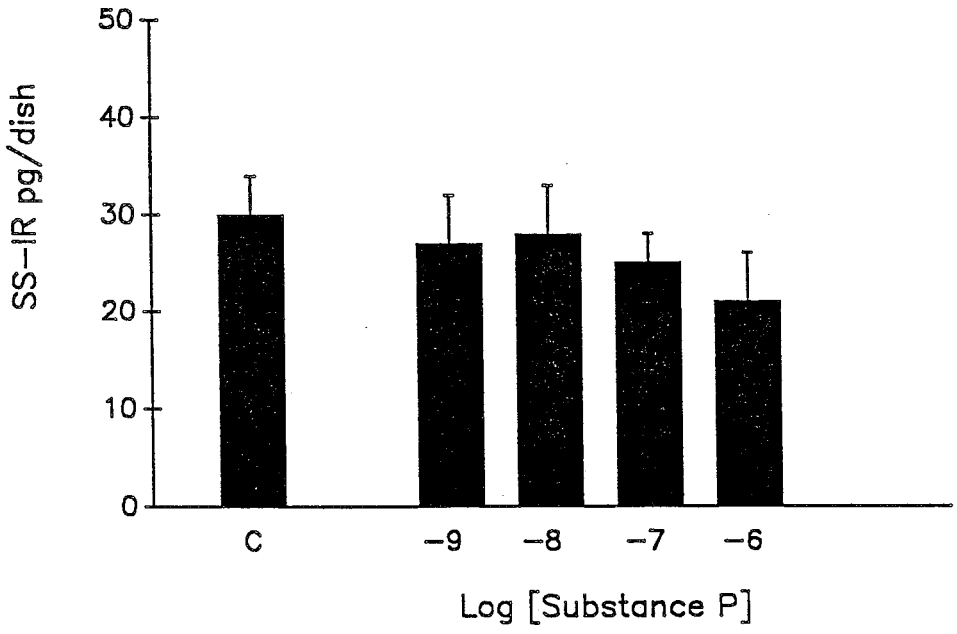

50
40
30
20
10
0
SS-IR pg/dish
C
-9
-8
-7
-6
Log [Substance P]

Figure 35. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with substance P for 120 min in the presence or absence of hexamethonium (hex) and atropine (atr) (n = 4 donors). Values are means ± S.E.

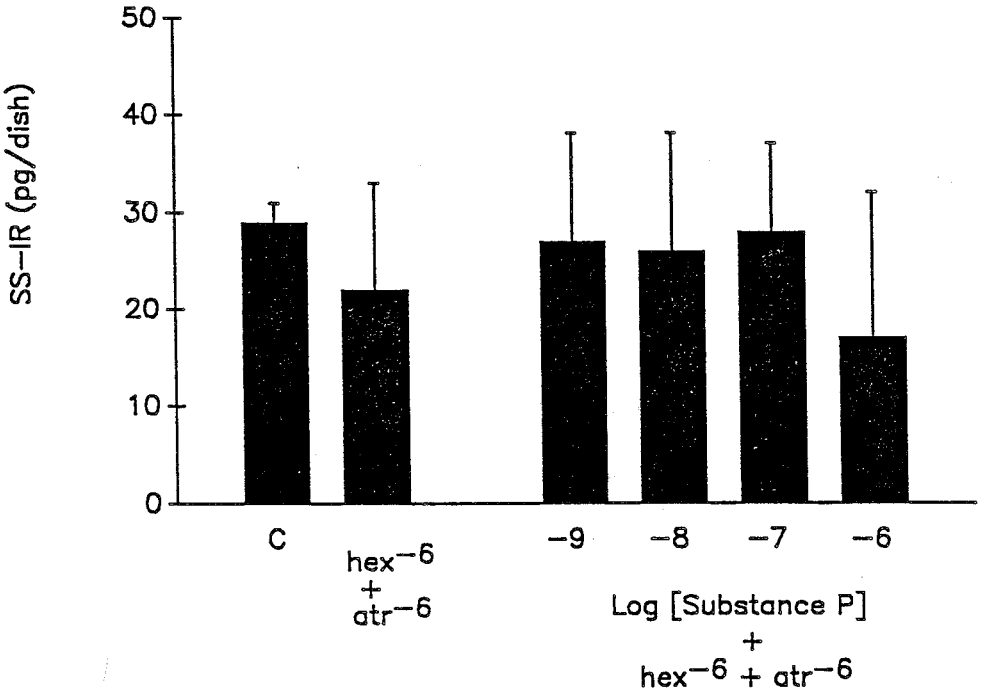

SS-IR (pg/dish)
50
40
30
20
10
0
C
hex−6 + atr−6
−9
−8
−7
−6
Log [Substance P] + hex−6 + atr−6

Figure 36. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with ß-PMA for 120 min in the presence or absence of substance P (n = 3 donors). Values are means ± S.E.

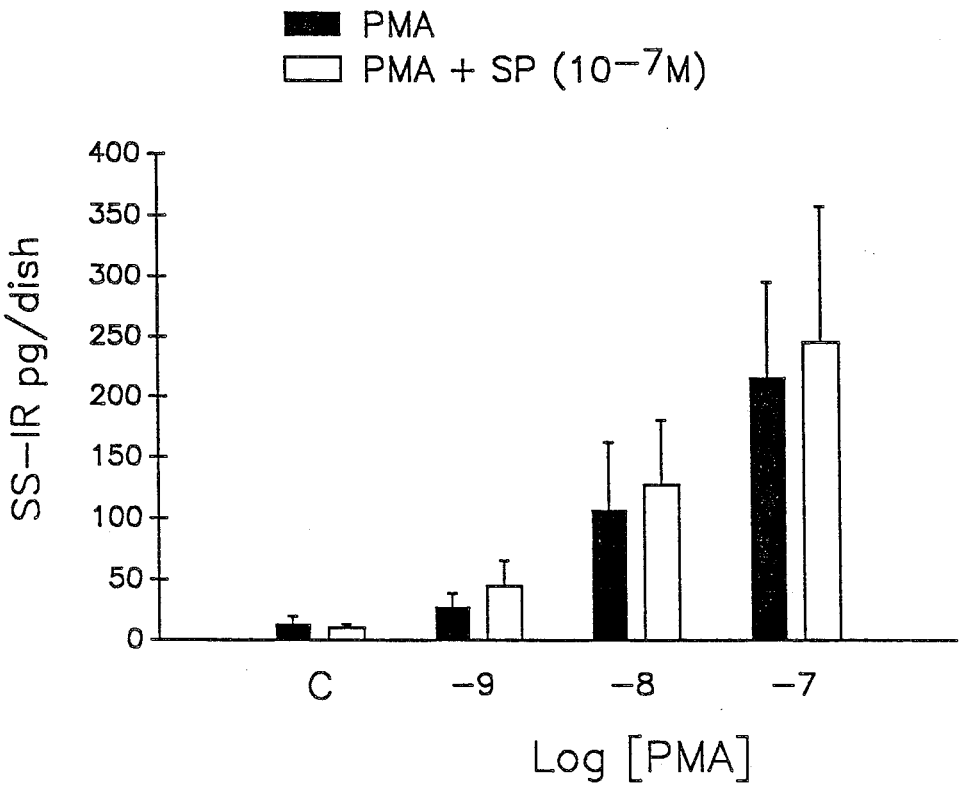

PMA
PMA + SP (10−7M)
SS−IR pg/dish
400
350
300
250
200
150
100
50
0
C
−9
−8
−7
Log [PMA]

Figure 37. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with ß-PMA for 120 min in the presence or absence of TTX (n = 1 donor).

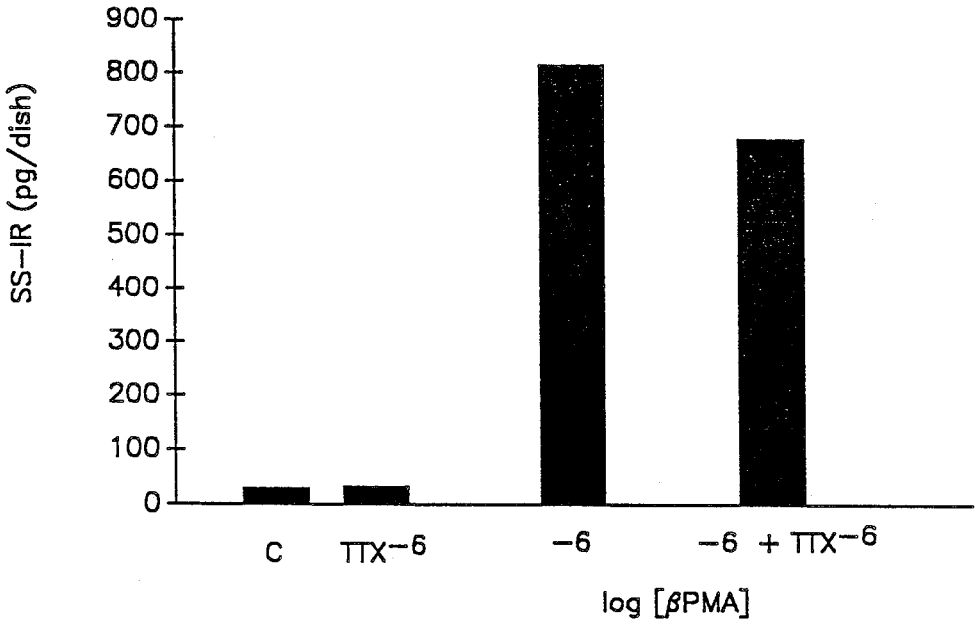

900
800
700
600
500
400
300
200
100
0
SS-IR (pg/dish)
C
TTX−6
−6
−6 + TTX−6
log [βPMA]

Figure 38. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with CGRP and methacholine for 120 min (n = 1 donor).

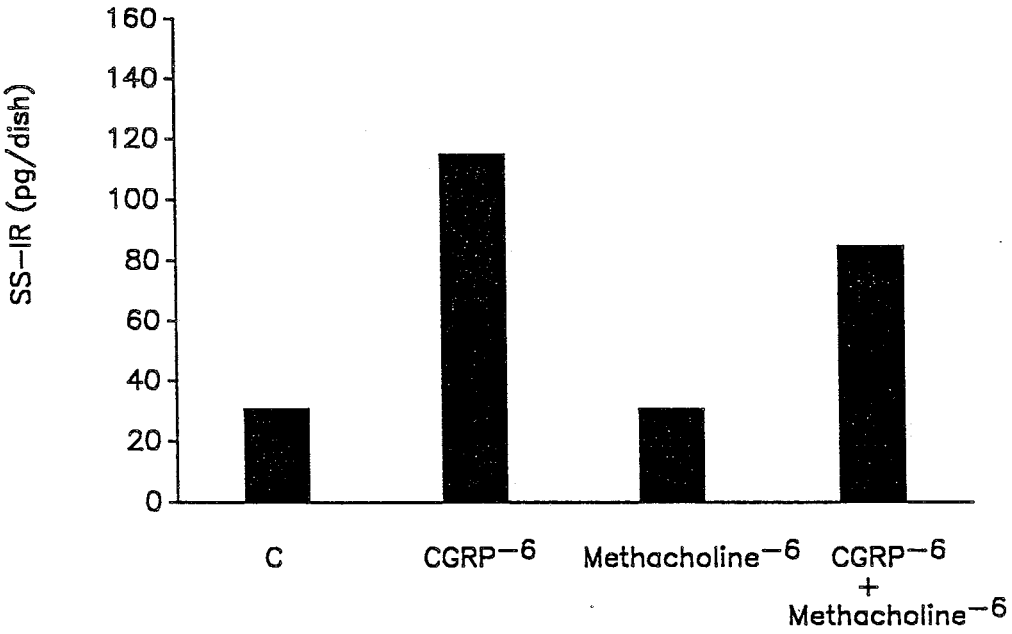

160
140
120
100
80
60
40
20
0
SS-IR (pg/dish)
C
CGRP−6
Methacholine−6
CGRP−6
+
Methacholine−6

Table 9. The effect of the calcium ionophore (A23187) on SS-IR secretion

	Somatostatin secretion (pg/dish)
control	21
A23187 10-6 M	22
" 5 x 10^{-6} M	39

The effects of carbachol (10^{-7} - 10^{-5} M) were tested in one donor (figure 39) and it appeared to increase the secretion of SS-IR.

b. Somatostatin Content (Table 9).

The amount of SS-IR per well was found to be 1990 ± 809 per well; however the variability of SS-IR content between wells within each donor was found to be < 3% (n = 6 donors), (Table 10). Also, incubation with different concentrations of ß-PMA, with or without increased KCL, did not significantly alter SS-IR content (n=6).

c. Somatostatin Recovery.

The recovery of SS-IR from release medium after incubation with the cultures was greater than 95% and after extraction with 2 N acetic acid was greater than 90%.

d. Characterization of molecular forms.

The majority (> 90%) of SS-IR in acid extracts of the neurones in culture and released in response to ß-PMA eluted with the same retention time as synthetic SS-14 on HPLC (figure 40, 41).

Figure 39. Release of SS-IR (pg/dish) from submucosal neurones in response to incubation with ß-PMA and carbachol for 120 min (n = 1 donor).

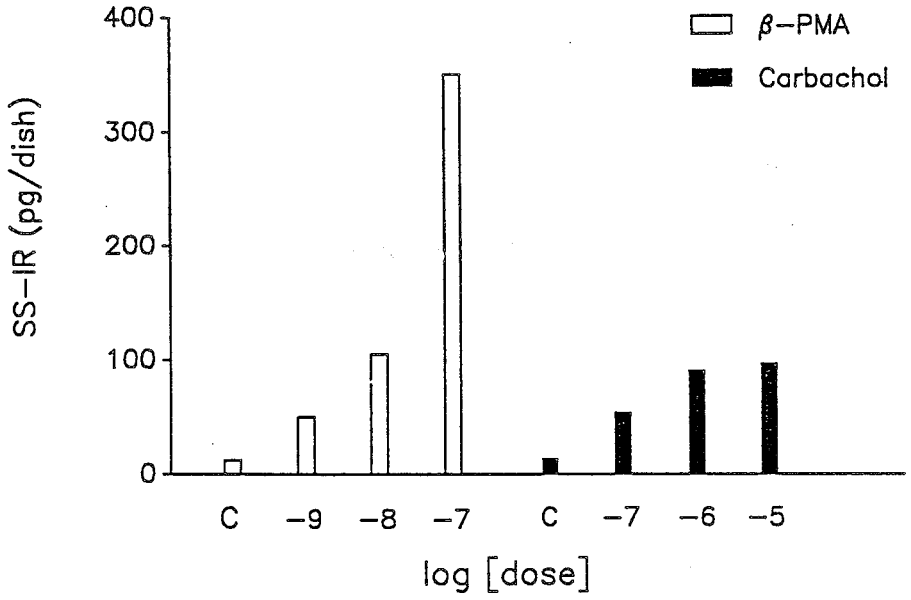

400
300
200
100
0
SS-IR (pg/dish)
β-PMA
Carbachol
C
−9
−8
−7
C
−7
−6
−5
log [dose]

Table 10. Variations in content of somatostatin immunoreactivity

	Somatostatin content of extracts (pg/dish)
control	3359 ± 1441
10 mM KCl	3131 ± 1253
ß-PMA 10^{-6} M	3483 ± 1429
10^{-7} M	3221 ± 1259
10^{-8} M	3276 ± 1332
10^{-6} M, 10 mM KCl	3475 ± 1367
10^{-7} M "	3286 ± 1327
10^{-8} M "	3307 ± 1444

Figure 40. HPLC profile of SS-IR released from submucosal neurones in response to ß-PMA (10^{-6} M). Sample represents pooled medium from 6 wells. SS-28 and SS-14 markers indicate the elution position of synthetic peptide.

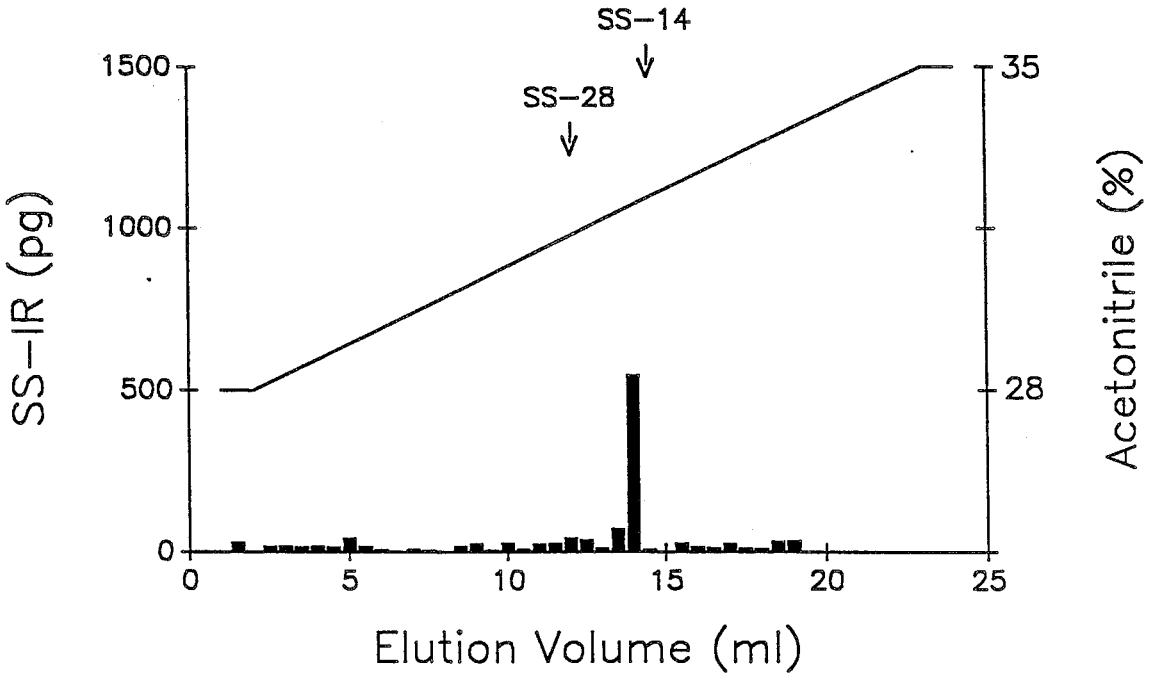
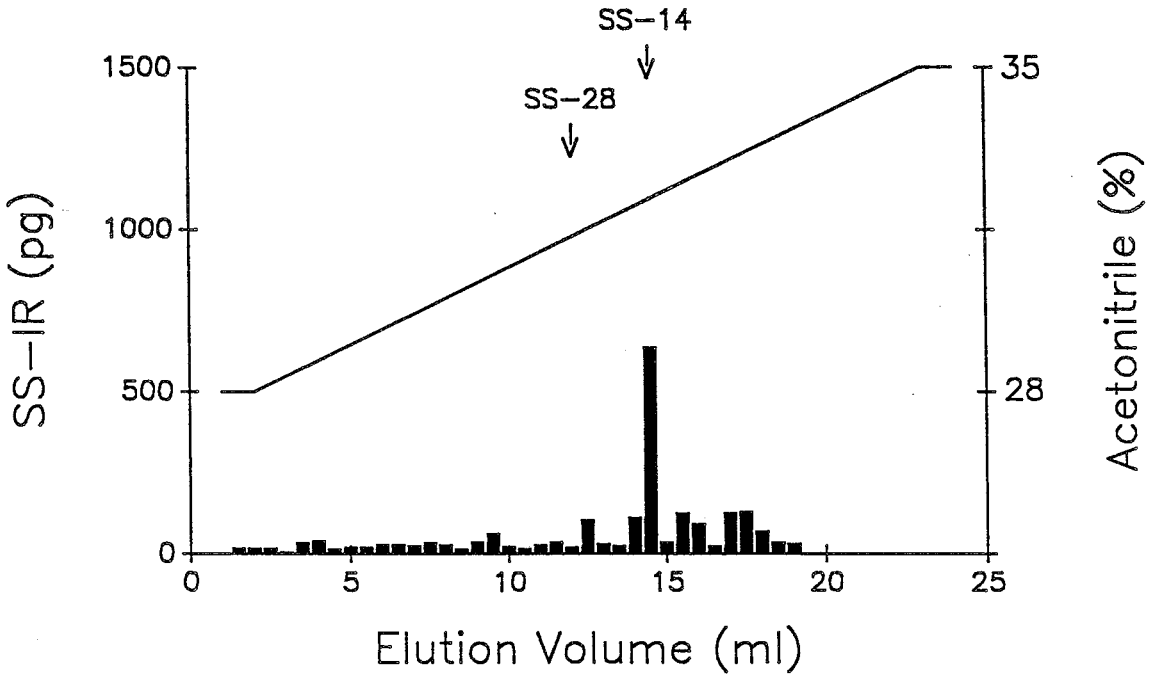

SS–14
SS–28
1500
1000
500
0
SS–IR (pg)
35
28
Acetonitrile (%)
0
5
10
15
20
25
Elution Volume (ml)

Figure 41. HPLC profile of SS-IR contained in acetic acid extracts in response to ß-PMA (10^{-6} M). Sample represents pooled extracts from 2 control wells. SS-28 and SS-14 markers indicate the elution position of synthetic peptide.


SS–14
SS–28
1500
1000
500
0
SS–IR (pg)
35
28
Acetonitrile (%)
0
5
10
15
20
25
Elution Volume (ml)

3. Somatostatin and Substance P Secretion from Canine Submucosal Neurones.

SS-IR and SP-IR were extracted according to the protocol used for SP-IR previously (Kwok and McIntosh, 1980) and were measurable by RIA. The total cell content of SS-IR was 1200 ± 210 pg/well and for SP-IR was 810 ± 107 pg/well (n=3). The basal release of SP-IR was 4.5 ± 0.4 (% TCC) and that for SS-IR was 8.1 ± 1.5 (% TCC) (n=3). The recovery of SS-IR was found to be > 90% using this procedure. The recovery of SP-IR was > 95 % with or without the presence of bacitracin and captopril. Depolarization by 55 mM K^+ did not increase SP-IR secretion (n=3).

D. Discussion

In the present study, submucosal neurones from human and canine small intestine were isolated and maintained in tissue culture and the localization and release of SS-IR were examined. A vigorous digestion procedure was required to obtain a high yield of neuronal ganglia from human tissue. Gelation and reaggregation of collagen occurred due to the large amount of collagen present in the human submucosa. Dimeric collagen has been shown to undergo a reversible gelation as temperature and pH increase (with significant changes in turbidity at pH=7.4 and T=28^{o}C), the process also being dependent on the concentration of free collagen (Yurchenko and Furthmayr, 1984). Therefore, the pH of the washing and incubation medium was kept below 7.4, and the cells were washed and centrifuged to reduce the concentration of collagen. If these procedures were not followed, the yield of viable neurones was insufficient to perform secretion studies. The method of short-term culture described was adapted from previous work carried out using canine small intestine (Barber *et al*, 1986; Buchan *et al*, 1989). In order to obtain neuronal ganglia, human tissue required a more vigourous digestion than canine tissue. A thicker submucosa in the human small intestine was the primary reason for this difference.

The average yield of neurones was two 12 well plates at a concentration of 1-2 x 10^6 cells/well. This meant that only 12 conditions could be tested in duplicate for each experiment.

The human and canine submucosal neurones attached overnight, were phase bright as demonstrated by phase contrast microscopy, sprouted processes which contained varicosities along their length and made anatomical connections with other cells. This indicated that after isolation and culture the neurones were viable and did not alter their normal morphologic phenotype. Time-course studies of canine cultures demonstrated that the cells in culture formed aggregates after 72 h in culture. This type of aggregate formation has been observed previously in explants of enteric neurones maintained in tissue culture (Jessen *et al*, 1983). Electron microscopic examination of the aggregates examined by these authors showed that the cellular contacts inside of them were similar to those seen in enteric ganglia *in situ*.

The staining pattern of SS-IR in human cultured submucosal neurones was similar to that observed in tissue sections, suggesting that peptide localization was unchanged after isolation and culture. Nerve fibres and varicosities positively staining for SS-IR and SP-IR were commonly observed in the cultures. The more intense staining of SS-

IR fibres in culture probably relates to the lack of obscuring collagen which surrounds the neurones *in situ*.

Somatostatin and SP-IR were co-localized in human, but not canine, submucosal neurones which parallels the location of these neuropeptides in tissue sections. Thus, the isolation of the ganglia and maintenance of submucosal neurones in tissue culture did not alter the expression of SP-IR and SS-IR.

The results presented have shown for the first time that activation of PKC by ß-PMA markedly stimulates the release of SS-IR from human enteric neurones. Release of SS-IR by activation of PKC using phorbol esters has been reported with dispersed cultures of fetal rat hypothalamus and cortex (Peterfreund and Vale, 1983) and canine jejunal submucosa (Buchan *et al*, 1989).

The predominant molecular form of SS-IR present in acid extracts of the cultures was SS-14. This supports the work of other groups that SS-14 is the the major molecular form in acid extracts of human enteric nerves (Baldissera *et al*, 1985; Keast *et al*, 1986; Penman *et al*, 1983) Furthermore, the predominant form of SS-IR released in response to ß-PMA was also found to elute with the same retention time as SS-14 using HPLC. Central neurones have been shown to contain and release SS-14 (Bonanno *et al*, 1988). Somatostatin

endocrine cells of the human gut have been shown to contain predominantly SS-28 (Baskin and Ensinck, 1984). The lack of measurable SS-28 in the human submucosal cultures further indicated the absence of endocrine cell contamination.

The release of SS-IR by ß-PMA was not due to non-specific membrane effects since the inactive 4α-phorbol did not significantly alter basal release. The secretion of SS-IR was probably due to activation of protein kinase C (Nishizuka, 1986) although effects other than PKC activation cannot be ruled out (Castagna *et al*, 1982).

The release of SS-IR in response to ß-PMA (10^{-6} M with 10 mM KCl) reached a plateau over the incubation period so that 59% of the SS-IR released after 120 minutes was present after 30 min and 80% of the total SS-IR released was present after one hour. Therefore, the rate of release of SS-IR decreased after the first 30 minutes of the incubation period. The decrease in SS-IR release may have been due to depletion of SS-IR from the cells, down-regulation of protein kinase C or autocrine regulation by SS. Autocrine regulation of SS has been demonstrated in.the pancreas and stomach (McIntosh, 1985). Phorbol esters have been shown to down-regulate PKC activity involved in norepinephrine release from rat brain synaptosomes (Oda *et al*, 1991). The release period was extended to 120 min to allow measurement

of basal SS-IR levels which were not detectable at earlier periods.

To examine whether neuronal depolarization would augment basal or phorbol ester-stimulated SS-IR secretion, the extracellular concentration of potassium was doubled to 10mM, which should have caused a small but sustained depolarization of neurones. Basal release of SS-IR was not increased, implying that attenuation of membrane polarity is not sufficient to generate release. However, there was an indication that the neurones were more sensitive to stimulation by ß-PMA after potassium depolarization although this effect was not statistically significant. Furthermore, preliminary experiments have demonstrated that TTX only partly attenuated ß-PMA-stimulated release of SS-IR, which suggests a mechanism only partly dependent on membrane depolarization. Previous studies demonstrated that even high levels of potassium (> 50 mM) did not evoke the release of vasoactive intestinal peptide or calcitonin gene-related peptide from enteric nerves (Belai *et al*, 1987; Belai and Burnstock, 1988; Besson *et al*, 1983). The results presented in this thesis imply that exocytosis of peptide-containing vesicles requires the activation of second messenger cascades (e.g. protein kinase C activation) in addition to membrane depolarization.

The release of SS-IR by ß-PMA from human neurones was not affected by the presence of the calcium ionophore A23187, unlike canine neurones in which stimulation of SS-IR secretion by ß-PMA occurred only in the presence of the ionophore (Buchan *et al*, 1989) (see Table 11). Interestingly, the basal secretion of SS-IR in the dog was 2-3 fold higher than that in the human cultures suggesting that there was a tonic basal stimulation of SS-IR secretion. It is probable that due to this background stimulation, the canine neurones required both activation of PKC (by ß-PMA) and influx of calcium (by A23187) to further stimulate SS-IR release.

The calcium ionophore on its own had no effect on SS-IR secretion in human cultures as was the case in canine neurones (Buchan *et al*, 1990). The ability of the calcium ionophore to stimulate the release of NT from canine cultures has suggested that different mechanisms must be involved in the release of different peptides.

It is possible that ß-PMA affected the release of other neurotransmitters present in the cultures which in turn may have altered the release of SS-IR. For example, SP has been shown to release NT from canine submucosal neurones (Barber *et al*, 1989). The release of SP by ß-PMA presumably would not have affected the release of SS-IR, since the present results have shown that exogenous SP had no effect. The

release of other neuropeptides from similar cultures in response to ß-PMA remains to be determined.

While SP-IR neurones were present in human submucosal cultures, the addition of exogenous SP did not inhibit SS-IR release. Substance P has been found to inhibit SS-IR release from neurones of canine submucosal cultures and endocrine cells of the perfused rat stomach (Buchan *et al*, 1990; Kwok *et al*, 1988) (see Table 11). Conversely, substance P has been shown to stimulate the release of SS-IR from the hypothalamus and pancreas (Reichlin, 1981). The variation in SP effect and co-localization of SP-IR and SS-IR observed in the human but not dog may reflect an underlying difference in the regulation of SS-IR secretion.

Different effects of SP, on canine versus human submucosal neurones, were probably due to a combination of direct and indirect effects on SS-IR containing neurones. Support for direct SP effects on submucosal neurones comes from experiments using isolated mucosa/submucosa preparations which have shown that SP-mediated increases in the secretion of Cl^- ions were TTX-sensitive (Keast *et al*, 1985; Perdue *et al*, 1987). In addition, substance P has been shown to release neurotransmitters such as acetylcholine from guinea pig myenteric neurones (Wiley and Owang *et al*, 1987) and

Table 11. Similarities and differences in the secretion of somatostatin immunoreactivity from canine and human submucosal neurones

	* Dog	Human
ß-PMA	no effect	increase
ß-PMA + A23187	increase	# increase
SP	decrease	no effect
SP + PMA	?	no effect

* Buchan et al, 1990

\# Data not shown

neurotensin from canine submucosal neurones (Barber *et al*, 1989).

The signal transduction mechanism for the SP-mediated slow EPSP in myenteric neurones was shown not to involve cAMP (Palmer *et al*, 1987) but substance P has been shown to increase levels of intracellular calcium in dorsal horn neurones and pancreatic acinar cells (Womack *et al*, 1988; Gallacher *et al*, 1990). In both cell types, SP was suggested to increase cytoplasmic calcium by activating protein kinase C. The activation of PKC has been shown to stimulate SS-IR secretion from a variety of neurones including submucosal neurones in this study.

Substance P has been shown to produce a slow EPSP in myenteric nerves (Katayama *et al*, 1979; Willard, 1989) and submucosal nerves (Surprenant, 1984; Mihara *et al*, 1985). The electrophysiological effects of SP in combination with its stimulatory effects on PKC activation and neurotransmitter secretion suggest that a direct action of SP on SS-IR containing neurones would have caused stimulation of SS-IR secretion. The data obtained in the present human study and the results previously shown in the dog do not support a direct action of SP on SS-IR containing neurones.

An indirect action of SP caused by the concommitant release of another neurotransmitter, for example Ach, is a more probable explanation for the results observed in both canine and human neuronal cultures. The release of Ach from enteric neurones by SP has been demonstrated (Keast *et al*, 1985) and also provides a possible explanation for differences in the effects of SP on SS-IR secretion between canine and human cultures. The release of various transmitters suggests that SP may have both excitatory and inhibitory effects (see section below on effects of Ach) which could be why no SP-effect was observed in the human cultures.

Acetylcholine has been shown to excite enteric neurones (Wood, 1970; North and Nishi, 1976). These neurones exhibited a postsynaptic fast nicotinic EPSP (Nishi and North, 1973; Hirst *et al*, 1974; Surprenant, 1984) and a slow EPSP by activation of a M_1 receptor (North *et al*, 1985). In addition, Ach was shown to produce a presynaptic IPSP by activation of a M_2 receptor (North and Tokimasa, 1982) which also inhibits the release of Ach and non-cholinergic neurotransmitters (Morita *et al*, 1982). The effects of Ach release on SS-IR secretion would then depend on the number and distribution of different cholinoceptor types. Further, cholinergic effects would depend on the anatomical relationship between SS-IR and Ach containing neurones. The results of this study do not provide evidence for a

difference in cholinergic receptor number and distribution, or in Ach distribution between the canine and human neurones. However, they have clearly demonstrated a difference in the anatomical relationship of SS-IR neurones between these species.

There was large variability in the basal SS-IR secretion of human submucosal neurones in the presence of hexamethonium and atropine. Preliminary experiments have demonstrated the release of SS-IR in response to the muscarinic agonist carbachol. However, the muscarinic agonist methacholine had no effect on its own and attenuated ß-PMA stimulated release of SS-IR. These experiments serve to illustrate that the effect of endogenous Ach and exogenous muscarinic agonists was variable. Further experiments using cholinergic agonists and antagonists are required to determine the precise role of cholinergic neurones in the secretion of SS-IR from submucosal neurones.

Immunohistochemical identification of neurones containing choline acetyl transferase (Chat) has not been carried out in human or canine enteric neurones but studies in the guinea pig indicated that at least 50 % of enteric neurones were cholinergic (Furness *et al*, 1984; Steele *et al*, 1991). The prevalence of fast postsynaptic EPSPs (mediated by Ach) during stimulation of presynaptic fibres (Wood, 1987) also argues in favor of a large population of

cholinergic enteric neurones. The lack of a suitable Chat antibody or antiserum has made it impractical to carry out studies to examine the localization of this enzyme in submucosal cultures.

The levels of added SP or SS, or secreted SP-IR or SS-IR in the present study may have been altered as a consequence of degradation by proteolytic enzymes, e.g. the lack of SP action on SS-IR secretion from human submucosal neurones could be attributed to the degradation of either added SP and/or secreted SS-IR. The recovery of > 95 % of exogenous SS-IR from release medium after incubation with human or canine submucosal cells suggested that significant immunoreactive SS was not lost during the 2 h incubation period. Substance P recovery was also found to be complete with or without the addition of bacitracin and captopril. These results indicate that minimal levels of proteolytic degradation occurred in the submucosal cultures. The peptidases responsible for degradation of SP and SS have been found in or on glia (Bunnett, 1987; Lentzen *et al*, 1983) and endothelial cells (Defendini *et al*, 1983; Takada *et al*, 1982). Endothelial cells were not present in the cultures while the amount of glia was small due to the presence of the mitotic inhibitor cytosine arabinoside. The basolateral membrane of enterocytes has been suggested to be a primary region for the degradation of both SS-28 and SS-14 (Weber *et al*, 1986) and it is probable that particular

capillary beds and circulatory enzymes contribute to the degradation of neuropeptides since their half-life in blood is short (Bunnett, 1987). These results have suggested that the use of dispersed neuronal cultures significantly reduced the levels of proteolytic enzymes. Previous studies have reported a lack of degradation of SS-IR in cultured rat brain neurones (Lucius and Mentlein, 1991) and neurotensin in cultured canine submucosal neurones (Barber *et al*, 1989).

Isolation methods utilizing collagenase have been shown to specifically damage muscarinic receptors on neurones and various cell separation methods using hypertonic solutions have been implicated in general receptor damage (Guarnieri *et al*, 1975). However, the lack of SP effect on SS-IR release from human cultures was not likely due to receptor damage for several reasons. Receptor damage due to hyperosmolality was avoided since cell separation in these experiments was carried out using elutriation which permits the use of isotonic solutions (Meinstrich, 1983; Guarnieri *et al*, 1975). Once the cells have been isolated, the receptors would have regenerated in culture, as has been shown for the nicotinic acetylcholine receptor (Hartzell *et al*, 1973). Further support comes from experiments using canine submucosal neurones which have been isolated and maintained in tissue culture in a similar fashion and were able to respond to receptor dependent secretagogues such as SP (Barber *et al*, 1989; Buchan *et al*, 1989). Finally,

preliminary experiments using CGRP and carbachol have shown increases in SS-IR secretion suggesting, but not confirming, the presence of functional receptors on the neurones.

As mentioned, the neurones in tissue culture sprouted processes which were associated with varicosities along their length. In addition, the proportion of human neurones containing SS-IR was similar to that in tissue sections. These results have suggested that the culture and isolation procedures did not alter their normal morphologic and immunocytochemical phenotype. Neurones dissociated from rat MYP have also been shown to retain morphological and immunocytochemical properties after having grown in culture for 4-8 weeks (Nishi and Willard, 1985). These authors have also demonstrated that dissociated myenteric neurones *in vitro* retain normal pharmacological and electrophysiological properties (Willard and Nishi, 1985a; 1985b).

E. Summary

These data have demonstrated that neurones of the SMP of the human small intestine can be isolated and maintained in tissue culture for 72 h. The neurones were viable and sprouted neurites which contained varicosities along their length and which made anatomical connections with other cells. This indicated that the neurones retained a 'normal' morphologic phenotype in culture. The cultures contained SS-IR, SP-IR and VIP-IR which were present in cell bodies, neurites and varicosities. The proportion of SS-IR neurones was similar in culture and *in situ* and these neurones contained SP-IR which reflected the co-localization observed *in situ*. This indicated that the isolation and culture conditions did not alter the expression of these neuropeptides. A major advantage of these cultures was that there were no SS-IR endocrine cells present and therefore experiments were carried out which examined SS-IR secretion from neurones exclusively. The presence of varicosities suggested that the release of SS-IR was regulated, since regulated peptide secretion from unmyelinated nerve fibres has been shown to occur at this level. The secretion of SS-IR from these neurones was stimulated by ß-PMA activation of PKC in a time-dependent fashion. Somatostatin-14 was the predominant form of SS-IR present in the neurones and released by ß-PMA. Potassium depolarization had no effect on SS-IR secretion but seemed to make the neurones more

sensitive to stimulation by ß-PMA, although this was not statistically significant. Preliminary experiments suggested that TTX could attenuate the ß-PMA-stimulated secretion of SS-IR. This would further support the contention that membrane depolarization was only partly responsible for the secretion of SS-IR from these neurones in response to activation of PKC. Further experiments are required to determine the role of membrane depolarization in the secretion of SS-IR. Unlike the situation in the dog, the calcium ionophore A23187 was not required to elicit the secretion of SS-IR in the human but, like the dog, the ionophore on its own did not release SS-IR. The inhibitory effect of SP on basal SS-IR secretion observed in canine cultures (Buchan *et al*, 1990) did not occur in the human cultures. In addition, SP did not have an effect on ß-PMA-stimulated SS-IR release. The difference in SP effect between the canine and human cultures is correlated to the difference in the co-localization of SP-IR and SS-IR between canine and human submucosal neurones . Preliminary experiments suggested that cholinergic agonists have mixed actions on the secretion of SS-IR from these neurones. The stimulation of SS-IR secretion by CGRP and carbachol indicated that the neurones were responsive to receptor dependent stimulation in addition to receptor independent stimulation by ß-PMA.

Human myenteric cultures exhibited characteristics similar to those observed in submucosal cultures. Neurones, neurites and varicosities contained SS-IR, SP-IR and VIP-IR.

The canine cultures exhibited similar characteristics to human cultures. These cultures contained SP-IR and SS-IR as demonstrated by RIA, and were present in distinct neurones as demonstrated by ICC, which reflected their localization *in situ*. The release of SP-IR was measurable but was not stimulated by potassium depolarization. The recovery of SP-IR incubated with canine cultures was close to 100 % and was not affected by the enzyme inhibitors bacitracin or captopril.

V. GENERAL SUMMARY AND CONCLUSIONS

Hypotheses 1. That interspecies variations in neuropeptide localization and enteric neuronal morphology exist between the human and canine small intestine.

A. Morphological Data from the Small Intestine

1. The human and canine small intestine contain two different sub-groups of submucosal ganglia based on morphological examination. One group was nearer the mucosa and the other was nearer the circular muscle. There were no differences noted in their morphology or chemical coding.

2. Canine and human submucosal ganglia contained neuronal types which were segregated into clusters of a predominant type. In canine submucosa, large ganglia were composed of several such clusters of different neuronal types. In human submucosa, the situation was more dramatic in that some small ganglia contained one neurone type exclusively. These results have both developmental and functional implications. From a developmental standpoint, the existence of such clusters suggests that neurones migrating into the enteric plexi could be pre-programmed to differentiate, for example, into SS-IR neurones rather than receive signals to differentiate after arrival. The embryonic neurone after arrival at a specific location would resume division to

produce clusters of a single type within the plexus. Alternatively, the existence of the clusters could result from the parallel tracking of axons growing out of the embryonic ganglia. In this case, the phenotype would be dependent on the target innervated but all neurones with axons in the same bundle would differentiate into the same type.

3. The canine, but not the human, small intestine has a characteristic deep muscular plexus that was separate from other sets of fibres in the circular muscle layer. This implies that neural control of circular muscle differs between the dog and human.

4. Immunocytochemical studies have demonstrated, for the first time, that SP-IR and SS-IR are co-localized in the human, but not canine, enteric neurones of the small intestine. Furthermore, canine ganglia were shown to be substantially larger than their human counterparts. The difference in chemical coding of neurones and the difference in ganglion size may be indicative of differences in the function of submucosal neurones.

Conclusions.

These data support the hypothesis that interspecies variations in neuropeptide localization and enteric neuronal

morphology exist between the human and canine small intestine. In addition, these studies have shown that there are differences between canine and human submucosal neurones with respect to ganglion size, chemical coding of submucosal neurones and the innervation of circular muscle. It has been suggested that interspecies differences in gut morphology are related to diet (Powell,1987; Gross, 1986) and size (Gabella, 1990). The interspecies differences observed in the present study support the contention that animals which have similar diets have similar enteric ganglia with respect to neuropeptide distribution and neuronal number. Moreover, the results have emphasized that comparisons of the results of physiological experiments carried out in different species should be made with caution.

Hypothesis 2. That differences in neuronal chemical coding and morphology will be reflected in neuronal function.

Hypothesis 3. That short-term cultures of human and canine SMP will provide a model system in which to examine such differences.

B. Culture Studies

1. These experiments have demonstrated, for the first time, that adult human submucosal neurones can be isolated and

maintained in tissue culture. Cell cultures eliminate the problems associated with peptides present in both submucosal and myenteric neurones, and enteroendocrine cells.

2. The neurones sprouted neurites which contained varicosities, suggesting that the secretion of neuropeptides is from varicosities and can be regulated. This provides an advantage over secretion studies carried out with acutely dissociated neurones which do not have neuronal fibres. Secretion from neurones which do not possess nerve fibres suggests that secretion of neuropeptides occurs from the cell body for which there is presently no evidence using *in vivo* experimental techniques. Previous studies and the present evidence have suggested that SS-IR secretion was independent of the cell body. Thus, the regulation of SS-IR secretion is suggested to be at the level of the varicosity. These data have also suggested that a small depolarization renders the varicosities more sensitive to a secretory stimulus.

3. The difference in neuronal localization of SS-IR and SP-IR between the canine and human small intestine also suggested a difference in function. Substance P did not inhibit the secretion of SS-IR from human neurones as was the case in canine neurones (Buchan et al, 1990). This supports the hypothesis that the differences observed in SP-

IR and SS-IR localization are related to differences in SS-IR secretion and SP actions.

4. The phorbol ester, ß-PMA, was able to stimulate the secretion of SS-IR from human neurones in the absence of the A23187, unlike canine neurones which required the presence of the calcium ionophore (Buchan et al, 1990). This further supports the hypothesis that canine and human submucosal neurones differ with respect to the regulation of SS-IR secretion.

5. The effects of CGRP and carbachol suggested that receptor dependent secretagogues were able to stimulate SS-IR secretion. This suggested that functional receptors were present on the neurones after isolation and tissue culture but further experiments are required to confirm this possibilty.

Conclusions.

These results support the hypothesis that dispersed cultures of submucosal neurones are useful models to examine neuronal function. The cultures provided advantages over *in vivo* experiments for studying the secretion of neural SS-IR. The regulation of SS-IR was shown to be different in canine and human submucosal neurones and this difference was

related to the difference in the localization of SS-IR and SP-IR i.e. a difference in neuropeptide phenotype.

VI. CONCLUSIONS AND FUTURE DIRECTIONS

A. Conclusions and Significance

The data presented and previous studies suggest that the morphology of the small intestine reflects diet i.e. structure and function are closely linked. The corollary to this statement is that animals having similar diets have similar gastrointestinal tracts. The similarity in structure is illustrated by the correspondence in the small intestine of omnivores (rat and human) compared to other groups (e.g. ruminants). Genetic similarity does not seem to be a prerequisite except where it confers a preference in diet. An example of this is seen in two new world monkeys similar in size, the howler and spider monkeys (Milton, 1986). Both species are plant eaters, but howler monkeys pass food through their digestive system at half the rate of spider monkeys. This reflects the larger colon of the howler monkey. Although the diets of both species are plant based, the spider monkey eats mostly fruit and meets its nutritional requirements by ingesting large volumes of food. The howler monkey eats less and is able to ferment quantities of plants present in the colon. There are many examples which show that species can rapidly respond to changes in dietary quality by altering the features of the gut (Gross *et al*, 1986). Alterations in the gut no doubt

include changes in the morphology of enteric neurones and the neurotransmitters they contain.

The experiments presented in this thesis have demonstrated that differences exist between canine and human small intestine with regard to the morphology of the SMP, the localization of SP, SS and VIP in enteric nerves and the actions of SP and PKC-activation on enteric nerves. Differences in the secretion and absorption of electrolytes and in the control of motility between the canine and human small intestine (outlined in the general introduction) are probably related to the differences observed in the present experiments. For example, SP increases the secretion of electrolytes in the small intestine by a mechanism which is TTX sensitive in some mammals but not the human (Hubel *et al*, 1984; Keast *et al*, 1987). It is possible that SP exerts its TTX sensitive effects by the indirect inhibition of neural SS-IR release similar to that which is observed in the canine small intestine.

The necessity and usefulness of combining morphological and physiological experimental techniques to study the function of the small intestine was exemplified by the results obtained in the present studies. Moreover, they have suggested possibilities for further examination of the morphology of the small intestine, characterization of the neuronal cultures themselves, differences in SP action and

SS-IR release from the cultures and regulation of other neurotransmitters present in the cultures. Finally, functional studies of the small intestine can be carried out which examine whether the differences in morphology correlate with neurotransmitter regulation.

B. Future Directions

1. Morphological Studies

The differences in neuropeptide distribution and plexus morphology between the human and canine enteric nervous system will probably be more extensive than reported in this thesis. Further characterization of the distribution of other known enteric neuropeptides will allow the neurone types to be identified in a manner similar to that which has been carried out previously in the rat and guinea pig (Furness *et al*, 1989; Pataky *et al*, 1990).

2. Functional Studies

With the use of dispersed neuronal cultures of the SMP, it has been possible to study the secretion of a peptide which is produced by several cell types. It is possible that the regulation of SS-IR secretion from neurones is different from that in endocrine cells. Furthermore, differences in the modulation of secretion could reflect

different physiological roles for endocrine and neuronal SS-IR. Parallel studies of SS-IR secretion from endocrine cell and neuronal cultures would provide an excellent model system in which to define different release patterns.

Although the data collected demonstrated both stimulation and inhibition, a role for cholinergic transmission in neural SS-IR release from human SMP neurones was indicated. Further experiments are required to elucidate the precise actions of cholinergic agonists and antagonists on SS-IR secretion. Testing the actions of the cholinergic agonists and antagonists on canine enteric neurones would be useful for comparison to human neurones. Access to an antibody for Chat would permit the determination of the proportion of cholinergic neurones present enteric neurones in culture and *in situ*.

The overall actions of SP on SS-IR secretion were suggested to be indirect, based on canine and human experiments. This hypothesis can be tested in the following way. First, the effects of SP on cellular events, such as ionic currents and calcium transients, can be examined in neurones known to contain SS-IR. Second, the effects of SP on the release of other neurotransmitters, such as Ach, can be determined. Neurotransmitters can then be tested for their ability to affect the cellular events of SS-IR neurones.

The present study and previous studies (Buchan et al, 1990) have suggested that somatostatin secretion from canine and human submucosal neurones is modulated differently by the phorbol ester. The secretion of SS-IR can be further characterized by examining the response to depolarization by levels of potassium higher than those used in the present experiments. It should be noted that depolarization of enteric neurones by potassium may not result in the secretion of SS-IR. The lack of neurotransmitter release in response to high potassium has been noted in other preparations where it has been suggested that calcium entry is mediated by receptor-operated calcium channels (e.g. Belai *et al*, 1987).

The cultures utilized in the present experiments were shown to contain neurones which possessed a morphologic phenotype similar to that observed *in situ*. It would be useful to compare ionic currents and pharmacological responses of neurones to known neurotransmitters in order to confirm the similarity of neurones maintained in tissue culture and *in situ*.

The calcium dependence of SS-IR secretion from submucosal neurones is necessary to support its role as a neurotransmitter. The examination of calcium transients in SS-IR neurones by the use of dyes such as Fura-2 would be

necessary to confirm the calcium dependence of SS-IR secretion.

The actions of PKC stimulation on SS-IR secretion suggests that neuropeptides which activate this pathway also stimulate SS-IR secretion. Gastrin-releasing peptide is an example of a neuropeptide, found in enteric neurones, which activates this pathway in a variety of cell types. The presence of this peptide in the human enteric nerves (Price *et al*, 1984) suggests that it influences the secretion of neural SS-IR.

The complexity of the enteric nervous system is such that the studies outlined will provide information in only a small fraction of the control systems. However, improvements in technology, such as calcium imaging systems, and experimental techniques, such as isolated cell culture systems, would allow concepts of enteric nervous function to be tested rigorously at the cellular level. Together with experiments carried out in the whole animal (e.g. the dietary habits of new world monkeys), the studies outlined will provide insight into the function of the small intestine under different conditions.

VII. REFERENCES

Ahmad, S., Allescher, H.D., Manaka, H., Manaka, Y. and Daniel, E.E. Biochemical studies on opioid and α_2-adrenergic receptors in canine submucosal neurones. Am. J. Physiol. 256: G957-965, 1989.

Allen, J.M., Hughes, J.and Bloom, S.R. Neuropeptide Y in the gastrointestinal tracts: distribution, chromatographic analysis and pharmacology. Can. J. Physiol. Pharmacol. Gastrointestinal Hormones Symposium, Vancouver, Canada. pp. 101-102, 1986.

Allescher, H.D., Ahmad, S., Kostka, P., Kwan, C.Y. and Daniel, E.E. Distribution of opioid receptors in canine small intestine: implications for function. Am J. Physiol. 256: G966-974, 1989.

Andersson, P.-O., Bloom, S.R. and Jarhult, J. Colonic motor and vascular responses to pelvic nerve stimulation and their relation to local peptide release in the cat. J. Physiol. 334: 293-307, 1983.

Baldissera, F.G.A., Holst, J.J., S.L. Jensen and T. Krarup. Distribution and molecular forms of peptides containing somatostatin immunodeterminants in extracts from the entire

gastrointestinal tract of man and pig. Biochim. Biophys. Acta 838: 132-143, 1985.

Bannerman, P.G., Mirsky, R. and Jessen, K.R. Analysis of enteric neurones, glia and their interactions using explant cultures of the MYP. Dev. Neurosci. 9: 201-227, 1987.

Barajos-Lopez, C., Berezin, I., Daniel, E.E. and Huizinga, J.D. Pacemaker activity recorded in interstitial cells of Cajal of the gastrointestinal tract. Am. J. Physiol. 257: C830-835, 1989.

Barber, D.L., A.M.J. Buchan, J.H. Walsh and A.H. Soll. Isolated canine ileal mucosal cells in short-term culture: a model for study of neurotensin release. Am. J. Physiol. 250: G374-G384, 1986.

Barber, D.L., A.M.J. Buchan, S.E. Leeman and A.H. Soll. Canine enteric submucosal cultures: transmitter release from neurotensin immunoreactive neurons. Neuroscience 32: 245-253, 1989.

Baskin, D.G. and Ensinck, J.W. Somatostatin in epithelial cells of intestinal mucosa is present primarily as somatostatin 28. Peptides 5: 615-621, 1984.

Belai, A. and G. Burnstock. Release of calcitonin gene-related peptide from rat enteric nerves is Ca^{2+} -dependent but is not induced by K^{+} depolarization. Reg. Pept. 23: 227-235, 1988.

Belai, A., V. Ralevic and G. Burnstock. VIP release from enteric nerves is independent of extracellular calcium. Reg. Pept. 19: 79-89, 1987.

Bell, J.D., I.L.O. Buxton, and L.L. Brunton. Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. J. Biol. Chem. 260: 2625-2628, 1985.

Berridge, M.J. The molecular basis of communication within the cell. Scientific American, 1985.

Besson, J., W. Rostene, A-M. Lhiaubet and G. Rosselin. Release of vasoactive intestinal peptide from rat jejuno-ileum *in vitro*. Effect of various depolarizing agents. Experentia. 39: 732-733, 1983.

Binder, H.J., Laurensen, J.P. and Dobbins, J.W. Role of opiate receptors in the regulation of enkephalin stimulation of active sodium chloride. Am. J. Physiol. 247: G432-436, 1984.

Blumberg, P. M. Protein kinase C as the receptor for the phorbol ester tumour promoters: sixth Rhoads Memorial award lecture. Cancer Res. 48: 1-8, 1981.

Bonanno, G., Raiteri, M. and Emson, P.C. *In vitro* release of somatostatin from cerebral cortical slices: characterization of electrically evoked release. Brain Res. 447: 92-97, 1988.

Bornstein, J.C. Substance P binding sites in guinea pig enteric ganglia. In: Substance P and Neurokinins, eds. J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion, pp. 59-70, 1987.

Bornstein, J.C. and Furness, J.B. Correlated electrophysiological and histochemical studies of submucosal neurones and their contribution to understanding neural circuits. J. Autonom. Nerv. Syst. 25: 1-13, 1988

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179: 77-79, 1973.

Brown, D.R., M.F. Overend and B.G. Treder. Neurohormonal regulation of ion transport in the porcine distal jejunum.

Actions of somatostatin-14 and its natural and synthetic homologs. J. Pharmacol. Exp. Therapeut. 252: 126-134, 1989.

Brown, J.C., Mutt, V. and Dryburgh, J. The further purification of motilin, a gastric motor activity stimulating polypeptide from the mucosa of the small intestine of hogs. Can. J. Physiol. Pharmacol. 49: 399-405, 1971.

Buchan, A.M.J., MacLeod, M.D., Meloche, R.M. and Kwok, Y.N. Muscarininc regulation of somatostatin release from primary cultures of human antral epithelial cells. Pharmacology 44: 33-40, 1992.

Buchan, A.M.J., A.D. Doyle and E.A. Accili. Canine jejunal submucosa cultures: characterization and release of neural somatostatin. Can. J. Physiol. Pharmacol. 68: 705-710, 1990.

Buchan, A.M.J. Neurofilament-M and Calbindin D_{28K} are present in two discrete populations of submucosal neurons. Brain Res. 538: 171-175, 1991.

Buchan, A,M.J. and Barber, D.L. Neurotensin-containing neurones in the canine enteric innervation. Neurosci. Lett. 76: 13-17, 1987

Bulbring, E., Lin, R.C.Y. and Schofield, G. An investigation of the peristaltic reflex in relation to anatomical observations. Q.J. Exp. Physiol. 43: 26-37, 1958.

Bunnett, N. Post Secretory metabolism of peptides. Am. Rev. Respir. Dis. 136: S27-34, 1987.

Cajal, S.R. Sur les ganglions et plexus nerveux de l'intestin. C.R. Soc. Biol. (Paris), 45: 217-223, 1893.

Carey, H.V., Cooke, H.J. and Zafirova, M. Mucosal responses evoked by stimulation of ganglion cell somas in the SMP of the guinea pig ileum. J. Physiol. 364: 69-80, 1985.

Castagna, M.Y. Takai, K. Kaibuci, K. Sano, U. Kikkawa and Y. Nishizuka. Direct activation of calcium-activated, phospholipid dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 13:7847-7851, 1982.

Cassuto, J., Siewert, A., Jodal, M. and Lundgren, O. The involvement of intramurals in cholera toxin-induced intestinal secretion. Acta Physiol. Scand. 115: 157-158, 1983.

Chang, M.M.., Leeman, S.L., and Niall, H.D. Amino acid sequence of substance P. Nature New. Biol. 232:86-87, 1971

Christensen, J. and Rick, G.A. Intrinsic nerves in the mammalian colon: confirmation of a plexus at the circular muscle-submucosal interface. J. Autonom. Nerv. Syst. 21: 223-231, 1987.

Cooke, H.J. Role of the "little brain" in the gut in water and electrolyte homeostasis. FASEB J. 3: 127-138, 1989.

Costa, M., Furness, J.B. and Llewellyn-Smith, I. Histochemistry of the enteric nervous system. In: Physiology of the Gastrointestinal Tract, second edition, edited by Leonard R Johnson. Raven Press, New York, pp. 1-40, 1987.

Couture, R., Mizrahi, J., Regoli, D. and Devroede, G. Peptides and the human colon.: an *in vitro* pharmacological approach. Can J. Physiol. Pharmacol. 59: 957-64, 1981.

Crema, A. On the polarity of the peristaltic reflex in the colon. In: Smooth Muscle ed. Bulbring, E., Brading, A.F., Jones, A.W. and Tomita, T. pp. 542-548, 1970.

Crowe, R., Kamm, M.A., Burnstock, G. and Lennard-Jones, J.E. Peptide-containing neurones in different regions of the SMP of human sigmoid colon. Gastroenterology 102: 461-467, 1992.

Daniel, E.E., , Furness, J.B., Costa, M. and Belbeck, L. The projections of chemically identified nerve fibres in canine ileum. Cell Tiss. Res. 247: 377-384, 1987.

Daniel, E.E., Costa, M., Furness, J.B. and Keast, J.R. Peptide neurons in the canine small intestine. J. Comp. Neurol. 237: 227-238, 1985.

Defendini, R., Zimmerman, E.A., Weare, J.A., Alhene-Gelas, F. and Erdos, E.G. Angiotensin converting enzyme in epithelial and non-epithelial cells. Neuroendocrinology 37: 32-40, 1983.

Dockray, G. Physiology of enteric neuropeptides. In: Physiology of the Gastrointestinal Tract, Leonard Johnson ed. vol. 1, pp. 41-66, 1987.

Donnerer, J. P. Holzer and F. Lembeck. Release of dynorphin, somatostatin and substance P from the vascularly perfused small intestine of the guinea pig during peristalsis. Br. J. Pharmac. 83: 919-925, 1984.

Doran, J.F., Jackson, P, Kynoch, P.A.M. and Thompson, R.J. Isolation of PGP 9.5, a new human neurone-specific protein detected by high-resolution two-dimensional electrophoresis. J. Neurochem. 40: 1542-1548, 1983.

Dubois, M.P. Immunoreactive somatostatin is present in discrete cells of the pancreas. Proc. Natl. Acad. Sci., USA 72: 1340, 1975.

du Vigneaud, V., Gish, D.T. and Katsoyannis, P.G. A synthetic preparation possessing biological properties associated with arginine-vasopressin. J. Amer. Chem. Soc. 75: 4751-4752, 1953.

du Vigneaud, V., Ressler, C., Swan, J.M., Roberts, C.W., Katsoyannis, P.G. and Gordon, S. The synthesis of an octapeptide amide with the hormonal activity of oxytocin. J. Amer. Chem. Soc. 76: 4879-4880, 1954.

Ekblad, E., Ekman, R., Hakanson, R. and Sundler, F. Projections of peptide-containing neurones in rat large intestine. Neuroscience 27: 655-674, 1988.

Ekblad, E., Ekman, R., Hakanson, R. and Sundler, F. Projections of peptide-containing neurones in rat small intestine. Neuroscience 27: 655-674, 1987.

Ferri, G.-L., Botti, P.L., Vezzadini, P., Biliotti, G., Bloom, S.R. and Polak, J.M. Peptide-containing innervation

of the human intestinal mucosa. Histochemistry 76 (1982) 413-420.

Feyrter, F. Uber die peripheren endokrinen (parakrinen) drusen des menschen, W. Maudrich, Wien-Dusseldorf pp. 1-231, 1953.

Furness, J.B., Lloyd, K.C., Sternini, C. and Walsh, J.H. Projections of substance P, vasoactive intestinal peptide and tyrosine hydroxylase immunoreactive nerve fibres in the canine intestine, with special reference to the innervation of the circular muscle. Arch. Hist. Cyt. 53: 129-140, 1990.

Furness, J., Morris, J.L and Gibbins, I.L Costa, M. Chemical coding of neurones and plurichemical transmission. Annu. Rev. Pharmacol. Toxicol. 29: 289-306, 1989.

Furness, J.B. and Costa, M. The enteric nervous system. Churchill- Livingstone. Edinburgh-London-Melbourne-New York, 1987.

Furness, J.B., Costa, M. and Keast, J.R. Choline-acetyltransferase and peptide immunoreactivity of submucosal neurones of the small intestine of the guinea pig. Cell Tiss. Res. 237: 329-336, 1984.

Furness, J.B. and Costa, M. VIP and enteric inhibitory nerves. In: Vasoactive Intestinal Peptide S. Said ed., New York:Raven pp. 391-406, 1982.

Furness, J.B. and Costa, M. Types of nerves in the enteric nervous system. Neuroscience 5: 1-20, 1980.

Gabella, G. On the plasticity of form and structure of enteric ganglia. J. Autonom. Nerv. Syst. 30: S59-66, 1990.

Gabella, G. Structure of muscles and nerves in the gastrointestinal tract. In Physiology of the Gastrointestinal Tract, second edition, edited by Leonard R Johnson. Raven Press, New York, 1987.

Gallacher, D.V., Hanley, M.R., Petersen, O.H., Roberts, M.L., Squire-Pollard, L.G. and Yule, D.I. Substance P and bombesin elevate cytosolic Ca^{2+} by different molecular mechanisms in a rat pacreatic acinar cell line. J. Physiol. 426: 193-207, 1990.

Gates, T.S., Zimmerman, R.P., Mantyh, C.R., Vigna, S.R., Maggio, J.E., Welton, M.L., Passaro Jr., E.P. and Mantyh, P.W. Substance P and substance K binding sites in the human gastrointestinal tract: localization by autoradiography. Peptides 9: 1207-1219, 1989.

Gershon, M.D. Erde, S.M. and Dreyfus, C.F. Long-term survival of serotoninergic neurones in tissue cultures of myenteric plexus dissected from guinea pig ileum and duodenum. Soc. Neurosci. Abstr. 6: 274, 1980.

Grider, J.R. Somatostatin release from isolated ganglia of the MYP. Am. J. Physiol. 257 (Gastrointest. Liver Physiol.): G313-G315, 1989.

Gross, J., Wang, Z. and Wunder, B.A. Adaptations to food quality and energy needs: changes ingut morphology and capacity of Microtitus Ochrogaster. J. Mammal. 66: 661-669, 1986.

Guarnieri, M., Krell, L.S., McKhann, G.M., Pasternak, G.W. and Yamamura, H.I. The effects of cell isolation techniques on neuronal membrane receptors. Brain Res. 93: 337-342, 1975.

Guillemin, R. Biochemical and physiological correlates of hypothalamic peptides. The endocrinology of the neuron. In: The Hypothalamus, S. Reichlin, R.J. Baldessarini and J.B. Martin, eds, New York: Raven Press, pp. 155-194, 1978.

Gulati, N., Mathison, R., Huggel, H., Regoli, D. and Beny, J.-L. Effects of neurokinins on the isolated pig coronary artery. European J. Pharmacol. 137: 149-154, 1987.

Gulbenkian, S., Wharton, J. and Polak, J.M. The visualization of cardiovascular innervation in the guinea pig using an antiserum to protein gene product 9.5 (PGP 9.5). J. Autonom. Nerv. Syst. 18: 235-247, 1987.

Gunn, M. Histological and histochemical observations on the myenteric and submucous plexus of mammals. J. Anat. 102: 223-239, 1968.

Hartzell, H.C. and Fambrough, D.H. Acetylcholine receptor production and incorporation into membranes of developing muscle fibres. Develop. Biol. 30: 153-165, 1973.

Hirst, G.D.S., Holman, M.E. and Spence, I. Two types of neurones in the MYP of duodenum in the guinea pig. J. Physiol. 236: 303-326, 1974.

Hokfelt, T. Neuropeptides in perspective: the last ten years. Neuron 7: 867-879, 1991.

Hubel, K.A. Intestinal nerves and ion transport: stimuli, reflexes and responses. Am. J. Physiol. 248: G261-271, 1985

Hubel, K.A., K.S. Renquist and S. Shirazi. Neural control of ileal ion transport: role of substance P (SP) in rabbit and man. Gastroenterology 86: 1118P, 1984.

Huizinga, J.D. Action potentials in gastrointestinal smooth muscle. Can J. Physiol. Pharmacol. 69: 1133-1142, 1991.

Inoue, K. and Nakazawa, K. ATP receptor operated Ca^{2+} influx and catecholamine release from neuronal cells. NIPS 7: 56-59, 1992.

Iversen, L. and Goodman, E., eds. Fast and Slow Chemical Signaling in the Nervous System. New York:Oxford University Press, 1986.

Jessen, K.R., Saffrey, M.J. and Burnstock, G. The enteric nervous system in tissue culture I. Cell types and their interactions in explants of the myenteric and SMPes from the guinea pig, rabbit and rat. Brain Research 262: 17-35, 1983.

Jessen, K.R., McConnell, J.D., Purves, R.D., Burnstock, G. and Chamley-Campbell, J. Tissue culture of mammalian enteric neurones. Brain Research 152: 573-579, 1978.

Johansson, O. and Hokfelt, T. TRH, somatostatin and enkephalin: Distribution studies using immunohistochemical techniques. J. Histochem. 28: 364-366, 1980.

Kaczmarek, L. The role of protein kinase C in the regulation of ion channels and neurotransmitter release. Trends in Neurosciences 10: 30-34, 1987.

Katayama, Y., North, R.A. and Williams, J.T. The action of substance P on neurones of the MYP of the guinea pig small intestine. Proc. R. Soc. Lond. B 206:191-208, 1979.

Keast, J.R. Mucosal innervation and control of water and ion transport in the intestine. Rev. Physiol. Biochem. Pharmacol. 109; 1-59, 1987.

Keast, J.R., Furness, J.B. and Costa, M. Distribution of certain peptide-containing nerve fibres and endocrine cells in the gastrointestinal mucosa in five mammalian species. J Comp Neurology 236: 403-422, 1985 .

Keast, J.R., J.B. Furness and M.Costa. Somatostatin in human enteric nerves: distribution and characterization. Cell Tissue Res. 237: 299-308, 1984.

Kimura, S., Okada, M., Sugita, Y., Kanagawa, K. and Munekato, E. Novel neuropeptides, neurokinin A and B,

isolated from porcine spinal cord. Biochem. Biophys. Res. Commun. 114: 533, 1983.

Korman, L.Y., Hassan, S., Bass, B., Moody, T.W. and Harmon, J.W. Distribution of vasoactive intestinal polypeptide and substance P receptors in human colon and small intestine. Dig. Dis. Sci. 34: 1100-1108, 1989.

Kottegoda, S.R. Peristalsis of the small intestine. In: Smooth Muscle ed. Bulbring, E., Brading, A.F., Jones, A.W. and Tomita, T. pp. 525-541, 1970.

Krejs, G. J. and Fordtran, J.S. Effect of VIP infusion on water and ion transport in the human jejunum. Gastroenterology 78: 722-777, 1980.

Krisch, B. Differing immunoreactivities of somatostatin in the cortex and the hypothalamus of the rat. Cell Tiss. Res. 212: 457-464, 1980.

Kulakowski, E.C., Lampson, W.G., Schafer, S.W. and Lovenberg, W. Action of substance P on the working rat heart. Biochem. Pharmacol. 32: 1097-1100, 1983.

Kwok, Y.N. and McIntosh, C.H.S. Release of substance P-like immunoreactivity from the vascularly perfused rat stomach. Eur. J. Pharmacology. 180: 201-207, 1990.

Kwok, Y,N., McIntosh, C.H.S., Pederson, R.A. and Brown, J.C. Effect of substance P on somatostatin release from the isolated perfused stomach. Gastroenterology 88:90-95, 1985.

Langley, J.N. The Autonomic Nervous System, Part 1. W. Heffer and Sons, Cambridge, England, 1921.

Larsson, L.I., Goltermann, N., DeMagistris, L., Reh, J.F. and Schwarz, T.W. Somatostatin cell processes as pathways for paracrine secretion. Science 205: 1393-1395, 1979.

Larsson, L.I. Ultrastructural localization of a new neuronal peptide (VIP). Histochemistry 54: 173-176, 1977.

Lauweryns, J.M. and Van Ranst, L. Protein gene product 9.5 expression in the lungs of humans and other mammals. Immunocytochemical detection in neuroepithelial bodies, neuroendocrine cells and nerves. Neurocience Lett. 85: 311-316, 1988.

Lentzen, H. and Palenker, J. Localization of the thiorphan-sensitive endopeptidase, termed enkephalinase A, on glial cells. FEBS Lett. 153: 93-7, 1983.

Lucius, R. and R. Mentlein. Degradation of the neuropeptide somatostatin by cultivated neuronal and glial cells. J. Biol. Chem. 28: 18907-18913, 1991.

Luft, R., Efendic, S., Hokfelt, T., Johansson, O. and Arimura, A. Immunohistochemical evidence for the localization of somatostatin-like immunoreactivity in a cell population of the pancreatic islets. Med. Biol. 52: 428-430, 1974.

McFadden, D., Zinner, M.J. and Jaffe, B.J. Substance P-induced intestinal secretion of water and electrolytes. Gut 27:267-272, 1986.

McIntosh, C.H.S., V. Bakich, Y.N. Kwok and J.C. Brown. A comparison of the inhibitory effects of somatostatin-14, -25 and -28 on motility of the guinea pig ileum. Can. J. Physiol. Pharmacol. 64: 303-306, 1987a.

McIntosh, C.H.S., Y.N. Kwok, C. Tang and J.C. Brown. The use of monoclonal antibodies in RIA for gastrointestinal hormones. J. Clin. Immunoassay 10: 79-84, 1987b.

McIntosh, C.H.S. Gastrointestinal somatostatin: distribution, secretion and physiological significance. Life Sciences 37: 2043-2058, 1985.

Maggi, C.A., Giuliani, S., Patacchini, R., Santicioli, P., Theodorsson, Barbanti, G., Turini, D. and Giachetti, A. Tachykinin antagonists inhibit nerve-mediated contractions in the circular muscle of the human ileum: Involvement of neurokinin-2 receptors. Gastroenterology 102: 88-96, 1992.

Manaka, H., Manaka, Y., Kostolanska, F., Fox, J.E.T. and Daniel, E.E. Release of VIP and substance P from isolated perfused canine ileum. Am. J. Physiol. G182-190, 1989.

Mantyh, P.W., Mantyh, C.R., Gates, T., Vigna, S.R. and Maggio, J.E. Receptor binding sites for substance P and substance K in the canine gastrointestinal tract and their possible role in inflammatory bowel disease. Neuroscience 25 (1988) 817-837.

Meinstrich, M.L. Experimental factors involved in separation by centrifugal elutriation. In Cell Separation Methods and Selected applications, vol 2. pp. 33-61, 1983.

Meissner, G. Uber dieNerven der Darmwand. Z. Ration. Med. N.F. 8: 364-366, 1857.

Millhorn, D.E. and Hokfelt, T. Chemical messengers and their co-existence in individual neurones. NIPS 3: 1-5, 1988.

Milner, P., Ralevic, V., Hopwood, A.M., Feher, E., Lincoln, J., Kirkpatrick, K.A. and Burnstock, G. Ultrastructural localization of substance P and choline acetyltransferase in endothelial cells of rat coronary artery and release of substance P and acetylcholine during hypoxia. Experentia 45: 121-125, 1989.

Mihara, S., North, R.A. and Surprenant, A. Somatostatin increases an inwardly rectifying potassium conductance in guinea pig SMP neurones. J. Physiol. 390: 335-355, 1987.

Milton, K. Digestive physiology in primates. NIPS 1: 76-79, 1986.

Morita, K., North, R.A. and Tokimasa, T. Muscarininc agonists inactivate potassium conductance of guinea pig myenteric neurones. J. Physiol. 333: 125-139, 1982.

Nishi, R. and Willard, A.L. Neurones dissociated from rat myenteric plexus retain differentiated properties when grown in cell culture. I. Morphological properties and immunocytochemical localization of transmitter candidates. Neuroscience 16: 187-199, 1985.

Nishi, R. and North, R.A. Intracellular recording from the MYP of the guinea pig ileum. J. Physiol. 231: 471-491, 1973.

Nishizuka, Y. Studies and perspectives of protein kinase C. Science 233: 305-312, 1986.

North, R.A. Receptors on individual neurones. Neuroscience 17: 899-907, 1986.

North, R.A., Slack, B.E. and Surprenant, A. Muscarinic M_1 and M_2 mediate depolarization and presynaptic inhibition in guinea pig enteric nervous system. J. Physiol. 368: 435 452, 1985.

North, R.A. and Tokimasa, T. Muscarininc synaptic potentials in guinea pig MYP neurones. J. Physiol. 333: 151-156, 1982.

Oda, T., M.S. Shearman and Y. Nishizuka. Synaptosomal protein kinase C subspecies: B. Down-regulation promoted by phorbol ester and its effect on evoked norepinephrine release. J. Neurochemistry 56: 1263-1269, 1991.

Palmer, J.M., Wood, J.D. and Zafirov, D.H. Transduction of aminergic and peptidergic signals in enteric neurones of the guinea pig. J. Physiol. 387: 371-383, 1987.

Pataky, D.M., Curtis, S.B. and Buchan, A.M.J. The co-localization of neuropeptides in the submucosa of the small

intestine of normal Wistar and non-diabetic BB rats. Neuroscience 36: 247-254, 1990.

Pearse, A.G.E. Peptides in the brain and intestine. Nature 262: 92-94, 1976.

Pearse, A.G.E. and Polak, J.M. Immunocytochemical localization substance P in mammalian intestine. Histochemistry 41: 373-375, 1975.

Penman, E., J.A.H. Wass, M.G. Butler, E.S. Penny, J. Price, P. Wu. and L.H. Rees. Distribution and characterization of immunoreactive somatostatin in human gastrointestinal tract. Reg. Peptides 7: 53-67, 1983.

Perdue, M.H., Galbraith, R. and Davison, J. Evidence for substance P as a functional neurotransmitter in the guinea pig small intestinal mucosa. Regul. Pept. 18: 63-74, 1987.

Peterfreund, R.A. and W.W. Vale. Phorbol diesters stimulate somatostatin secretion from cultured brain cells. Endocrinol. 113: 200-208, 1983.

Polak, J., Pearse, A.G.E., Grimelius, L., Bloom, S.R. and Arimura, A. Growth-hormone release-inhibiting hormone in gastrointestinal and pancreatic D cells. Lancet 1: 1220-1224, 1975.

Powell, D.W. Intestinal water and electrolyte absorption. In: Physiology of the Gastrointestinal Tract, second edition, edited by Leonard R Johnson. Raven Press, New York, pp. 1267-1306, 1987.

Price, J., Penman, E., Wass, J.A.H. and Rees, L.H. Bombesin-like immunoreactivity in human gastrointestinal tract. Regulatory Peptides 9: 1-10, 1984.

Quirion, R. and Gaudreau, P. Strategies in neuropeptide receptor binding research. Neuroscience and biobehavioural rev. 9: 413-420, 1985.

Reichlin, S. Systems for the study of regulation of neuropeptide secretion. In Advances in Biochemical Psychopharmacology. Vol. 28. Neurosecretion and brain peptides, implications for brain function and disease. Edited by J.B. Martin, S. Reichlin and K.L. Bick. Raven Press:New York, 1981.

Rogers, D.C. and Burnstock, G. The interstitial cell and its place in the concept of the autonomic ground plexus. J. Comp. Neurol. 126: 255-286, 1966.

Rumesson, J.J. and Thuneberg, L. Interstitial cells of Cajal in human small intestine. Ultrastructural

identification and organization between the main smooth muscle layers. Gastroenterology 100: 1417-1431, 1991.

Said, S. and Mutt, V. Potent peripheral and splanchnic vasodilator peptide from normal gut. Nature 225: 863-864, 1970

Sanders, K.M. and Smith, T.K. Motoneurones of the submucosal plexus regulate electrical activity of the circular muscle of canine proximal colon. J. Physiol. 380: 293-310, 1986.

Schally, A.V., Arimura, A. and Kastin, A.J. Hypothalamic regulatory hormones. Science 179, 341-350, 1973.

Scheuermann, D., Stach, W., Timmermans, J.-P., Adriansen, D. and De Groodt-Lasseel. Neuron-specific enolase and S-1-protein immunohistochemistry for defining the structure and topographical relationship of the different enteric nerve plexuses in the small intestine of the pig. Cell Tiss. Res. 256: 65-75, 1989.

Schultzberg, M., Hokfelt, T., Nilsson, G, Terenius, L., Rehfeld, J.F., Brown, M., Elde, R., Goldstein, M. and Said, S. Distribution of peptide- and catecholamine-containing neurones in the gastrointestinal tract of rat and guinea pig: Immunohistochemical studies with antisera to substance

P, vasoactive intestinal peptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine-ß-hydroxylase. Neuroscience 5: 689-744, 1980.

Smith, T.K., Furness, J.B., Costa, M. and Bornstein, J.C. An electrophysiological study of the projections of motor neurones that mediate non-cholinergic excitation in the circular muscle of the guinea pig small intestine. J. Autonom. Nerv. Syst. 22: 115-128, 1988.

Stach, W. Der plexus submucosus externus (Schabadasch) im Dunndarm das Schweins. I. Form, Struktur und Verbindungen der Ganglien und Nervenzellen. Z. Mikrosk.-Anat. Forsch 91: 737-755, 1977.

Surprenant, A. Slow excitatory synaptic potentials recorded from neurones of guinea pig submucous plexus. J. Physiol. 351: 343-361, 1984.

Takada, Y., Hiwada, K., Unno, M. and Kokubu, T. Immunocytochemical localization of angiotensin converting enzyme at the ultrastructural level in the human lung and kidney. Biomed. Res. 3: 169-74, 1982.

Tange, A. Distribution of peptide containing endocrine cells and neurons in the gastrointestinal tract of the dog: immunohistochemical studies using antisera to somatostatin,

substance P, vasoactive intestinal polypeptide, met-enkephalin and neurotensin. Biomed. Res. 4: 9-24, 1983.

Tapia-Arancibia, L., Pares-Herbute, N., Astier, H., Reichlin, S. and Nathanson, J. Adenylate cyclase activation is not sufficient to stimulate somatostatin release from dispersed cerebral cortical and diencephalic cells in glia free cultures. Brain Research 450: 101-110, 1988.

Thuneberg, L. Interstitial cells of Cajal: intestinal pacemaker cells? Adv. Anat. Embryol. Cell. Biol. 71: 1-130, 1982

Unverferth, D.V., O'Dorisio, T.M., Muir, W.W.III, White, J., Miller, M.M., Hamlin, R.L. and Magorien, R.D. Effect of vasoactive intestinal polypeptide on the canine cardiovascular system. J. Lab. Clin. Med. 106: 542-550, 1985.

Vigna, S.R., Mantyh, C.R., Giraud, A.S., Soll, A.H., Walsh, J.H. and Mantyh, P.W. Localization of specific binding sites for bombesin in canine gastrointestinal tract. Gastroenterology 93: 1287-95, 1987.

von Euler, U.S. and Pernow, B. An unidentified depressor substance in certain tissue extracts. J. Physiol. 72: 74-87, 1931.

Wang, H.-L, Bogen, C., Reisine, T. and Dichter, M. Somatostatin-14 and somatostatin-28 induce opposite effects on potassium currents in rat neocortical neurones. Proc. Natl. Acad. Sci. USA 86: 9616-9620, 1989.

Wattchow, D.A., Furness, J.B. and Costa, M. Distribution and co-existence of peptides in nerve fibres of the external muscle of human gastrointestinal tract. Gastroenterology 95: 32-41, 1988.

Weber, M. Cole, T. and Conlon, J.M. Am. J. Physiol. 250: G679-685, 1986.

Wiley, J. and Owang, C. Somatostatin inhibits cAMP-mediated cholinergic transmission in the MYP. Am. J. Physiol. 253: G607-612, 1987.

Willard, A.L. Substance P mediates synaptic transmission between rat myenteric neurones in cell culture. J. Physiol. 426: 453-471, 1990.

Willard, A.L and Nishi, R. Neurones dissociated from rat MYP retain differentiated properties when grown in cell culture. II. Electrophysiological properties and responses to neurotransmitter candidates. Neuroscience 16: 201-211, 1985a.

Willard, A.L and Nishi, R. Neurones dissociated from rat MYP retain differentiated properties when grown in cell culture. III. Synaptic interactions and modulatory effects of neurotransmitter candidates. Neuroscience 16: 213-221, 1985b.

Wilson, A.J., Llewellyn-Smith, I.J., Furness, J.B. and Costa, M. The fine structure of the SMP of the guinea pig ileum. I. The ganglia, neurones, Schwann cells and neuropil. J. Neurocytol. 10:759-784, 1981.

Wilson, P.O.G., Barber, P.C., Hamid, Q.A., Power, B.F., Dhillon, A.P., Rode, J., Day, I.N.M., Thompson, R.J. and Polak, J.M. The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. Br. J. Exp. Path. 69: 91-104, 1988.

Womack, M.D., MacDermott, A.B. and Jessell, T.M. Sensory neurotransmitters regulate intracellular calcium in dorsal horn neurones. Nature 334: 351-353, 1988.

Wong, H.C., J.H. Walsh, H. Yang, Y. Tache and A.M.J. Buchan. A monoclonal antibody with potent immunoneutralizing activity. Peptides 11: 707-712, 1990.

Wood, J.D. Physiology of the enteric nervous system. In: Physiology of the Gastrointestinal Tract, second edition, edited by Leonard R Johnson. Raven Press, New York. pp. 67-110, 1987.

Wood, J.D. Electrical activity from single neurones of Auerbach's plexus. Am. J. Physiol. 219: 159-169, 1970.

Yamada, T. Local regulatory actions of gastrointestinal peptides. In: Physiology of the Gastrointestinal Tract, second edition, edited by Leonard R Johnson. Raven Press, New York, 1987.

Yau, W.M., Dorsett, J.A. and Youther, M.L. Inhibitory petidergic neurones: functional difference between somatostatin and enkephalin in the MYP. Am. J. Physiol. 250: G60-63, 1986.

Yurchenko, P.D. and H. Furthmayr. Self-assembly of basement membrane collagen. Biochemistry 23: 1839-1850, 1984.

Zappia, L., Molina, E., Sianesi, M. and Bertaccini, G. Effects of natural analogues of substance P on the motility of the human gastrointestinal tract *in vitro*. J. Pharm. Pharmacol. 30: 593-594, 1978.

Zimmerman, R.P., Gates, T.S., Mantyh, C.R., Vigna, S.R., Boehmer, C.G. and Mantyh, P.W. Vasoactive intestinal peptide (VIP) receptors in the canine gastrointestinal tract. Peptides 9: 1241-1253, 1989.

APPENDIX I. CHEMICAL SOURCES

Chemical	Source
Acetic acid	BDH
Acetone	BDH
Ammonium acetate	Baker
Ammonium chloride	Fisher
Amphotericin B	Gibco
Aprotinin	Miles
Basal medium eagle (powder)	Gibco
Bovine serum albumin (fraction V)	Sigma
Bovine serum albumin (RIA grade)	Sigma, Miles
Calcium chloride	Fisher
Calcium ionophore (A23187)	Sigma
Carbon decolourizing neutral (Activated charcoal)	Fisher
Chloramine T	Sigma
Collagenase (type I, XI)	Sigma
Cytosine ß-D-arabinoside	Gibco
Dextran T-70	Pharmacia
Diaminobenzidine	BDH
Dimethylsufoxide	Sigma
Dulbecco's modified Eagle Medium	Gibco
Ethanol	Commercial Alcohol
Fetal calf serum	Gibco
Formaldehyde (histology grade)	Fisher
Forskolin	Sigma
Gelatin	Sigma
Gentamycin sulphate	Sigma
Glucose (50 % commercial solution)	Abbott
Glucose oxidase	Sigma
Glutamine	Sigma
Hank's balanced salt solution (powder)	Gibco
Hematoxylin	Fisher
HEPES	Fisher
Hydrocortisone	Sigma
Hydrogen peroxide	Fisher
Imidazole	Sigma
Insulin	Sigma
Lithium carbonate	Fisher
Magnesium sulphate	Fisher
Nerve growth factor	Collaborative Research
Normal swine serum	Gibco
Parraffin (paraplast)	Monoject
Permount	Fisher
Petroleum ether	Fisher
Phenol red	Sigma
Phorbol esters (ßPMA, 4α-phorbol)	Sigma

Chemical	Source
Picric acid	BDH
Potassium chloride	Fisher
Potassium phosphate (monobasic)	Fisher
Sephadex CM-52	Pharmacia
Sodium acetate	Fisher
Sodium barbital	Baker
Sodium bicarbonate	Fisher
Sodium chloride	Fisher
Sodium hydroxide	Fisher
Sodium ^{125}iodide	Amersham
Sodium merthiolate	Eastman Kodak
Sodium metabisulphite	Fisher
Sodium pentobarbital	Gibco
Sodium phosphate	Fisher
Sodium pyruvate	Gibco
Somatostatin	Peninsula
Substance P	Peninsula
Tris-HCL	Sigma
Triton X-100	Fisher
Xylene	Fisher

APPENDIX II. Eurocollins buffer (mmol/L)

Na 10 , K 115, Cl 15, HCO_3 10, PO_4 57.7, glucose 19.5

Osmolarity = 330 mOsm/kg

pH = 7.0