INVESTIGATION OF PEPTIDERGIC AND NITRERGIC INNERVATION OF THE HUMAN ANTRUM.

by

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ABSTRACT

Immunocytochemical studies were carried out to determine the normal peptidergic and nitrergic innervation, as well as the cellular expression of the neurokinin 1 receptor in the human antrum.

Antral tissue was obtained from 36 multiple organ donors and processed for immunocytochemical studies using antibodies/antisera for vasoactive intestinal polypeptide, nitric oxide synthase, calcitonin gene-related peptide, substance P, gastrin releasing peptide, the neurokinin 1 receptor, c-kit, s100, fibronectin, von Willebrand factor, gastrin, somatostatin and serotonin.

The significant findings from these studies are as follows: first, the human antrum has a prominent submucosal plexus containing nerve fibers and neurons which showed immunoreactivity for a majority of the neuromodulators investigated; second, the distribution of NOS was extended to include mucosal fibers innervating the surface of the antral glands; third, within the mucosal layer vasoactive intestinal polypeptide, nitric oxide synthase and gastrin releasing peptide immunoreactive nerve fibers were observed to be in close apposition to gastrin cells; fourth, within the human antrum somatostatin immunoreactivity was confined to endocrine cells in the mucosal layer.

In order to determine the targets of the neuromodulators in the human antrum knowledge of the location of their respective receptors is required. Due to the species specificity of receptor sequences these studies were confined to the neurokinin 1 receptor. Two antibodies for the NK-1r were utilized. Western blot analysis of the two antibodies showed that both antibodies detected a band at 46 kDa, the molecular

weight of the NK-1r. Both antibodies immunostained cell bodies in the submucosal and myenteric plexuses. Many of these cell bodies were often surrounded by SP-IR nerve fibers. Endothelial cells lining the major blood vessels were also immunostained with both the NK-1r antibodies. However, the monoclonal antibody, mAb12, was found to immunostain numerous other cell types. Double labeling experiments demonstrated that c-kit-IR interstitial cells of Cajal in the circular muscle were also NK-1r-IR as were gastrin-IR endocrine cells in the mucosal layer.

In the human antrum, the peptidergic and nitrergic innervation are considerably different from that reported in other animals. The presence of abundant mucosal innervation along with a well defined large submucosal plexus suggests that, in the human antrum, gastric functions may be regulated by alternate mechanisms than those proposed from animal investigations. In addition, the distribution of the NK-1r is also significantly expanded and different from that reported in other species suggesting that the mechanism of physiological regulation of the human antrum cannot be extrapolated from information obtained in animal studies.

In conclusion these data strongly suggest that human antral gastrin cells will be regulated by vasoactive intestinal polypeptide, nitric oxide, gastrin releasing peptide and substance P.

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LIST OF ABBREVIATIONS

aa	amino acids
ANS	autonomic nervous system
CGRP	calcitonin gene-related peptide
Cy3	cyanine dye 3
DMEM	Dulbecos modified eagle medium
ENS	enteric nervous system
ECL	enhanced chemiluminescence
FITC	fluorescein isothiocyanate
G cell	gastrin cell
GRP	gastrin releasing peptide
HCA	highly cross adsorbed
ICC	immunocytochemistry
IgG	immunoglobulin G
IR	immunoreactivity
LSCM	laser scanning confocal microscope
mRNA	messenger RNA
NADPH-d	NADPH-diaphorase
NDN	non-synaptic diffusion neurotransmission
NK-1r	neurokinin 1 receptor
NK2	neurokinin 2 receptor
NK3	neurokinin 3 receptor
NKA	neurokinin A
NKB	neurokinin B
NO	nitric oxide
NOS	nitric oxide synthase
NPK	neuropeptide K
NPγ	neuropeptide γ
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PPT-A	preprotachykinin A gene
PPT-B	preprotachykinin B gene
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
SP	substance P
SS	somatostatin
TBST	Tris buffered solution with Tween 20
TR	texas red
TX-100	triton-X 100
VIP	vasoactive intestinal polypeptide

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1. INTRODUCTION

1.1 The Stomach

The stomach is found between the distal end of the esophagus and the proximal end of the duodenum. The stomach can be divided into three main regions based on the types of mucosal cells present and the relationship of the stomach to surrounding structures in the abdominal cavity. These regions are the cardia, body or corpus and the antrum (Figure 1). The cardiac region is located next to the gastroesophogeal junction. The corpus is found between the cardia and the incisura angularis, from which the antrum begins and continues distally until the pyloric sphincter.

1.2 Innervation of the Stomach

The innervation of the stomach is a complex network of nerves involving all three branches of the autonomic nervous system (ANS); the parasympathetic, sympathetic and the gastro-enteric. While the parasympathetic and sympathetic nervous systems provide the stomach with extrinsic innervation, the gastro-enteric nervous system is a network of intrinsic ganglia that has the ability to mediate neural pathways independent of input from the central nervous system. This system contains approximately 10^7 to 10^8 neurons (Furness & Costa, 1980; Goyal & Hirano, 1996) and innervates the entire gastrointestinal system. The gastric nervous system is made up of one main ganglionated plexus, the myenteric, and a submucosal plexus that has

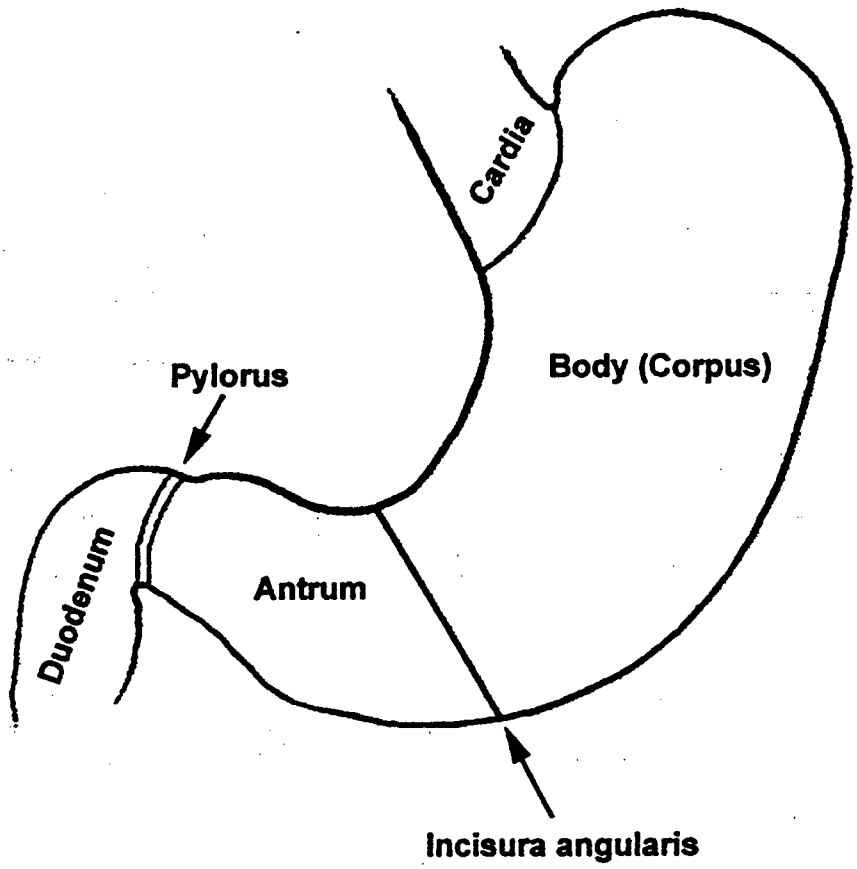


FIGURE 1. The anatomy of the human stomach (modified from Dunn, D.H. and Eisenberg, M.M., 1985).

not been as clearly defined (Figure 2). The myenteric plexus is the larger of the two and is found between the two external muscle layers. The submucosal plexus is smaller, contains very few ganglia and lies between the circular muscle layer and the muscularis mucosa. While each plexus contains numerous intraganglionic connections, the two plexuses are also interconnected.

Although the gastro-enteric nervous system can act on its own without extrinsic innervation, there are extrinsic inputs from the other two branches of the autonomic nervous system. Parasympathetic innervation of the stomach is provided by the vagus nerve, while the sympathetic innervation originates from the anteromediolateral column of the 7^{th}, 8^{th} and 9^{th} thoracic roots of the spinal cord (Dunn & Eisenberg, 1985). These extrinsic inputs are present throughout all layers of the stomach wall, however, they are especially abundant in the myenteric plexus (Berthoud & Powley, 1992; Berthoud, 1996; Gershon *et al.* 1994; Holst *et al.* 1997; Berthoud *et al.* 1997). Vagal motor fibers appear to be confined to the myenteric plexus whereas numerous vagal afferent axons enter both external muscle layers. However, these fibers do pass through the myenteric ganglia before entering the muscle (Berthoud & Powley, 1992). Vagal afferent fibers have also been shown to project to both the submucosa and mucosal layers of the gut unlike the vagal efferent axons (Berthoud & Powley, 1992; Gershon *et al.* 1994). As there are very few vagal efferent fibers in the submucosa, vagal secretory effects may be due to activation of interneurons within the myenteric plexus (Gershon *et al.* 1994). While vagal regulation of gastric functions (Berthoud, 1996; Schubert & Shamburek, 1990; Lloyd & Walsh, 1994; Furness & Costa, 1987a; Furness

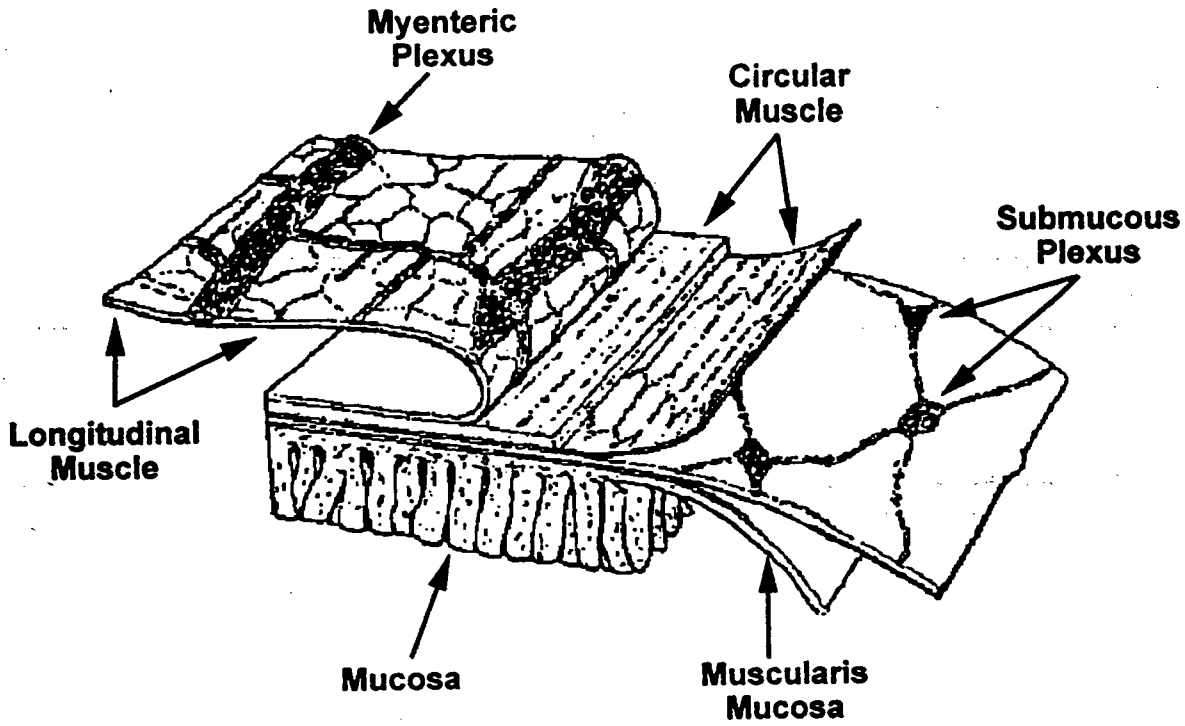


FIGURE 2. Representation of the stomach wall including the myenteric and submucosal plexuses (modified from Furness, J.B. and Costa, M., 1987c).

& Costa, 1987c) is extremely important, the contribution of the intrinsic innervation to the regulation of these functions cannot be ignored.

1.3 Neuromodulators in the Stomach

The innervation of the gastro-enteric nervous system has become of great interest in the past few decades, predominantly due to the realization that this branch of the ANS produces a large variety of neurotransmitters and neuromodulators. The initial finding of multiple peptides in neurons was the result of research carried out within the enteric nervous system (ENS). For many years the vagal innervation of the stomach was believed to be the driving force for most gastric functions. Cholinergic transmission was considered to be the major influence on gastric functions during digestion (Schubert *et al.* 1982; Schubert & Shamburek, 1990; Noto *et al.* 1997; Debas & Carvajal, 1994; Konturek *et al.* 1977). The consequence of this is that studies of the importance of intrinsic innervation have been concentrated on the ENS , mainly in the small intestine. With respect to the intrinsic peptidergic innervation of the stomach the majority of the information available is from animal models.

1.3.1 Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid peptide that belongs to the same family as secretin and glucagon. In mammals VIP is found in neurons in many different regions including the gut, brain, spinal cord and urogenital tract. The actions of VIP throughout the gut are widespread and include stimulation of intestinal

secretion and intestinal blood flow as well as relaxation of gastrointestinal smooth muscle. In the stomach the actions of VIP include smooth muscle relaxation and stimulation of gastric secretion. Studies have shown that within the guinea pig fundus (Dockray, 1987), rat stomach (Krowicki & Hornby, 1996), rabbit corpus (Baccari *et al.* 1994) and the dog antrum (Dockray, 1987) VIP can either have a direct relaxant effect or a depressant effect on cholinergic stimulation of gastric smooth muscle. The presence of VIP immunoreactivity (IR) within the ganglionic plexuses and circular muscle layer of the gastrointestinal tract (Furness & Costa, 1980) provides neuroanatomical support for the effect of VIP in the muscle layer.

Recent studies have demonstrated a co-localization of VIP and nitric oxide synthase (NOS), the enzyme responsible for the synthesis of nitric oxide (NO), in inhibitory neurons (Murthy *et al.* 1995; Keef *et al.* 1994) suggesting that NO and VIP may be released from the same neuron, thereby contributing to the inhibitory responses elicited in the muscle layers (this will be discussed in detail in the section below on Nitric Oxide).

Gastric secretion has also been shown to be mediated by the release of VIP. While VIP has been shown to stimulate acid secretion in the cat (Vagne *et al.* 1982) and inhibit acid secretion in the mouse (Schubert, 1991) or dog (Makhlouf *et al.* 1978), it has no effect on acid secretion in humans (Holm-Bentzen *et al.* 1983). In support of a role for VIP in mucosal gastric regulation immunocytochemical studies have identified VIP-IR nerve fibers in the mucosal layer of the human fundus and antrum (Ferri *et al.* 1984), fetal and adult pig stomach (van Ginneken *et al.* 1996; Holst *et al.* 1992) and the

rat glandular stomach (Berthoud, 1996). Although VIP and NO are known to be co-localized in the innervation of the muscle, secretomotor neurons supplying the mucosa are thought to release VIP only (Keef *et al.* 1994; Guo *et al.* 1997), suggesting that VIP alone may be involved in the modulation of epithelial functions.

1.3.2 Nitric Oxide

In the early 1980's Furchgott and Zaawadski demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium and suggested that this effect was mediated by a labile humoral factor (Moncada *et al.* 1991). This factor later became known as the endothelium-derived relaxing factor (EDRF). It was first suggested in 1986 by Furchgott that EDRF may be NO (Furchgott, 1988).

Nitric oxide is a gaseous, bioregulatory free radical which has proven to be a ubiquitous physiological regulator. It is synthesized from the reaction of L-arginine and molecular oxygen by the enzyme, NO synthase (NOS). Originally only two types of NOS were identified; constitutive and inducible NOS. Constitutive NOS is Ca^{2+}/calmodulin and NADPH dependent and produces a small amount of NO for short periods of time in response to stimulation. Constitutive NOS is found within endothelial cells and neurons (Moncada *et al.* 1991). The second type, inducible NOS, is NADPH dependent, however it is not Ca^{2+}/calmodulin dependent. Inducible NOS may be stimulated by certain cytokines and bacterial products and produces larger quantities of NO for longer periods of time. It is found within macrophages, neutrophils,

endothelial cells, fibroblasts, hepatocytes and smooth muscle cells. A more recent terminology is used to describe three distinct isoforms of the enzyme: neuronal (constitutive) NOS, endothelial (constitutive) NOS and inducible NOS.

Nitric oxide has been found in neuronal cell bodies of the submucous and myenteric plexuses and in nerve fibers in the circular and longitudinal muscle layers of the pig gastric fundus (Lefebvre *et al.* 1995). Since nitrergic nerve cells contain NADPH-diaphorase (NADPH-d), these cells can be localized using NADPH-d histochemistry. Within the human stomach, a study in 1998 on the distribution and neuron density of NADPH-d positive neurons demonstrated that NADPH-d positive neurons in the submucous plexus were predominantly found in the ganglia in close association with the circular muscle (Manneschi *et al.* 1998). They observed that NADPH-d positive fibers were present in the innermost portion of the circular muscle layer of the corpus and a few fibers were also present in the muscularis mucosa and lamina propria, but most were localized to the glandular crypts and blood vessels.

Nitric oxide has been shown to be of importance as a mediator of transmission from enteric inhibitory neurons to gastrointestinal smooth muscle (Allescher & Daniel, 1994). In 1977, two independent groups showed that organic nitrites induced dose-dependent increases in the levels of cyclic guanosine monophosphate (cGMP) in smooth muscles. Subsequently, studies showed that all of the vasodilators and NO activate soluble guanylate cyclase. It is now widely accepted that NO induces smooth muscle relaxation by the activation of this enzyme and consequent increase of cGMP levels that lead to protein phosphorylation and muscle relaxation (McDonald & Murad,

1996; Moncada *et al.* 1991).

While it is clear how NO acts to cause relaxation within the smooth muscle itself, the precise contribution of either NO and/or VIP to elicit this relaxation remains unclear. One hypothesis is that NO released from nerve terminals regulates VIP release which in turn regenerates NO by activating NOS present in the smooth muscle cells (Figure 3). Evidence to support this theory comes from studies on isolated myenteric ganglia and smooth muscle strips. Studies of isolated myenteric ganglia have shown that NOS inhibitors abolish the release of both VIP and NO, implying a dependence of VIP release on NO synthesis (Murthy *et al.* 1995; Jin *et al.* 1996) and that addition of NO stimulates VIP release but the addition of VIP has no effect on neuronal NO synthesis.

This ability of NO to regulate the release of VIP is also evident in innervated muscle strips. Upon electrical field stimulation of gastric muscle strips, inhibitors of NOS abolished VIP release and relaxation at low intensities but only partially inhibited it at higher intensities, suggesting that VIP release is facilitated by NO release but can occur independently if the stimulus is of a high intensity (Murthy *et al.* 1995; Grider *et al.* 1992). On the other hand, a VIP antagonist, VIP-(10-28), strongly inhibited neurally induced NO synthesis and relaxation, but augmented VIP release in both rabbit and rat gastric muscles (Murthy *et al.* 1995; Jin *et al.* 1996). As VIP had no effect on the production of NO in neurons, as shown in the isolated ganglia, yet NO production in gastric smooth muscles was inhibited by the VIP antagonist, these data suggest that a major component of NO is formed in muscle cells by the actions of VIP (Murthy *et al.* 1995; Jin *et al.* 1996).

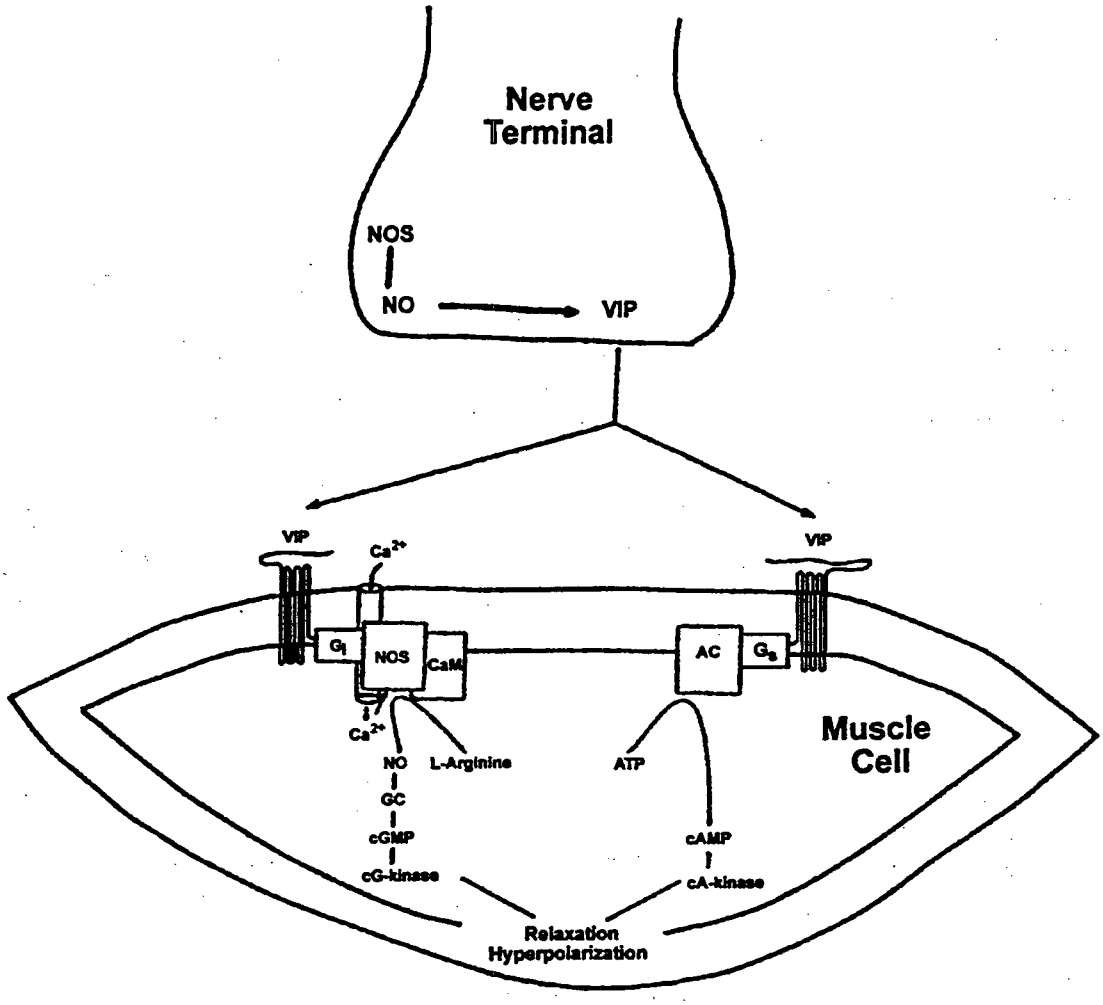


FIGURE 3. Proposed model depicting the origin, synthesis and function of NO in neurons and smooth muscle of the gut and its interaction with VIP (modified from Murty, K.S., Grinder, J.R., Jin, J.G. and Makhlouf, G.M., 1995).

Evidence to support the regulation of NO synthesis in muscle cells by VIP is shown in studies of dispersed muscle cells in which the addition of VIP initiates a sequence of events including increases in cytosolic Ca^{2+}, increases in NO, cGMP and cAMP as well as an increase in cAMP-dependent and cGMP-dependent protein kinase activity. Although there is significant data to support the interactions of VIP and NO (Figure 3), there are other studies to the contrary. Pharmacological studies have shown that antagonism of both NO and VIP is necessary to abolish the nerve-mediated relaxation of the intestinal muscles (Keef *et al.* 1994). Studies also show that in the rat pylorus there does not appear to be any interaction between NO and VIP (Willis *et al.* 1996; Holzer-Petsche & Moser, 1996).

1.3.3 Gastrin Releasing Peptide

Gastrin releasing peptide (GRP) was originally isolated from the porcine stomach by McDonald *et al* in 1979 using a gastrin bioassay (McDonald *et al.* 1979). It is the mammalian counterpart to bombesin, first isolated from the skin of two European amphibians, *Bombina bombina* and *Bombina variegata* (Dockray *et al.* 1979). Sequencing studies showed that the C-terminus of GRP and bombesin are identical in 9 out of ten amino acid residues (Reeve, Jr. *et al.* 1988). The mammalian peptide is a 27 amino acid peptide (Reeve, Jr. *et al.* 1988; McDonald *et al.* 1979) whose biologically active region is found in the C-terminal tail (Furness *et al.* 1988; Vigna *et al.* 1988; Battey *et al.* 1988).

Within the brain GRP has been shown to modulate body temperature, satiety,

and other homeostatic functions (Battey *et al.* 1988). Outside of the central nervous system, GRP has been localized to peripheral nerves in the gut (Miller *et al.* 1989; Dockray *et al.* 1979) where it has been shown to stimulate the release of gastroenteropancreatic hormones and modulate gastrointestinal motility (Battey *et al.* 1988).

One of the primary functions of GRP released from excitatory nerve fibers in the stomach is to stimulate the release of gastrin from the antral G cells (Koop *et al.* 1997; Buchan & Meloche, 1994; Makhlouf & Schubert, 1988; Holst *et al.* 1987; McDonald *et al.* 1979; Mccoll & Elomar, 1995; DuVal *et al.* 1981; Walsh *et al.* 1981; Giraud *et al.* 1987). In most species an increase in gastrin release is associated with an increase in gastric acid secretion. However, in rats intravenous bombesin stimulated gastrin release but did not stimulate acid secretion (Walsh *et al.* 1988; Bertaccini *et al.* 1973). Gastrin releasing peptide not only stimulates gastrin release but it has also been shown to inhibit gastric emptying of solid food in the rat and human and to increase antral contraction (Walsh *et al.* 1988).

The biological activity of GRP is supported by the anatomical location of GRP-IR nerves and nerve fibers. In the stomach, GRP-IR has been localized to neurons and nerve fibers in the myenteric plexus as well as nerve fibers within the ganglia, in the two external muscle layers and in the mucosa in several species (Berthoud, 1996; Mccoll & Elomar, 1995; Holst *et al.* 1987; Dockray *et al.* 1979; Ekblad *et al.* 1985; Miller *et al.* 1989). One study has looked at the anatomical relationship between GRP-IR nerve fibers and antral G cells in the guinea pig, rat, dog and human (Miller *et al.*

1989). They showed that in the guinea pig and rat stomach there was a very sparse innervation of GRP-IR nerve fibers of the antral mucosa. The majority of GRP positive fibers were observed around the basal mucosa, next to the muscularis mucosa. From the basal mucosa fibers projected up into the glandular region. In the dog, GRP-IR nerve fibers were observed in the glandular and apical mucosal lamina propria. In the human, GRP positive fibers formed a subglandular plexus with small nerve bundles within the lamina propria of the basal mucosa (Miller *et al.* 1989). A majority of antral G cells were localized to the middle portion of antral glands, however in rats the G cells are located in the lower portion (Mccoll & Elomar, 1995; Miller *et al.* 1989). Studies of the relationship between GRP fibers and G cells demonstrated that the nerve fibers were at least 2 μm away from G cells (Miller *et al.* 1989). These findings suggest that either GRP released from nerve fiber varicosites must diffuse long distances through the interstitium and/or enter the local microcirculation before affecting gastrin release from the G cells.

The presence of GRP-IR nerve fibers in the mucosal region and evidence that electrical stimulation of the vagus nerve resulted in an increase in GRP-IR in the perfused rat antrum (Berthoud, 1996) suggests that GRP stimulated antral gastrin release is regulated by the vagus nerve.

A study by Berthoud (Berthoud, 1996) found that a portion of the GRP-IR neurons are directly contacted by vagal preganglionic efferent nerves in both the myenteric and the rare submucosal plexuses ganglion of the rat antrum. They also found that VIP was frequently co-localized with GRP in the myenteric plexus and noted

that neurons containing different neurochemical phenotypes display distinct projection patterns in the rat antrum.

1.3.4 Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid polypeptide derived from the calcitonin gene by alternative mRNA splicing (Tan *et al.* 1994; Green & Dockray, 1987; Tache *et al.* 1984; Emeson *et al.* 1992; Rosenfeld *et al.* 1992). CGRP was the first peptide identified using molecular techniques in the absence of prior biological information. It has been localized in neurons of the brain, spinal cord and peripheral nervous system (Hokfelt *et al.* 1992) and has been shown to have numerous biological effects; vasodilation, increased cardiac contractility, relaxation of nonvascular smooth muscle, immunological and trophic actions such as increased cell proliferation (Lundberg, 1996); reduction of gastric acid secretion, regulation of calcium metabolism, increased body temperature and decreased food intake (Quirion *et al.* 1992).

Within the peripheral nervous system CGRP has been localized to the capsaicin-sensitive sensory nerves and is often co-localized with members of the tachykinin family (Lundberg, 1996; Kinoshita *et al.* 1993; Hokfelt *et al.* 1992). A study on the gastric mucosa noted considerable differences when comparing the distribution of CGRP-IR nerve fibers in nine different species. While fibers immunoreactive for CGRP were found in the antral mucosa of the mouse, rat, mole, hamster, ferret and cat, fibers were rarely observed in the pig and guinea pig mucosa and no fibers were found

in the human antral mucosa (Sundler *et al.* 1991). The precise distribution of CGRP-IR fibers in each species was slightly different.

Within the stomach a majority of the major blood vessels are located within the submucosa. In the stomach, CGRP is a more potent vasodilator than in the duodenum (Holzer & Lippe, 1992). Maintaining or increasing blood flow is important for protection of gastric mucosal integrity (Holzer & Lippe, 1992). Accordingly, abundant CGRP-IR nerve fibers have been observed in this layer surrounding vascular structures (Sternini, 1992). However the density of fibers around arteries is usually higher than that around veins (Holzer & Lippe, 1992). It has been shown that CGRP-IR fibers surrounding the submucosal vessels are located in the adventitial layer of the vessel wall (Sternini, 1992). A high density of CGRP receptor binding sites in association with arterial vessels and the presence of abundant CGRP-IR fibers surrounding the arteries supports the vasodilatory role of the peptide (Sternini, 1992).

Studies have also shown CGRP to be associated with an inhibition of gastric acid secretion. The exact mechanism by which CGRP exerts its effects is still somewhat controversial. It remains to be determined to what extent the central, peripheral or vascular effects of CGRP mediate gastric acid secretion. The central action of CGRP to inhibit acid secretion in rats and dogs is through modulation of parasympathetic outflow to the stomach (Tache, 1992). Injections of CGRP into the cerebral spinal fluid of rats resulted in a decrease in cholinergic and histaminergic input to the parietal cells of the corpus which may also contribute to the inhibition of acid secretion. (Tache, 1992). Similarly, in dogs it was observed that meal-induced gastrin

release was not altered after injection of CGRP into the third ventricle, however, there was an inhibition of acid secretion (Lenz *et al.* 1986b; Lenz *et al.* 1986a). Studies have also demonstrated that the peripheral actions of CGRP in the rat, dog, rabbit and human involve the release of somatostatin through an interaction with a CGRP receptor on D cells (Manela *et al.* 1995; Tache, 1992; Inui *et al.* 1991).

1.3.5 Substance P

Substance P (SP) was discovered in 1931 by von Euler and Gaddum and was the first of the gut neuropeptides to be discovered. Ever since its discovery the gut has been a model system in which to study the actions and physiological significance of SP.

Nearly 40 years after the discovery of SP, Leeman and her colleagues isolated and sequenced the peptide from the bovine hypothalamus (Dockray, 1987; Tache *et al.* 1991; Chang & Leeman, 1970). Studies have shown that SP is an 11 amino acid peptide (Tache *et al.* 1991; Chang *et al.* 1971). Previous to the isolation of the mammalian peptide, Erspamer had isolated several peptides with similar biological activity from the skin of various amphibians and the salivary gland of the mollusc *Eledone* (see Dockray, 1987 for references). This family of peptides shares a common C-terminal sequence: Phe-X-Gly-Leu-Met-NH_2. The X is a Phe in SP but in the other related peptides it is either a Tyr, Ile or Val. These peptides were called tachykinins and the name is now used to describe the entire group of SP-related peptides (Dockray, 1987). The term tachykinin was used to describe the rapid development of

the contraction produced by these peptides in smooth muscles (Maggi, 1995).

The main members of the mammalian tachykinin family are substance P (SP) neurokinin A (NKA) and neurokinin B (NKB). Later N-terminally extended forms of NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ) were added to the family (Holzer & Holzer-Petsche, 1997a). All members of the mammalian tachykinin family, except NKB, are derived from alternative splicing of the primary transcripts of the preprotachykinin (PPT)-A gene, while NKB is derived from the PPT-B gene (Figure 4) (Holzer & Holzer-Petsche, 1997a; Regoli *et al.* 1994).

Substance P is widely expressed throughout the central and peripheral nervous systems (Maggi, 1995). In the periphery SP has been shown to stimulate as well as inhibit smooth muscle contraction, increase blood flow, inhibit acid secretion and intestinal absorption (Holzer & Holzer-Petsche, 1997a; Holzer & Holzer-Petsche, 1997b; Dockray, 1987). Stimulation of contractile activity in the smooth muscle layers of the gut by SP has been shown to be not only a direct effect on the muscle but also an indirect action on other motility regulating pathways. Differences in the excitatory motor responses in various species and muscle layers is a result of differences in receptor subtypes present as well as the transduction mechanisms participating in the motor responses (Holzer & Holzer-Petsche, 1997a). While the excitatory responses have been shown to be the predominant motor effect elicited by SP, it has been reported to inhibit gastrointestinal motility as well. These inhibitory effects are the result of SP acting on other neural pathways either by stimulating inhibitory neurons or by interrupting excitatory relays (Holzer & Holzer-Petsche, 1997a).

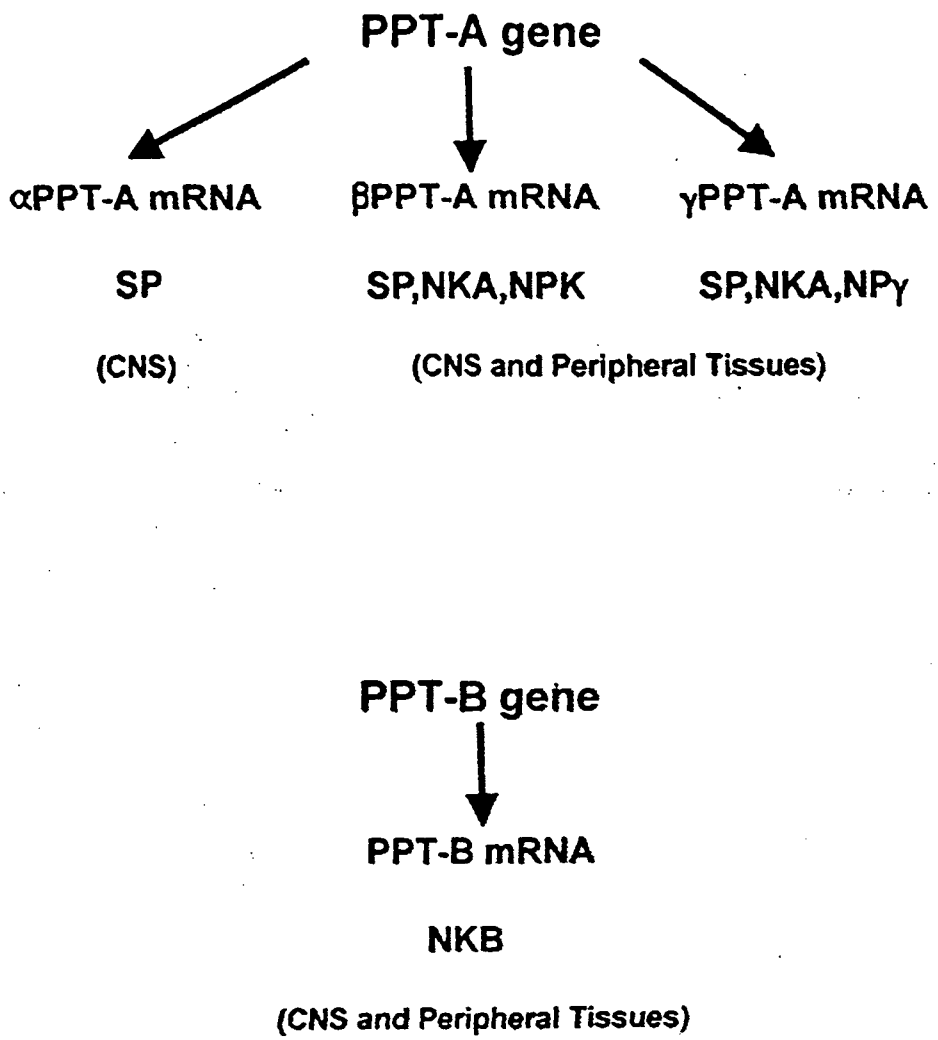


FIGURE 4. Molecular biology of mammalian tachykinins (modified from Regioli, D., Boudon, A. and Fauchere, J.L., 1994).

The discovery of pharmacologically and physiologically different responses to SP among various species is supported by the immunohistochemical data in which there are also considerable alterations in the chemical coding (Dockray, 1994). In the human SP-IR fibers have been located in both external muscle layers throughout the stomach with the highest innervation occurring in the circular muscle of the antrum and pylorus (Wattchow *et al.* 1988). In the rat and mouse stomach numerous SP-IR fibers were observed in the myenteric plexus and the external muscle layers. Again more fibers were observed in the circular muscle layer than in the longitudinal layer. In the mucosa fine SP positive fibers were found to extend up to the mid-portion of the glands. A few fibers were observed in the submucous ganglia (Ekblad *et al.* 1985). In the canine corpus, SP-IR was observed in cell bodies of the myenteric plexus and nerve fibers in the muscle and mucosal layers (Furness *et al.* 1991). In the rat stomach, SP-IR nerve fibers were observed throughout the wall of the body and antrum (Sharkey *et al.* 1984).

One study demonstrated intense labeling of fibers in the myenteric plexus surrounding non-SP-IR cell bodies with abundant SP-IR in the circular muscle region, but sparse innervation of the longitudinal muscle. This study also reported a fine plexus of nerve fibers in the submucosal region often associated with blood vessels as well as SP-IR fibers in the mucosa running between the gastric glands (Sharkey *et al.* 1984).

A study by Sundler *et al.* in 1991, comparing the distribution of SP nerve fibers in the gastric mucosa of several species, observed that in the gastric mucosa of the

mouse and rat, SP-IR nerve fibers were few to moderate in number with scattered fibers running up into the glands. They also found that there were more SP-IR fibers in the antrum than in the body of the stomach of these species. In the ferret and the cat, there were very few weakly immunostained fibers in the corpus. However, in the pig SP-IR fibers were numerous in the mucosa of both the corpus and antrum. In addition, in the pig SP containing nerve cell bodies were frequently observed in little ganglia found within the mucosa, underlying the glands and a few SP-IR cell bodies were also found in the submucosal plexus. In the human Sundler *et al.* found that SP-IR nerve fibers were greater in number in the antrum than in the corpus. They were unable to locate any SP-IR cell bodies within the mucosa, however, SP positive neurons were found within the submucosa (Sundler *et al.* 1991).

1.4 Neuropeptide Receptor Interactions

Neural communication is classically considered to occur by synaptic transmission. In this process the transmitter is released from the synaptic bouton of the presynaptic neuron into the synaptic cleft. The transmitter binds to the receptor site on the postsynaptic density. However, in the past few decades it has been shown that neurotransmission within the central and peripheral nervous systems can be achieved by non-synaptic diffusion neurotransmission (NDN) (Dismukes, 1979; Bach-y-Rita, 1994b; Bach-y-Rita, 1993; Bach-y-Rita, 1994a). Neurotransmission of this type occurs by diffusion of neurotransmitters through the extracellular fluid to the target cells. The result is activation of extrasynaptic receptors, intrasynaptic receptors reached by

diffusion into the synaptic cleft and the diffusion of substances through both the extracellular fluid and cellular membranes (Bach-y-Rita, 1993). It has also been referred to as volume transmission by Fuxe and Agnati and they have emphasized the role of neuropeptides as volume transmission signals (Fuxe & Agnati, 1991).

To date no synaptic contacts have been seen between axons of gastric interneurons and their targets outside of the ganglia. Therefore, volume transmission must be the mode of communication. It would be reasonable to expect that a system as large as the gastrointestinal tract, would be organized so as to minimize the number of axons and dendrites, by reducing the requirement for synaptic contact with each target cell.

Once neuromodulators are released into the extracellular fluid the question then becomes, how far can they travel in order to reach their respective target cells? The sphere of influence for a neuropeptide depends on the distance it crosses by diffusion, the rate at which it is inactivated and the number of available receptors. Unfortunately, not much is known about how far these neuropeptides can spread. A study on frog sympathetic ganglia indicated that a peptide resembling luteinizing-hormone-releasing hormone (LHRH) probably diffuses tens of micrometers before it reaches a target (Zupanc, 1996). In 1990 a study by Gally *et al.* (Gally *et al.* 1990) suggested that nitric oxide, a neurotransmitter, may diffuse up to 100 μm in 5 seconds. One of the arguments made against NDN pertains to the amount of extracellular space available for diffusion through the brain. Although this may be a concern in the central nervous system, it is not a limiting factor in the stomach where there is extensive interstitial

space through which neuromodulators can diffuse.

The distribution of potential neuropeptide receptors provides further support for the concept of volume transmission. Numerous studies have provided evidence of mismatches between the ultrastructural localization of synapses and receptor sites. There appears to be a distinct mismatch in the distribution of substance P fibers and the density of its binding sites in the substantia nigra (Zupanc, 1996). In cortical neurons it has been shown that 68% of the surface membrane is immunoreactive for the substance P receptor yet only 9% of these receptor sites are located next to synaptic profiles (Liu *et al.* 1994). Studies by Herkenham, who has reviewed the evidence for mismatches for a large number of neurotransmitters demonstrated that in the brain mis-matches are the rule rather than the exception (Herkenham, 1987). Although mis-matches have been shown in the central nervous system there is currently no indication of mis-matches in the gastrointestinal system. However, NDN is undoubtedly the mode of communication within the gastroenteric nervous system due to absence of synapses outside the ganglia.

The characterization of the distribution of peptidergic receptors in the stomach has predominantly been confined to animal models. While there are numerous antibodies available to detect peptideregic receptors in various animal tissues, these antibodies have not been successfully used to immunolabel the receptors in human tissues. Due to the non-conservation of receptor sequences for the neuromodulators examined between species, we only had access to antibodies capable of detecting the human NK-1r.

1.5 The Neurokinin 1 Receptor

There are three primary tachykinin receptor subtypes which have been identified neurokinin 1 (NK1), neurokinin 2 (NK2) and neurokinin 3 (NK3). Of the three receptors, NK1 preferentially binds SP while NK2 has a high affinity for NKA and NK3 selectively binds NKB (Stratowa *et al.* 1995; Regoli *et al.* 1994; Maggi, 1995). All three receptors belong to the G-protein coupled receptor superfamily. This family of receptors have seven transmembrane spanning segments with an extracellular amino terminus and an intracellular carboxy terminal tail (Regoli *et al.* 1994; Maggi, 1995). Studies have shown that the receptors are discriminatory for their agonists and antagonists but not for the second messengers. All three receptors interact with G-proteins which leads to increased IP_3 production thereby causing increases in intracellular Ca^{2+} (Regoli *et al.* 1994; Boyd *et al.* 1991).

The human NK-1 receptor (NK-1r) was first cloned in 1991 by two independent groups (Figure 5) (Takeda *et al.* 1991; Gerard *et al.* 1991). These studies showed that the human receptor is approximately 92-94% homologous to the rat NK-1r. The receptor is comprised of 407 amino acids (Takeda *et al.* 1991; Gerard *et al.* 1991) and has a molecular weight of 46 kDa (Regoli *et al.* 1994). A study in 1996 demonstrated that the NK-1r interacts specifically with the α subunit of G_q and G_{11} (Macdonald *et al.* 1996) to elicit increases in intracellular Ca^{2+}.

The widespread distribution of SP throughout the body and its diverse biological functions have been well documented. In the past decade a lot of research has focused on the distribution of the NK-1r in a variety of species and tissue types. The

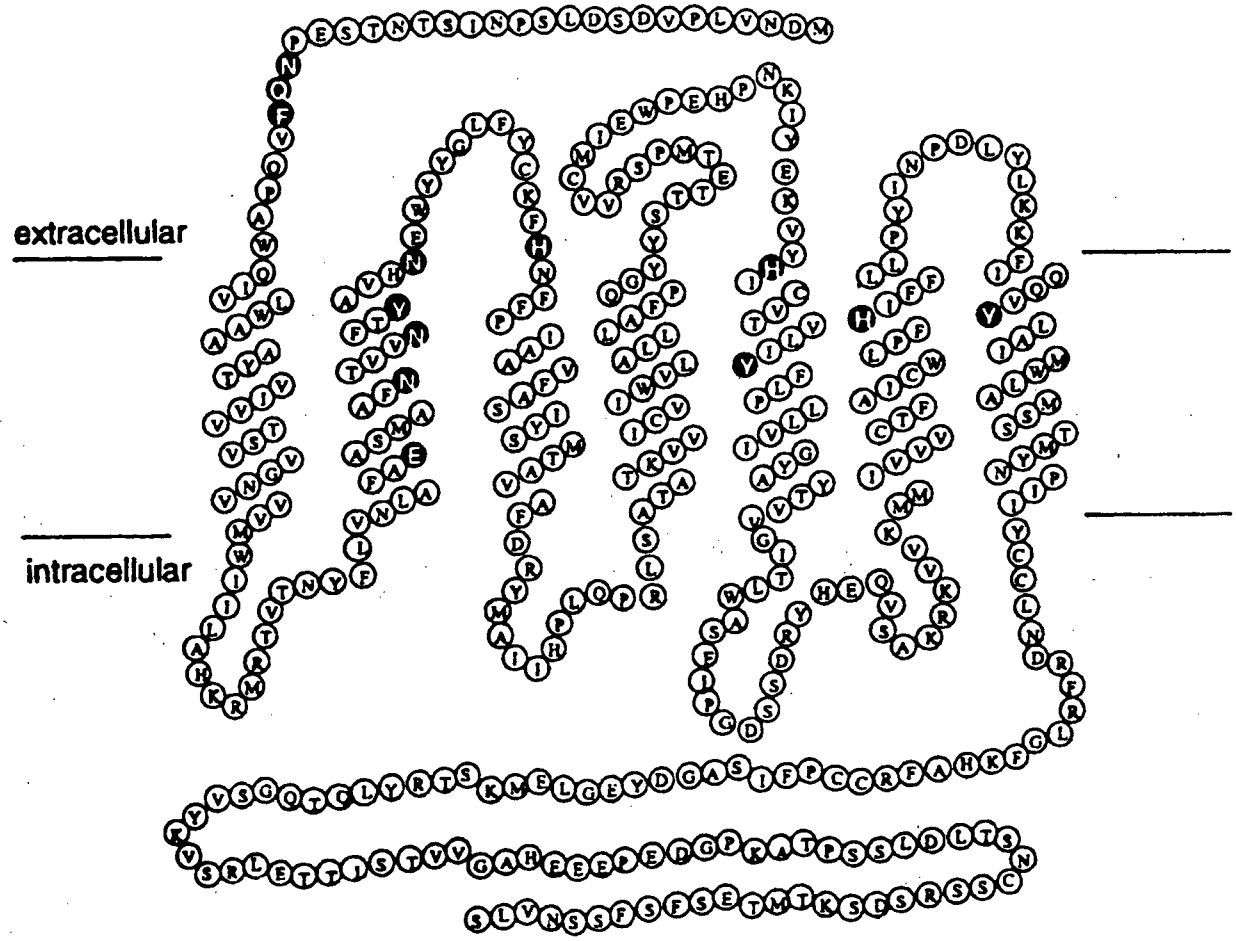


FIGURE 5. Representation of the human NK-1r (modified from Huang, R.R., Yu, H., Strader, C.D. and Fong, T.M., 1994).

receptor has been localized to various structures of the rat central nervous system including the dorsal and lateral horns of the spinal cord (Liu *et al.* 1994; Zerari *et al.* 1995; Bret-Dibat *et al.* 1994; Pollock *et al.* 1997), the olfactory bulb, the subfornical organ, the hippocampus, superior colliculus (Zerari *et al.* 1995), the locus coeruleus (Zerari *et al.* 1995; Liu *et al.* 1994), the cerebral cortex, the cerebellum (Liu *et al.* 1994) and the dorsal motor nucleus of the vagus (DMV) (Ladic & Buchan, 1996). Immunohistochemistry, *in situ* hybridization or polymerase chain reaction (PCR) experiments have identified the NK-1r-IR in human striatum (Aubry *et al.* 1994; Kowall *et al.* 1993), nucleus basalis and hippocampus, as well as in deep neocortical layers and the cortical subplate (Kowall *et al.* 1993). Outside the central nervous system, the NK-1r has been localized to endothelial cells of the rat tracheal mucosa (Bowden *et al.* 1996) and human small bronchi (Naline *et al.* 1996). Binding sites for SP have been found on the endothelium of bronchial microvessels in both the human and guinea pig lung. However, in the guinea pig lung SP binding sites were also observed on bronchial smooth muscle and pulmonary arteries (Walsh *et al.* 1994). Reverse transcriptase PCR (RT-PCR) has demonstrated the presence of NK-1r mRNA in human peripheral blood monocytes and macrophages (Ho *et al.* 1997).

Within the gastrointestinal tract immunocytochemical studies of the guinea pig small intestine have demonstrated NK-1r-IR on neurons in the submucosal (Southwell *et al.* 1996; Portbury *et al.* 1996; Moore *et al.* 1997) and myenteric plexuses (Southwell *et al.* 1996; Portbury *et al.* 1996; Grady *et al.* 1996b; Southwell *et al.* 1998) as well as the interstitial cells of Cajal (Portbury *et al.* 1996). In the guinea pig gastric corpus, NK-

1r-IR was observed only on the myenteric neurons, as submucosal ganglia were rare or absent in this region (Portbury *et al.* 1996). In the rat NK-1r-IR was localized to the neurons and varicose nerve fibers of the submucosal and myenteric plexuses as well as the interstitial cells of Cajal of the ileum (Vannucchi *et al.* 1997; Grady *et al.* 1996a; Sternini *et al.* 1995), the duodenum, jejunum, and colon (Grady *et al.* 1996a; Sternini *et al.* 1995). In the stomach, NK-1r-IR was limited to neurons in the myenteric plexus (Grady *et al.* 1996a; Sternini *et al.* 1995) as in the guinea pig. In human antrum, binding sites for SP were expressed by arterioles, venules, circular muscle, the submucosa and the germinal centers of lymph nodules and epithelial cells in the mucosa (Gates *et al.* 1988). Throughout the rest of the gut SP binding sites have also been localized to the longitudinal muscle and muscularis mucosa (Gates *et al.* 1988). While SP binding sites have been localized within the human antrum, the distribution of the NK-1r, specifically, has not been identified.

1.6 Thesis Proposal

During studies of the regulation of gastrin release from human antral G cells it became evident that the response of these cells to neural inputs differed significantly from animal models. Differences in anatomical size, diet and nutritional requirements would also be expected to demand species specific regulation of gastric functions, which might be reflected in different neural distributions. While neural pathways in animal models have been well characterized in other regions of the gut, very few studies have examined the neuromodulator distribution in the human stomach. Our

laboratory has a unique advantage in that we have samples of the human antrum readily available for study.

The objective of this thesis is to establish the chemical coding of the human antral innervation and the cellular expression of the neurokinin 1 receptor. In order to fulfill this objective the following specific aims were undertaken; 1) to establish the peptidergic and nitrergic innervation of human antral tissue with respect to the major gut neuromodulators: vasoactive intestinal polypeptide, nitric oxide, gastrin releasing peptide, calcitonin gene-related peptide and substance P; 2) to determine the effect of colchicine treatment on the intensity and distribution of neuromodulator-IR; 3) to establish the distribution of the NK-1r in the human antrum; 4) to characterize the cellular expression of the NK-1r in the human antrum.

2. METHODS

2.1 Tissue

All experiments were carried out on human antral tissue which was obtained from multiple organ donors in association with the Pacific Organ Retrieval for Transplantation Program. All tissue was retrieved with ethical approval from the Clinical Research Ethics Board of the University of British Columbia. Human antral tissue was obtained from 36 multiple organ donors (see Table 1: 16 females, 20 males, ranging in age from 12-59 yrs)

2.2 Immunocytochemistry (ICC)

2.2.1 Tissue Preparation

Upon receiving the tissue, a section of antrum was fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature. Two different batches of PFA (Fisher Scientific, Fairlawn, NJ) were used. The first 19 donors were fixed in the first batch while the last 17 donors were treated with the second batch. The tissue was washed three times in 0.1 M phosphate buffer solution (PBS) and stored in PBS containing 20% sucrose for 24 h at 4°C before being frozen in 2-methylbutane cooled in liquid nitrogen to -60 °C. Cryostat sections (40-90 μm) were cut and placed in wells containing PBS.

TABLE 1: AGE AND SEX OF DONORS

Age	Males	Females
Under 20	116,123,126,127,128,143,145	125,132,133,149
20 - 29	119,121,131,136,142	
30 - 39	114,122,124,138,141	129
40 - 49	130,148	118,120,137,139
50 - 59	117	115,134,135,140,144,146,147
Total	20	16

2.2.2 Immunofluorescence

All tissue sections were incubated in 10% normal horse serum in PBS containing 0.3% Triton X-100 (TX-100) for 60 min at room temperature. Incubation in normal horse serum is required to reduce the amount of non-specific binding of antisera. Triton X-100 was used to increase permeability of the tissue and thereby allow for better penetration of the antibodies through the thick sections. Once the tissues were blocked the sections were incubated with a combination of antibodies and antisera in 5% normal horse serum in PBS with 0.3% TX-100 for 48-72 h at 4 °C. After incubation the tissue sections were washed in PBS (3 x 5 min) at room temperature. The tissues were subsequently incubated in secondary antibodies for 60-90 min at room temperature. Secondary antibodies were chosen based on the species in which the primary antibody was raised. All secondary antibodies were conjugated to a different fluorophore and were diluted in 5% normal horse serum in PBS with TX-100 to prevent non-specific binding of the secondary layer. Tissue sections were washed in PBS and mounted on glass slides. Coverslips were applied with PBS/glycerine (1:9) adjusted to a pH of 8.6 to prevent excess fading of the fluorophore.

Sections were pre-screened using a Zeiss Axiophot fluorescent microscope and were chosen for subsequent investigation based on the intensity of the fluorescence and the level of background. Chosen sections were then scanned under a Bio-Rad MRC 600 laser scanning confocal microscope.

2.2.3 Controls

All antisera were prescreened to reduce the possibility of cross-reactivity. In cases were the primary antisera had not been previously characterized primary antibodies were preabsorbed with the parent peptide for 24 h before application to the tissues. In all cases the immunoreactivity of each antisera was determined to be specific. The secondary labels were either antisera purchased from Jackson Immunocytochemical Laboratories (West Grove, PA) or fluorescent Alexa dyes purchased from Molecular Probes (Eugene, OR). Antisera from Jackson was considered suitable for multi-labeling experiments while the Alexa dyes from Molecular Probes are highly cross adsorbed.

Control incubations were carried out in which the primary antibody was omitted from the serum solution. Sections were also incubated with a primary antibody, followed by a secondary antibody raised against a different species to determine if there was cross reactivity between the species. All controls were carried out in parallel to the experimental sections such that antibody/antisera concentrations and the timing of incubations were consistent.

2.3 Colchicine Treatment

A small piece of tissue (N= 6, 1 female, 5 males, ranging in age from 19-49 yrs) was taken from the antrum and treated with colchicine. This was to prevent further axonal transport of peptides and increase the concentration of peptides in the cell bodies (Ekblad *et al.* 1996; Arluison *et al.* 1994; Boyer *et al.* 1994). Tissue sections

were placed 5 ml of growth media (Dulbecos modified eagle medium (DMEM) with penicillin and streptomycin, fetal bovine calf serum, insulin, hydrocortisone, pen/strep glutamine, neural growth factor and cytosine furanoside) with 500 µl of colchicine (1 mg/ml media) for 24 h at 37 °C in 5% CO_2. The tissue sections were subsequently fixed in 4% PFA for 2 h at room temperature. The tissue was washed three times in 0.1 M PBS and stored in PBS containing 20% sucrose for 24 h at 4 °C before being frozen in 2-methylbutane cooled in liquid nitrogen to -60 °C. Cryostat sections (40-90 µm) were cut and placed in wells containing PBS. The tissues were used for immunocytochemical studies as described in section 2.2.2 Immunofluorescence.

2.4 Confocal

Once the tissue sections were prescreened using a Zeiss Axiophot fluorescent microscope. Sections with a high intensity of fluorescence and a low level of background immunostaining were then scanned under a Bio-Rad MRC 600 laser scanning confocal microscope (LSCM). The confocal microscope allows for examination of a thick specimen with an increased resolution. This is achieved by the ability of the LSCM to optically scan in the z plane at sub-micron increments. The confocal microscope also discriminates against out of focus light thereby improving the resolution of the final scanned image.

The collected image stacks were processed using the NIH image program to produce maximum intensity projections. A maximum intensity projection is a single image generated from a consecutive series of images. A computer algorithm

determines the intensity of a pixel in one image and compares it to the corresponding pixel in the previous image. The pixel with the greatest intensity is retained and used as a basis for comparison with the next image. This procedure is repeated for all images in the series. The final maximum intensity projection represents the cumulative result of all of the maximum intensities from all the images in the stack. Once the maximum intensity projection images were obtained they were imported into Adobe Photoshop 4.0® for further processing to determine the overall distribution of the nerve populations and the plurichemical coding.

2.5 Westerns

Proteins were isolated from intact full thickness tissue immediately after dissection by rapid homogenization in boiling lysis buffer (5% SDS and 10 mM Tris-HCl pH 7.4) and microwaving for 15-20 seconds. The homogenate was centrifuged (12,000 X g, 15 °C) for 5 min, diluted 1:1 with electrophoresis sample buffer (125 mM Tris-HCl ph 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue and 1% β-mercaptoethanol). While a 10% Tris/Glycine SDS-Polyacrylamide Gel (4.0 ml dH2O, 3.3 ml 30% Acrylamide Solution, 2.5 ml 1.5M Tris (pH 8.8), 0.1 ml 10% SDS, 50 µl 10% Ammonium Persulphate (APS), 5.0 µl Temed) polymerizes, the protein sample was denatured at 94 °C for 3-5 min. Once the gel was set, 10-15 µl of the sample was loaded into each well. The gel was then run for 45-60 min at a constant voltage of 150 V. Following electrophoresis the gel was equilibrated in transfer buffer (10X Transfer Buffer: 25 mM Tris pH 8.3, 192 mM glycine, 20 % methanol, dH2O) for 15 min to remove excess

buffer salts and detergents. The proteins were transferred onto a nitrocellulose membrane for 1 h at a constant current of 300 mA. The membrane was blocked in 2% skim milk powder and 50% normal horse serum in Tris Buffered Solution with 0.05 % Tween 20 (TBST) overnight at 4 °C to prevent non-specific binding of the antibodies. Membrane was rinsed three times in TBST before incubation with the primary antibody for 1 h at room temperature and constant agitation. Following incubation with the primary antibody, the membrane was washed three times in TBST and incubated in the secondary antibody for 1 h at room temperature. Bound antibody was detected using either the alkaline phosphatase method in which the membrane is developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 16.5 µl / 5 ml buffer) and nitro blue tetrazolium (NBT; 33 µl/ 5 ml buffer) from Gibco Life Technologies (Grand Island, NY), or by utilizing the ECL Western Blotting Detection system (Amersham Pharmacia Biotech, Piscataway, NJ) which detects immobilized antigens that have been labeled with horseradish-peroxidase conjugated secondaries antibodies (Figure 6). Visualization of antigens detected with ECL system was on Kodak diagnostic film, processed in a Kodak M35A X-OMAT processor.

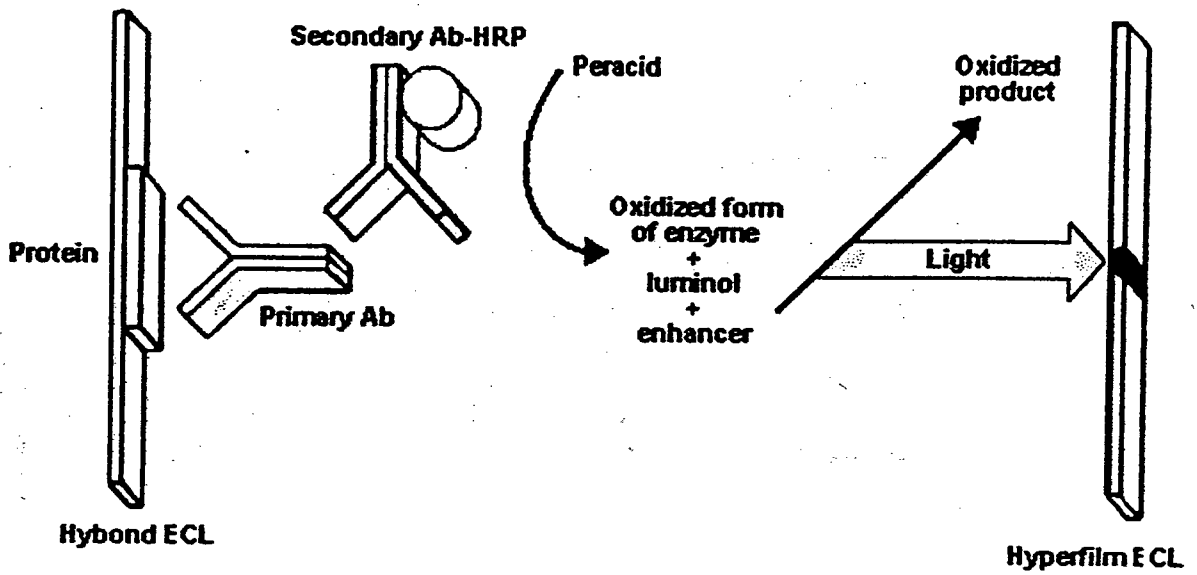


FIGURE 6. Principles of enhanced chemiluminescence (ECL) Western blotting (from the manufacturer's handbook, Amersham Pharmacia Biotech, 1998).

3. Peptidergic and Nitrergic Innervation of the Human Antrum

3.1 Introduction

Pyloric stenosis, achalasia of the esophagus, motility disorders and ulcers are often the result of abnormal innervation in the corresponding regions of the stomach. Both adult and developmental disorders can be attributed to alterations in normal neuronal pathways. In order to understand the pathologies of these various disease states, the normal chemical coding of the human stomach must be defined. Although a little is known about the innervation of the human antrum most research has focused on the innervation of laboratory animals. However, studies indicate that there are vast species differences, therefore, extrapolation to human innervation should not be made in the absence of direct evidence.

Innervation of the human stomach involves a complex network of nerves, the neuropeptide content of which has yet to be characterized. The distribution of individual neuropeptides in the mammalian stomach has been examined in several studies (Schubert, 1991; Leslie *et al.* 1982; Sharkey *et al.* 1984; Wattchow *et al.* 1988; Berthoud & Powley, 1992; Kinoshita *et al.* 1993; Mccoll & Elomar, 1995; Berthoud, 1996), however very few studies have illustrated the distribution in the human stomach (Yacoub *et al.* 1996; Timmermans *et al.* 1994; Burrell *et al.* 1996).

Studies on the regulation of human antral endocrine cells by neurotransmitters have shown inter-species variation (Koop *et al.* 1997; Furness *et al.* 1991), some of which were described in more detail in section 1.3 Neuromodulators in the Stomach.

In the human antrum, it has been shown that muscarinic agonists failed to have a direct stimulatory effect on gastrin release (Koop *et al.* 1997), yet cholinergic agonists have a direct stimulatory effect on somatostatin release (Buchan *et al.* 1992). These results suggest that cholinergic input increases somatostatin release which in turn has an inhibitory effect on gastrin release. In contrast numerous studies have shown that cholinergic agonists have an inhibitory effect on somatostatin secretion in the perfused rat stomach (Schubert *et al.* 1982; Koop *et al.* 1982; Saffouri *et al.* 1980) thereby increasing gastrin release and, in canine antral gastrin cell (G cell) cultures, it has been demonstrated that cholinergic agonists directly stimulate gastrin release (Yokotani *et al.* 1995).

Given the observations of significant species differences in the neural regulation of gastric functions, it is logical that the chemical coding of the antral regions will be altered depending on the specific requirements of a particular species. The present study was carried out to elucidate the innervation and pleurichemical coding of the human antrum. Samples of normal human antrum, obtained from transplant organ donors, were used to examine the distribution of VIP, NOS, CGRP, SP, GRP, somatostatin (SS) and gastrin.

3.2 Materials and Methods

Sections to be used for both Western blot analysis and immunocytochemical studies were processed according to the protocols outlined in Chapter 2. Detection of the bound antibody after Western blot analysis was by the alkaline phosphatase

method also outlined in Chapter 2. The immunocytochemical staining protocol used a combination of rabbit- or guinea pig-generated antisera with murine monoclonal antibodies. In all cases the primary antisera/antibodies and secondary antisera were prescreened to eliminate any possible cross reactivity. Details of the antisera/antibodies used are given in Table 2.

To ensure that the cell bodies for a particular neuromodulator were observed, 6 donor antral sections were treated with colchicine as described in section 2.3. This protocol prevents further axonal transport of the peptides and subsequently increases the neuromodulator concentration within the cell bodies.

Materials from the first 19 donors were treated as outlined in Chapter 2, however, tissue sections from the latter 17 donors to be immunostained for NOS were incubated in 1% sodium dodecyl sulphate (SDS) for 5 min and rinsed in PBS three times prior to incubation with the primary antibody. Incubation with SDS prior to the immunostaining protocol was used as a method of antigen retrieval (Brown *et al.* 1996). This was required after switching batches of fixative in order to restore the level of immunostaining observed with the NOS antibody.

3.3 Results

3.3.1 Western Blot Analysis

Western blot analysis was carried out to determine if the NOS antibody was detecting a protein of the correct molecular weight. Western blot analysis demonstrated that the NOS antibody detected a protein of 155 kDa.

TABLE 2. DETAILS OF ANTISERA/ANTIBODIES

Antigen	Species	Source[a]	Specificity	Dilution
VIP	Mouse Monoclonal	RPG	C terminal	1:100
	Rabbit	Milab	C terminal	1:1000
NOS	Rabbit	Transduction	aa 1095-1289	1:500
CGRP	Mouse Monoclonal	CS	ND	1:1000
	Rabbit	Polak		1:1000
SP	Rabbit	SL	C terminal	1:4000
	Guinea Pig	KK		1:500
GRP	Rabbit	TMcD	ND	1:750
SS	Mouse Monoclonal	CURE		1:500
	Rabbit	DAKO		1:500
Gastrin	Mouse Monoclonal	Walsh		1:5000
	Rabbit	DAKO		1:500
Rabbit IgG	Donkey FITC	Jackson		1:500
	Donkey Cy3	Jackson		1:3000
Mouse IgG	Goat FITC	Jackson		1:500
	Goat Cy3	Jackson		1:2000
Mouse Alexa	Donkey HCA 488	MP		1:2000
Rabbit Alexa	Donkey HCA 488	MP		1:2000
Guinea Pig IgG	Donkey Texas Red	Jackson		1:1000

aa, amino acids; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; Cy3, cyanine dye 3; ND, not determined; HCA, highly cross adsorbed.

[a]RPG, Regulatory Peptide Group; Milab, Milab (Sweden); Transduction, Transduction Laboratories (Lexington, KY) ; CS, Dr. C. Sternini (Center for Ulcer Research and Education, VA Wadsworth, Los Angeles, CA); Polak, Dr J Polak (Hammersmith Hospital, London, England) ; SL, Professor S. Leeman (Department of Physiology, University of Massachusetts, Boston, MA); KK, Dr Yin Nam Kwok (Department of Physiology, University of British Columbia, Vancouver, BC); TMcD, Dr. T. Macdonald (Department of Medicine, University of Western, London, ON); CURE, Center for Ulcer Research and Education (VA Wadsworth, Los Angeles, CA); DAKO, DAKO Diagnostics Canada (Missisauga, ON); Walsh, Dr J.H. Walsh (Center for Ulcer Research and Education, VA Wadsworth, Los Angeles, CA); Jackson, Jackson Immunocytochemical Laboratories (West Grove, PA); MP, Molecular Probes (Eugene, OR).

3.3.2 Single Immunostains

The results of the immunostaining in nerve fibers throughout the antral wall are summarized in Table 3. Due to the size of the submucous plexus this layer was subdivided into three regions. Zone 1 was directly underneath the muscularis mucosa, zone 2 was in the central region of the plexus while zone 3 was directly above the circular muscle layer.

Treatment of the antrum with colchicine prior to fixation allowed for better visualization of the neuronal cell bodies. It was observed that in most colchicine treated sections the level of immunostaining in the cell bodies was higher than in non-colchicine treated tissue sections. No comparisons were made of the number of immunoreactive cell bodies in colchicine treated versus non-colchicine treated tissue due to the fact that the orientation of the tissue sections were not identical and the sections were not consecutive.

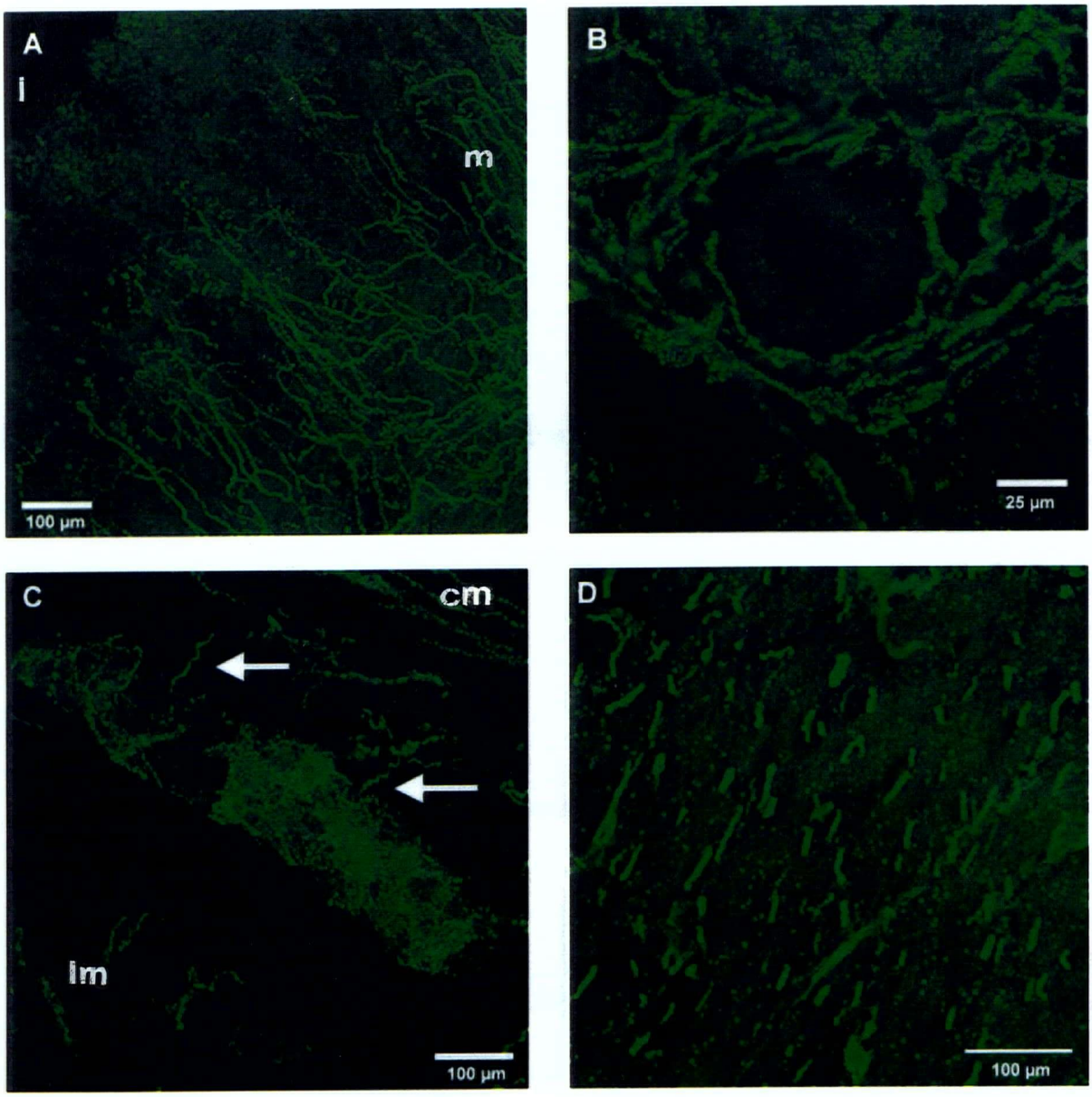
Intense VIP-IR was observed in all layers of the antrum (Figure 7). Immunostaining for VIP demonstrated that VIP-IR cell bodies were abundant in all three zones of the submucous plexus. Within the mucosa these fibers ran up through the lamina propria, a loose connective tissue underlying the epithelium, forming a network of nerve fibers (Figure 7a). Abundant VIP-IR nerve fibers were seen throughout the muscularis mucosa and the submucous plexus. Within the submucosal layer VIP-IR nerve fibers were observed to encircle some of the major blood vessels localized to this region (Figure 7b). The myenteric plexus showed VIP positive cell bodies as well as intense networks of VIP-IR fibers that penetrated into both of the

TABLE 3. DENSITY OF IDENTIFIED NERVE FIBERS IN DIFFERENT REGIONS OF THE ANTRUM

Antigen	Mucosa	Submucous Plexus[a]	Circular Muscle	Myenteric Plexus	Longitudinal Muscle
VIP	++++	+++	++++	+++	++++
NOS	+++	+++	++++	++++	++++
CGRP	++	+++	+++	+++	++
SP	+++	+++	++	+++	++
GRP	+++	+++	+++	+++	++

++++, abundant fibers; +++, moderate fibers; ++ sparse fibers.
[a]Zones 1, 2 and 3.

FIGURE 7. VIP immunoreactivity in all layers of the human antrum. A) VIP-IR fibers in the lamina propria of the mucosal layer. Note the fibers running up towards the tips of the antral glands at the top left hand corner of the figure, B) VIP-IR fibers surrounding a submucosal blood vessel, C) a network of intensely labeled VIP-IR nerve fibers in the myenteric plexus. Note the two VIP-IR fibers penetrating into the longitudinal muscle layer from the plexus (arrows) and D) VIP-IR fibers in the longitudinal muscle layer. (l, lumen; m, mucosa; cm, circular muscle; lm, longitudinal muscle)

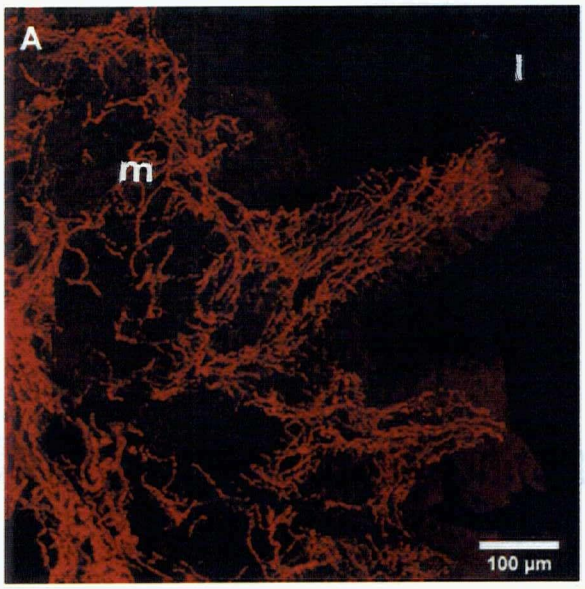

A
l
m
100 µm
B
25 µm
C
cm
lm
100 µm
D
100 µm

external muscle layers (Figure 7c), each of which displayed numerous VIP-IR fibers coursing throughout (Figure 7d).

Immunoreactivity for NOS was also present throughout all layers of the antral wall (Figure 8). Cell bodies that were NOS positive were seen in all zones of the submucosa as well as in the myenteric plexus. Upon the first examination of NOS immunoreactivity in the mucosal layer, it was observed that there was abundant NOS innervation in this region. The NOS-IR fibers were observed to run up through the lamina propria forming a meshwork of fibers (Figure 8a). Interestingly, upon staining the twentieth donor sample for NOS-IR, the mucosal labeling was absent as it was in subsequent antral sections (Figure 8b). However, pretreatment of the tissues with a simple antigen retrieval method (5 min incubation in 1% SDS) brought back the intense mucosal staining (Figure 8c). In all sections, both prior to and after the twentieth antral section, substantial NOS-IR fibers were also found throughout the myenteric plexus (Figure 9a) and in both the longitudinal (Figure 9b) and circular muscle layers (Figure 9c). As with VIP, NOS-IR fibers were observed to surround some of the blood vessels in the submucosal layer. While fine varicose fibers were observed throughout the antrum, NOS-IR fibers were also observed in very large fiber tracts in the muscle layers (Figure 9c) and in the submucous plexus.

Immunostaining for CGRP demonstrated the most abundant CGRP-IR cell bodies were in zone 2 (Figure 10a). While zone 1 had fewer CGRP positive cell bodies, no CGRP-IR cell bodies were observed in zone 3. Nerve fibers which were CGRP-IR were observed in the mucosal region running throughout the lamina propria

FIGURE 8. Examples of NOS immunoreactivity before and after antigen retrieval. A) Abundant NOS-IR fibers in the lamina propria of the mucosal layer, prior to the use of a new batch of PFA, B) after the new batch of PFA is used, NOS-IR mucosal fiber staining is lost, however NOS immunostaining of fibers within the muscularis mucosa remains strong and C) after antigen retrieval techniques are used mucosal NOS-IR fibers are clearly observed. Note the fibers are running right up to the tips of the antral glands. (mm, muscularis mucosa)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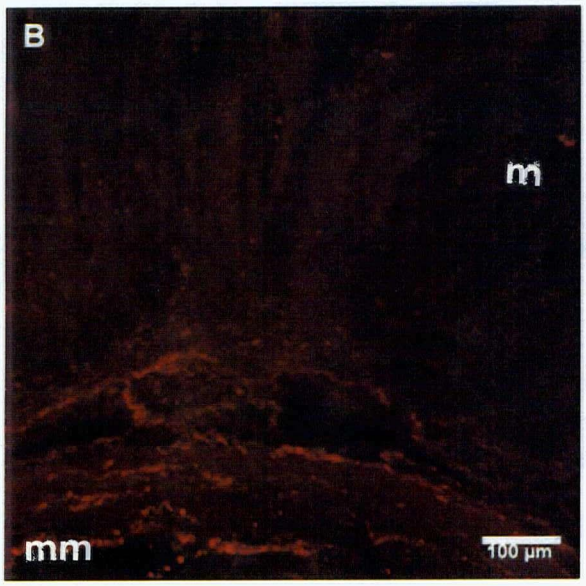
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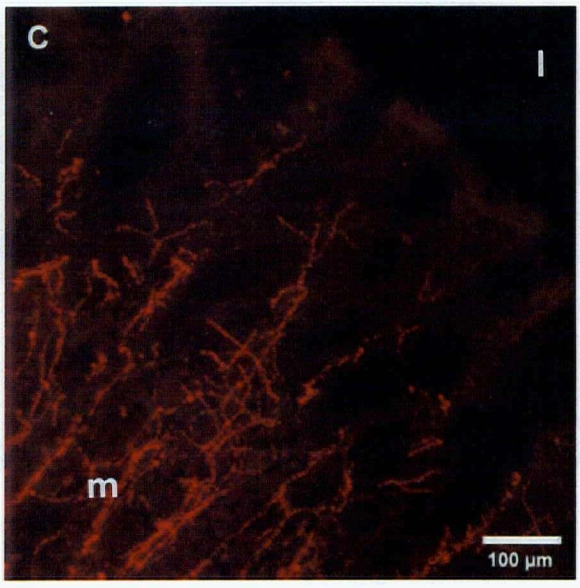
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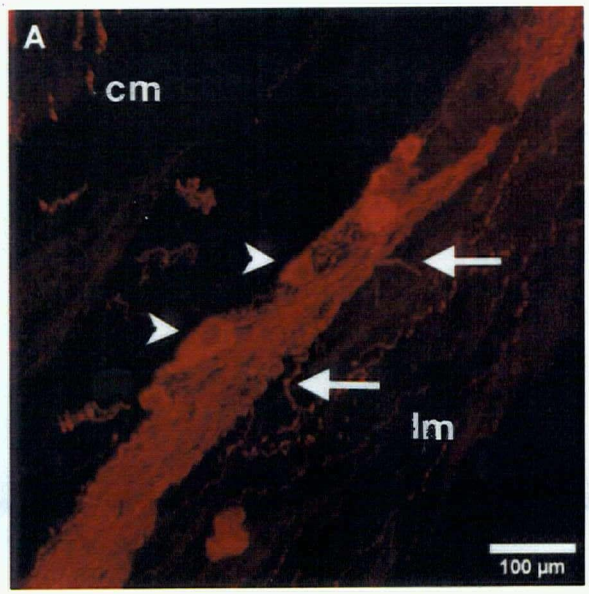
FIGURE 9. NOS immunoreactivity in the human antrum. A) NOS-IR cell bodies (arrowheads) in the myenteric plexus. Note the fibers penetrating into the longitudinal muscle layer (arrows), B) NOS-IR fibers in the longitudinal muscle layer and C) NOS-IR nerve fibers in the circular muscle layer. Note the presence of fine NOS-IR varicose fibers (arrowhead) and bundles of fibers in large nerve tracts (arrow).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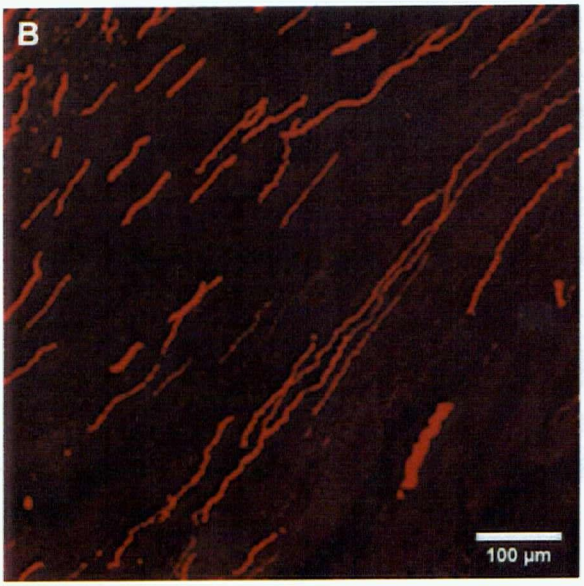
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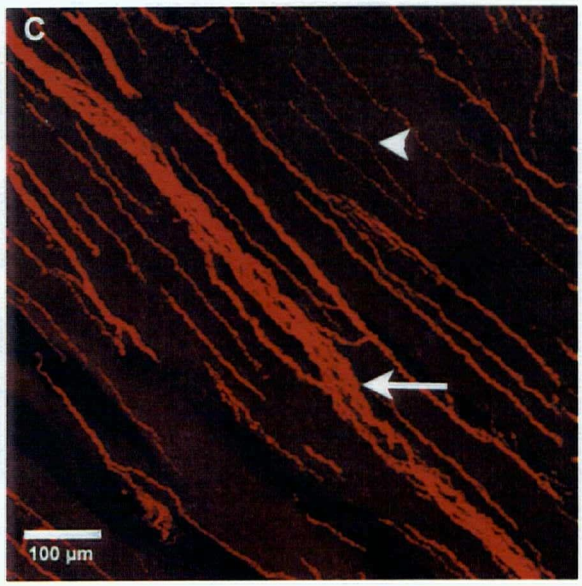
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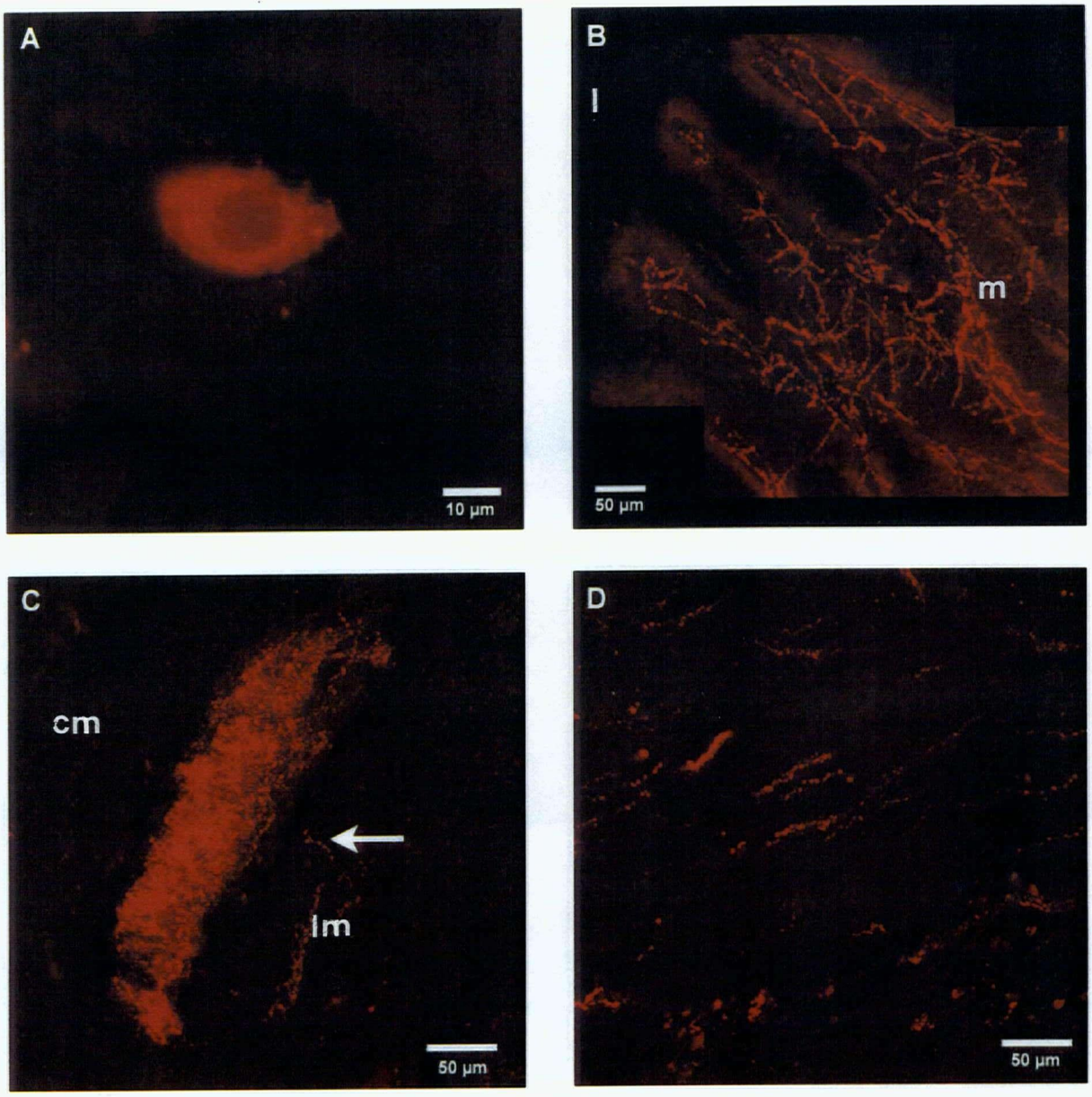
100 µm

FIGURE 10. Examples of CGRP immunostaining throughout the human antrum. A) a CGRP-IR cell body within zone 2 of the submucosal plexus, B) networks of CGRP-IR nerve fibers in the lamina propria of the mucosal layer, C) intense CGRP-IR nerve fibers within the myenteric plexus. Note the varicose fiber extending into the longitudinal muscle layer from the plexus (arrow) and D) circular muscle layer demonstrating CGRP-IR fibers running throughout, although not to the same extent as either VIP- or NOS-IR fibers.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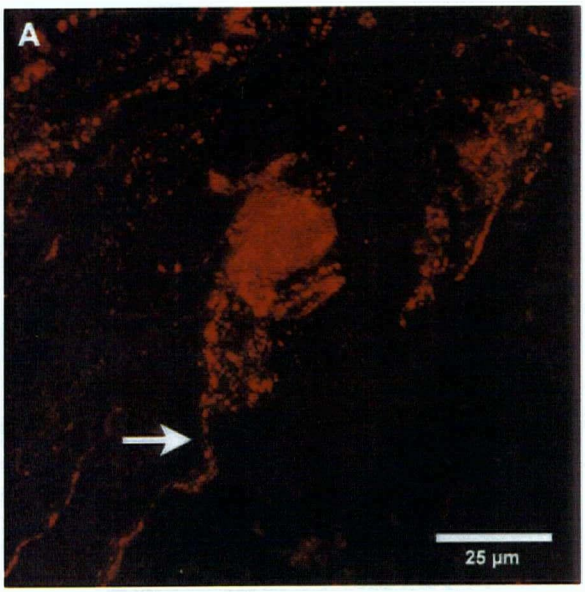
and down into the muscularis mucosa (Figure 10b). At the tips of the antral glands CGRP-IR nerve fibers branched out into networks of fine nerve fibers. In the submucous plexus CGRP-IR fibers were observed to run in close spatial proximity and sometimes surround the major blood vessels. In the myenteric plexus very few CGRP-IR cell bodies were found, however, intense CGRP-IR nerve fibers were observed (Figure 10c). Both external muscle layers contained CGRP-IR (Figure 10d) fibers running parallel to the muscle cells, although not to the same extent as either VIP- or NOS-IR fibers.

Substance P-IR was also observed in all layers of the antrum. The most abundant SP-IR cell bodies were found in zone 2, with fewer SP-IR cell bodies observed in zone 1 and zone 3 (Figure 11). Substance P-IR fibers were most abundant in the mucosal region creating a network of fibers within the lamina propria (Figure 12a). As the fibers approached the tips of the antral glands they appeared to branch into tiny little networks of fibers to a greater extent than did the CGRP-IR fibers. The circular muscle layer displayed more SP-IR fibers (Figure 12b) than the longitudinal muscle. In the myenteric plexus very few SP-IR cell bodies were found, however, numerous fibers were observed to form a meshwork (Figure 12c) and encircle non-SP-IR cell bodies.

In all three zones of the submucous plexus GRP-IR cell bodies were observed (Figure 13a), however, there were very few localized to zone 3. Similarly, in the myenteric plexus, there were very few GRP positive cell bodies found. GRP-IR fibers were abundant in the mucosal region and were observed to run up through the lamina

FIGURE 11. Substance P immunoreactive cell bodies in the submucosal region. A) a SP-IR cell body in the submucous plexus. Note the nerve fiber running into the submucosal layer (arrow), B) submucosal ganglia with three SP positive cell bodies (arrowheads) and C) the same submucosal section at a different focal plane (z step). Note the SP-IR cell body at this level is not shown in B (arrow) and that the positive cell bodies of the larger ganglia are out of focus at this focal plane (arrowheads).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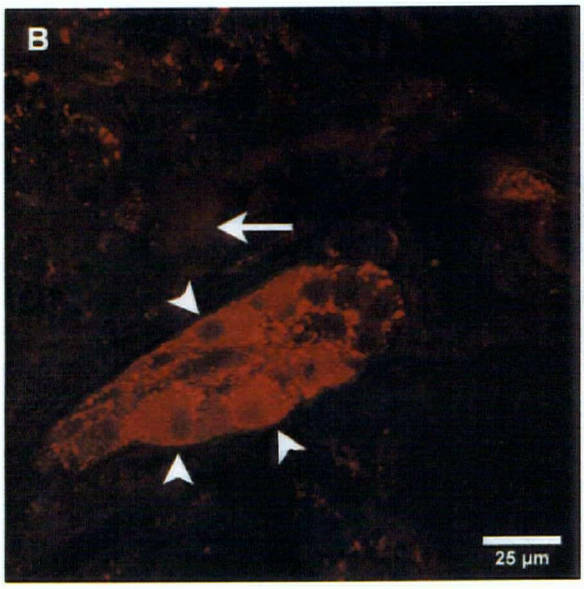
B
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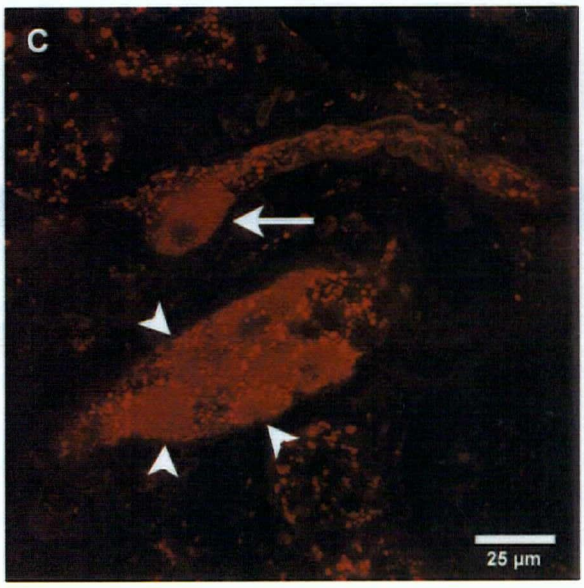
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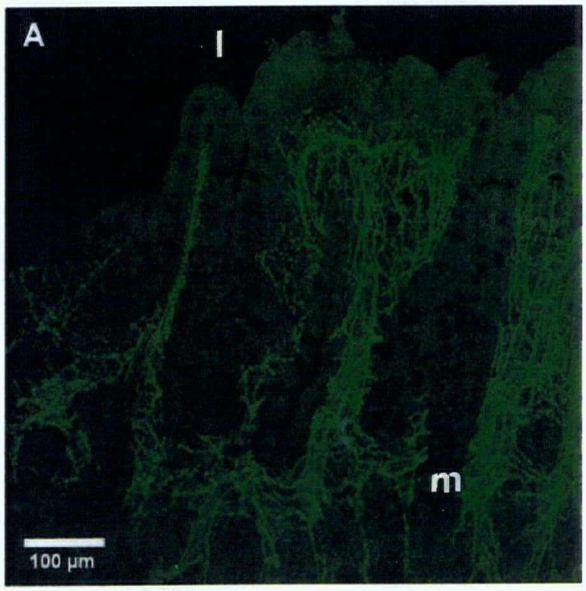
FIGURE 12. Representative immunostains for SP in the human antrum. A) Abundant SP-IR nerve fibers in the lamina propria of the mucosal region. Note the branching of the fibers at the tips of the antral glands at the top of the figure, B) SP-IR nerve fibers running throughout the circular muscle layer and C) a myenteric plexus, demonstrated rich innervation of SP-IR fibers (arrow). Note the adjacent longitudinal muscle layer also contains SP-IR nerve fibers throughout.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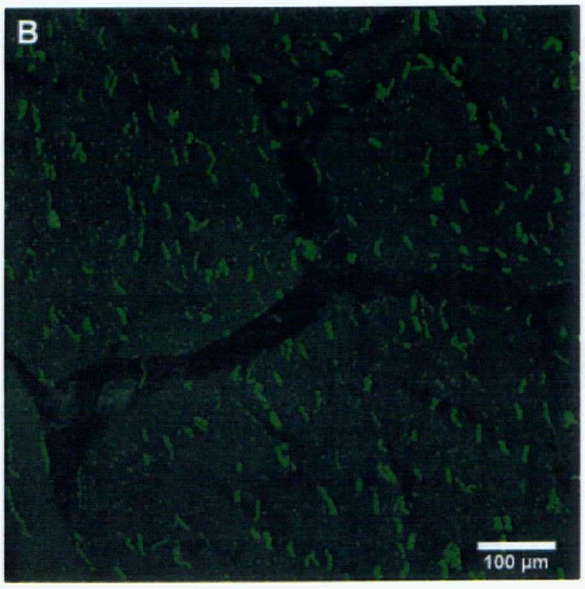
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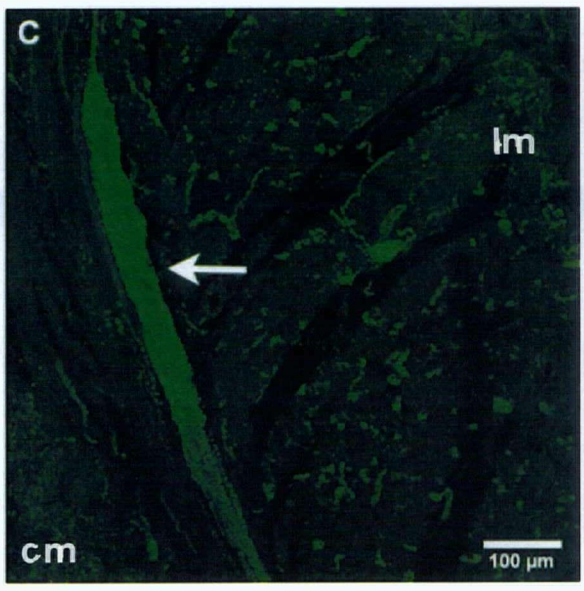
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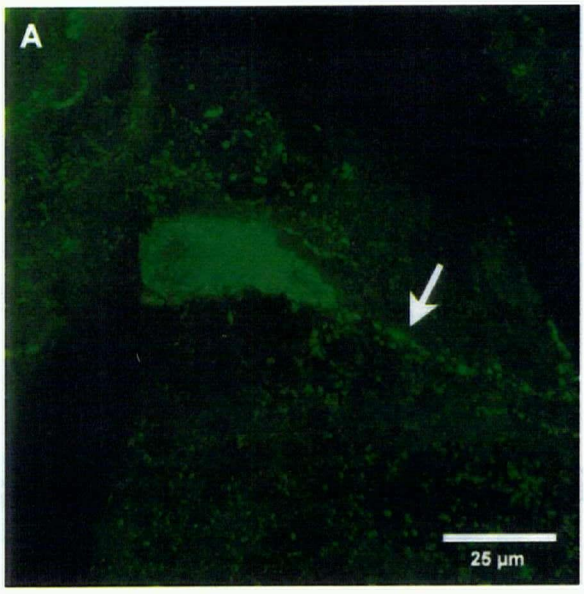
FIGURE 13. GRP immunoreactivity in the human antrum. A) a GRP-IR submucosal cell body. Note the GRP-IR nerve fiber running into the submucosal layer from the cell body (arrow), B) abundant innervation of the mucosal layer by GRP-IR nerve fibers within the lamina propria and C) a network of GRP-IR nerve fibers in the myenteric plexus.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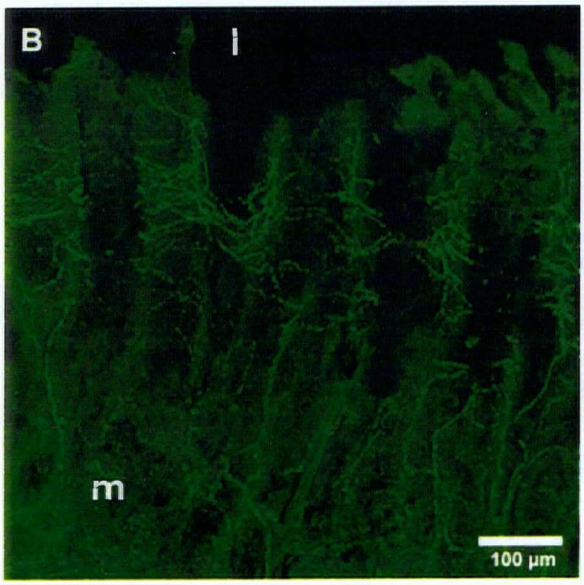
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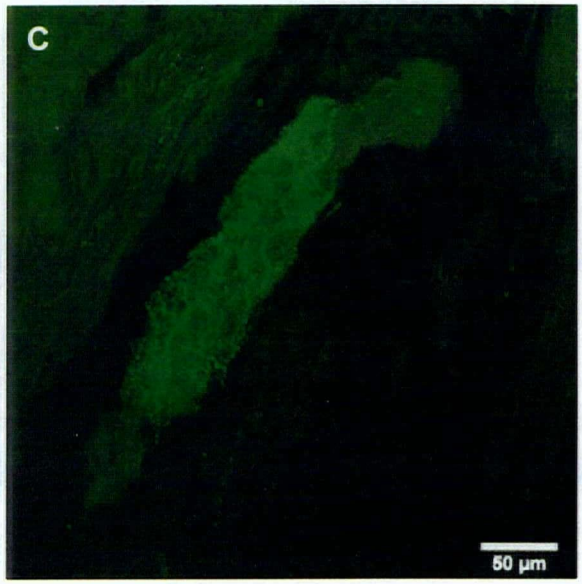
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propria underneath the epithelial layer towards the lumen (Figure 13b). These fibers formed a dense meshwork of interconnecting fibers in the lamina propria. Within the myenteric plexus numerous GRP-IR fibers were observed to run throughout the plexus forming a large network of intrinsic innervation (Figure 13c). As was observed with NOS-IR in the circular muscle layer, GRP-IR nerve fibers were observed in both fine varicose fibers and in large fiber tracts (data not shown). These large nerve tracks were also observed in the submucosal layer.

Gastrin-IR was observed on epithelial cells within the mucosa (Figure 14a-b). The gastrin-IR cells were localized to the lower half of the antral glands. Somatostatin-IR was confined to epithelial endocrine cells within the mucosal layer (Figure 14c-d). These cells were found in the same layer of the mucosa as the gastrin-IR cells.

3.3.3 Double Labeling Experiments

All the neuromodulator antibodies in the present study were used to double label the antral tissue in combination with each of the other antibodies as well as with SS and gastrin. These studies demonstrated that VIP-IR cell bodies within both plexuses were co-labeled with NOS-IR. Nerve fibers within all regions of the antrum were found to be co-labeled for VIP and NOS (Figure 15). While the number of NOS-IR nerve fibers within the two external muscle layers and the myenteric plexus was greater than that of VIP-IR nerve fibers, the opposite was true in the mucosal region, VIP-IR nerve fibers exceeded that of NOS-IR fibers. Neither neuromodulator was found to be co-labeled with any of the other neuropeptides examined. While NOS-IR cell bodies were

FIGURE 14. Gastrin and somatostatin distribution within the human antrum. A) gastrin-IR cells in the lower half of the antral glands, B) magnification of gastrin-IR cells in another section of the mucosal layer, C) SS-IR cells also found in the lower portion of the antral glands and D) magnification of SS-IR within another mucosal region.

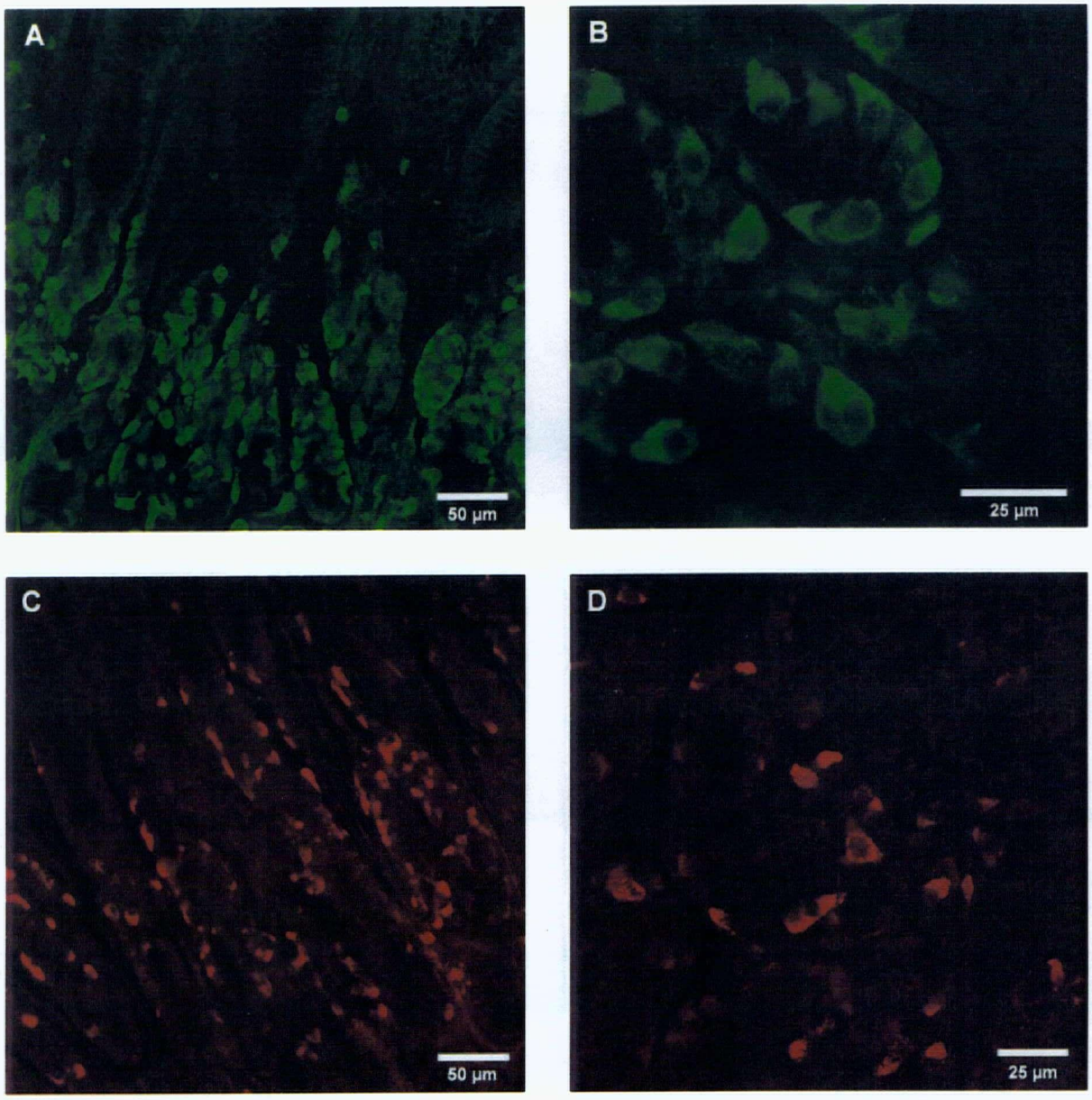
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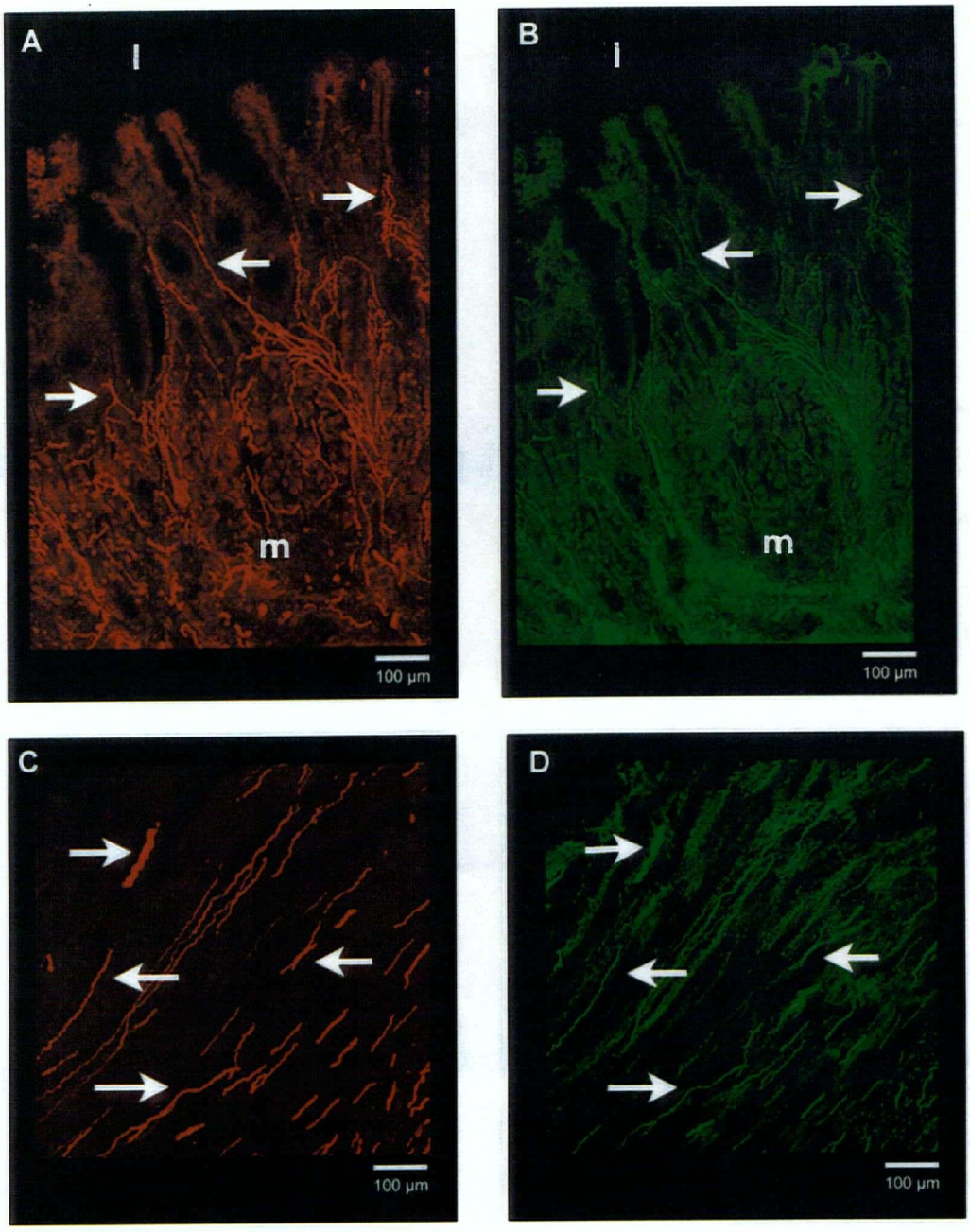
FIGURE 15. Double labeled sections of the human antrum with antibodies for VIP and NOS. NOS-IR nerve fibers in the mucosa (A) and the circular muscle layer (C). VIP-IR nerve fibers in the same section of mucosa (B) and circular muscle (D). Note the arrows highlighting a few of the double immunostained nerve fibers within both regions.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not shown to be co-immunostained with any of the other peptides, except VIP, it was observed that some of the NOS-IR cell bodies in both plexuses were surrounded by SP- or CGRP-IR nerve fibers (Figure 16). Double immunostains of gastrin or SS with VIP showed that the VIP-IR fibers within the mucosal region were in close spatial arrangement to the gastrin cells. As a majority of VIP-IR nerve fibers in the mucosa were also NOS-IR, it was also observed that double stains of NOS with either gastrin or SS showed a similar spatial arrangement as that of the VIP-IR fibers (Figure 17).

Only two other neuropeptides in this study were found to be co-labeled in all regions of the antrum, SP and CGRP. A large percentage of cell bodies in both plexuses were observed to be co-labeled by the two peptides (Figure 18) as were the nerve fibers in all layers. The numerous SP- and CGRP-IR nerve fibers in the mucosal region were not observed to be in close spatial proximity to gastrin- or SS-IR cells. It was also observed that neither CGRP- nor SP-IR neurons or fibers were co-labeled with any of the other neuromodulators studied.

During initial screening of sections it appeared that CGRP/SP positive nerve fibers were co-stained with VIP/NOS positive nerve fibers. However, further inspection of overlays of single z plane confocal images immunostained for either CGRP or SP with either NOS or VIP clearly showed that individual nerve varicosities in a single fiber bundle were not co-labeled (Figure 19). Therefore, while it may seem as though the two nerve fibers are double labeled, in fact, they are two distinct fibers running in the same fiber tract.

FIGURE 16. Myenteric neurons surrounded by SP-IR nerve fibers in the human antrum. Two NOS-IR cell bodies (Cy3, red) completely encircled by SP-IR nerve fibers (FITC, green)(arrows). Note that the yellow areas represent SP-IR nerve terminals overlying the NOS-IR cell body. They appear yellow due to the overlay of red and green images and are an artifact of the creation of a maximum intensity projection consisting of 10 z plane images (0.5 μm between images) flattened into one image.

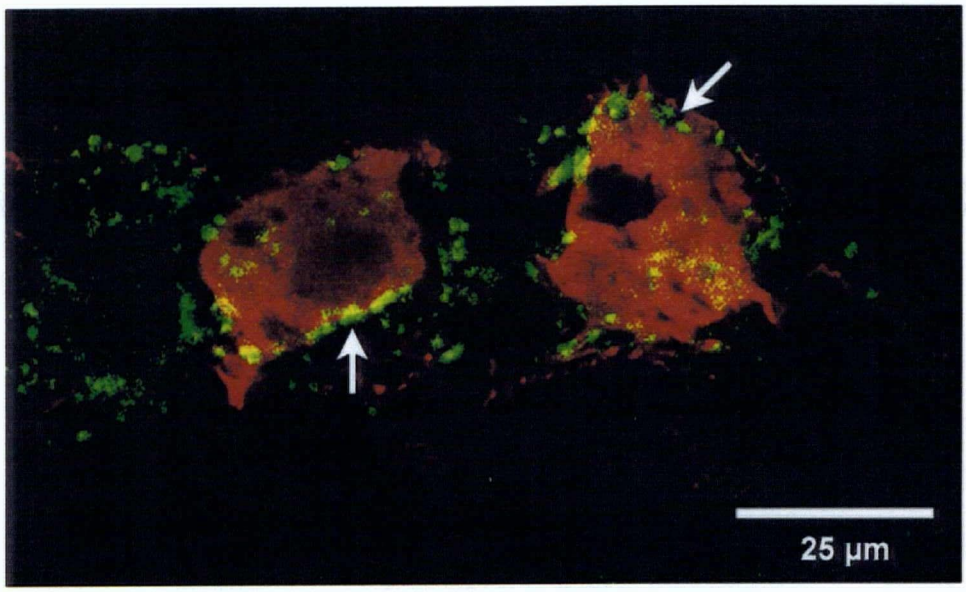
25 µm

FIGURE 17. Gastrin-IR cells in close spatial proximity to NOS-IR fibers in the human antrum. In the mucosal layer, NOS-IR fibers (Cy3, red) were observed to be in close association to the gastrin-IR endocrine cells(FITC, green). This image is a maximum intensity projection of 23 z plane images (1.8 μm between images). Yellow areas represent NOS-IR fibers overlying gastrin-IR cells and are created as an artifact of merging the red and green images.

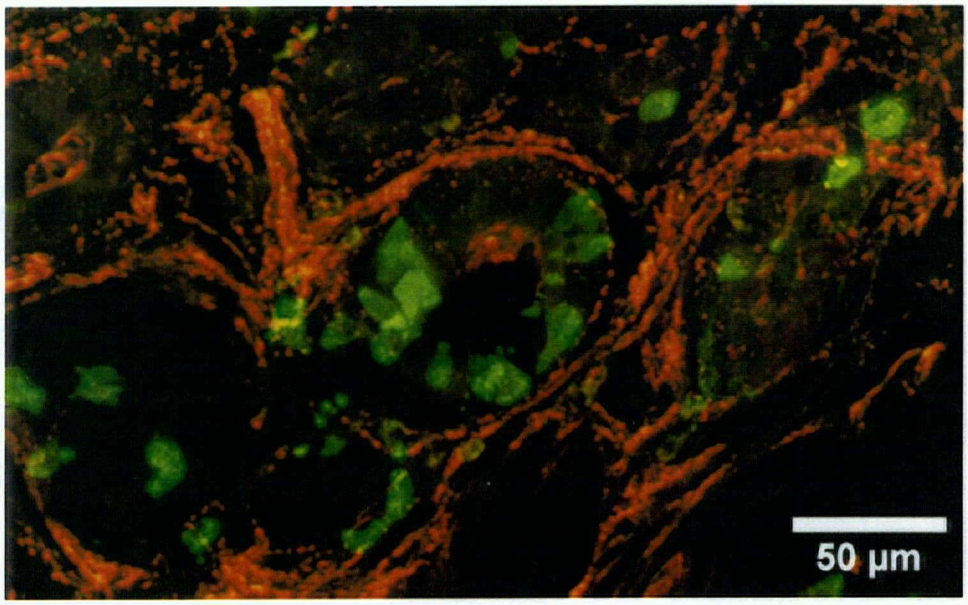
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FIGURE 18. Example of a double immunostained submucosal cell body with mouse anti-CGRP and guinea pig anti-SP antibodies. A) a submucosal cell body positive for CGRP, B) the same submucosal cell body expressed SP-IR and C) an overlay of the two images from (A) and (B) demonstrated co-labeling of the cell body with the two antibodies.

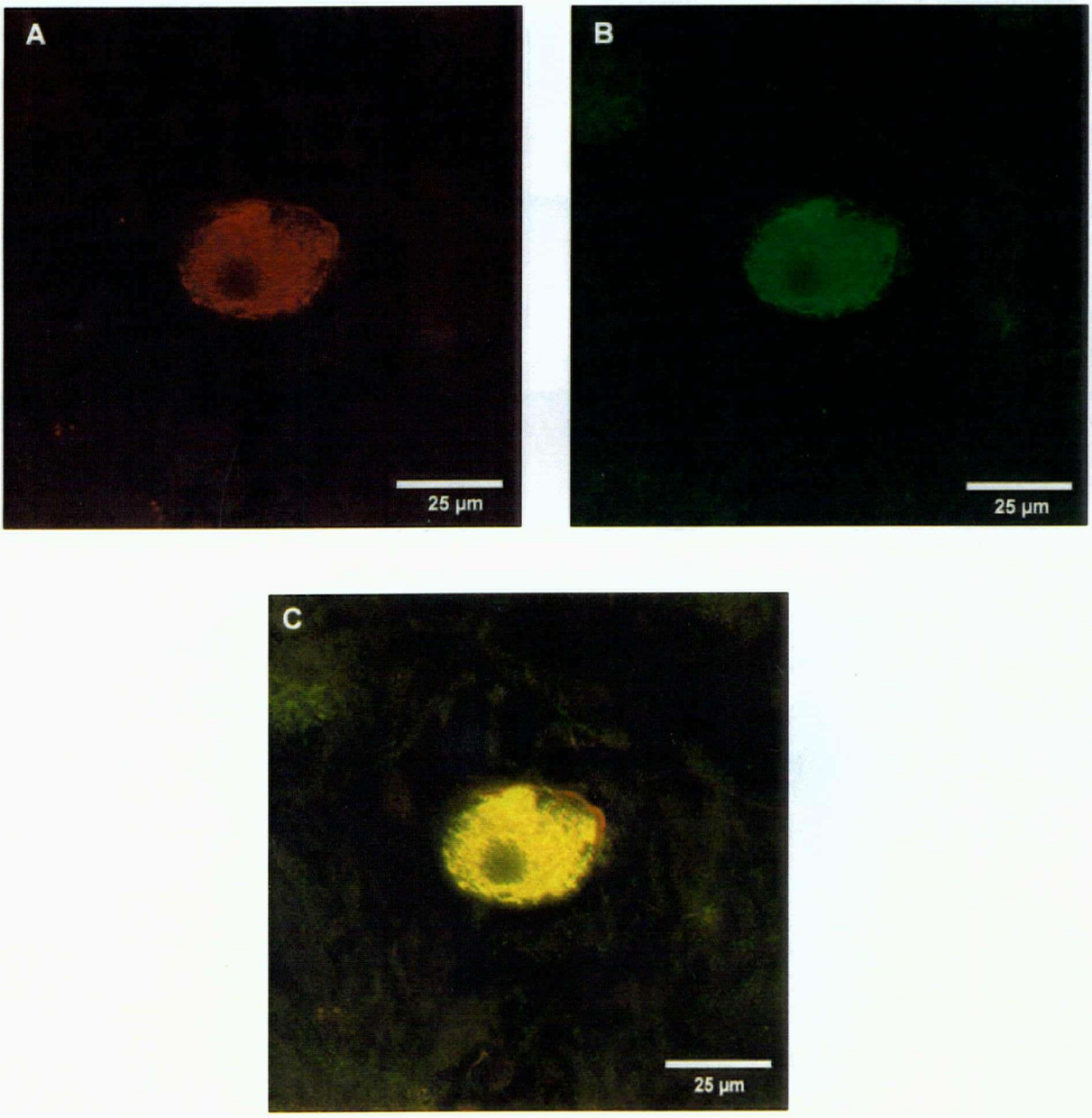
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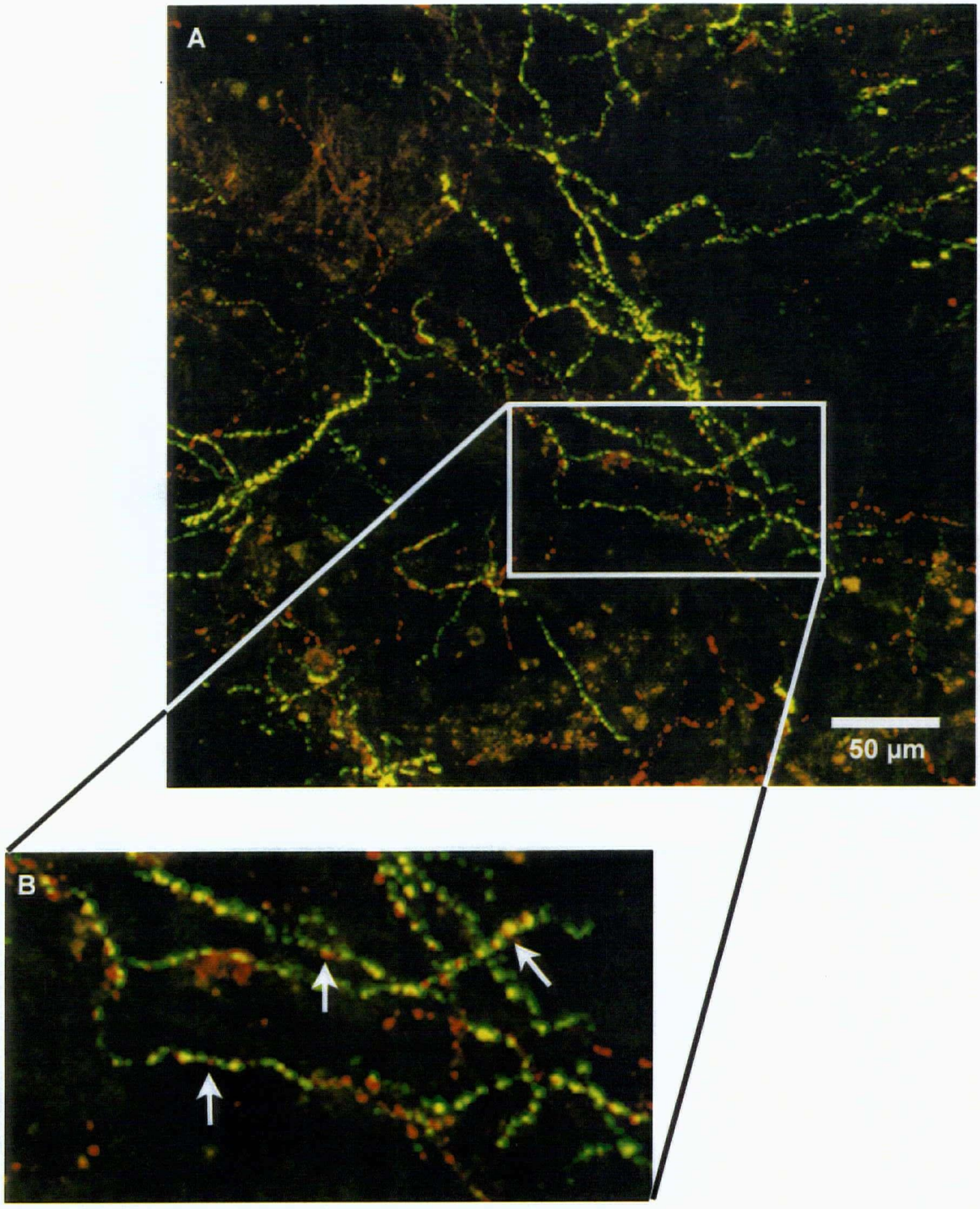
FIGURE 19. Mucosal nerve fibers in the human antrum immunostained for SP and VIP. In the mucosa, VIP-IR nerve fibers (FITC, green) and SP-IR fibers (TR, red) first appeared to be co-labeled, however, individual nerve varicosities were not co-labeled (arrows). A maximum intensity projection created from 26 z plane images (0.9 µm between images). Note that regions which appear yellow are due to the overlay of red and green images and do not represent areas of co-localization.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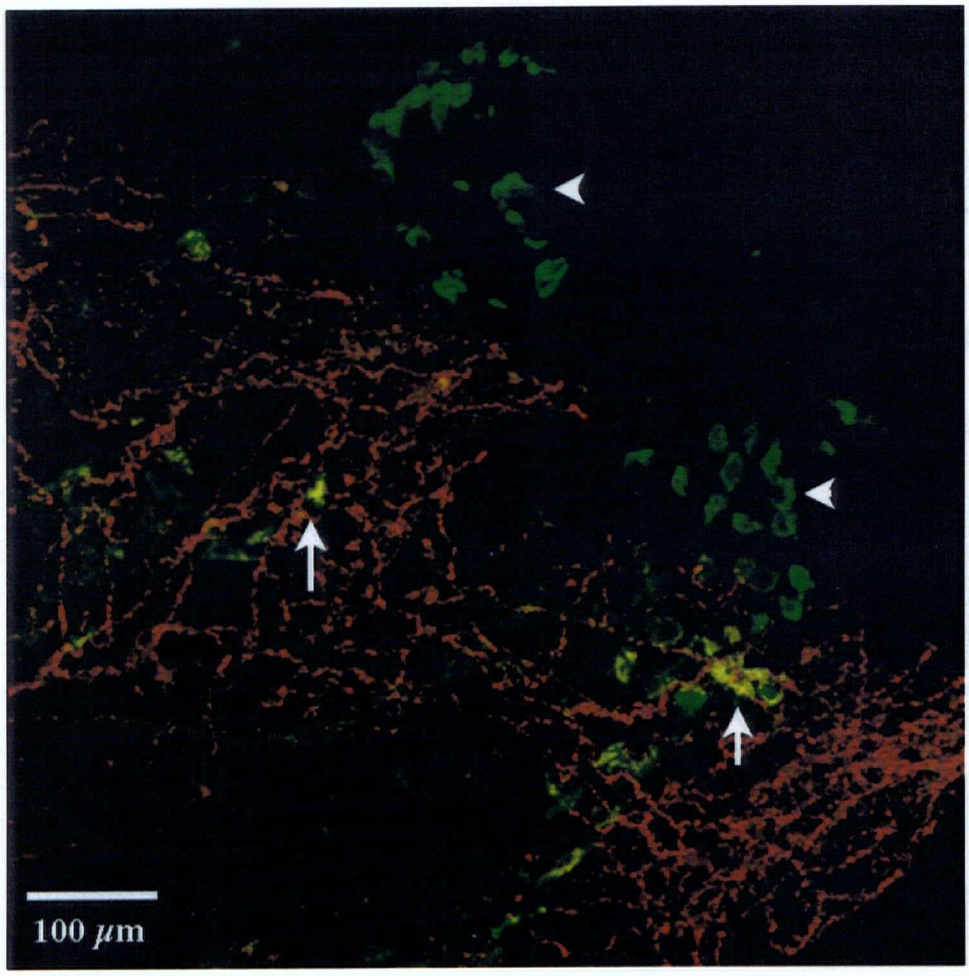
Double labeling experiments showed that neither GRP-IR neurons nor nerve fibers were co-labeled by the other substances studied. However, GRP-IR nerve fibers within the mucosa were in close spatial proximity to the gastrin-IR epithelial endocrine cells (Figure 20).

3.4 Discussion

The purpose of this study was to determine the chemical coding of the human antrum as a basis for understanding the neural regulation of gastric physiology. The results presented describe the location of neurons and fibers within the antral region for VIP, NOS, CGRP, SP, and GRP. The human antrum has an extremely thick submucosal region that is predominantly collagen with blood vessels and nerve ganglia.

The thickness of the submucosal region allowed division into three regions as previously described (Dhatt & Buchan, 1994). In the canine ileum, submucosal cell bodies lying next to the circular muscle (zone 3) were shown to innervate that layer of muscle, whereas the remaining neurons within the plexus were found to innervate the mucosa (Daniel *et al.* 1987). In a later study on the human ileum it was observed that these remaining neurons could further be subdivided into two regions: one region that lay in the middle of the plexus and innervated both the mucosa and the vascular supply (zone 2) and a second region of ganglia that lay just below the muscularis mucosa (zone 1) and provided innervation to the mucosa (Dhatt & Buchan, 1994). In the present study these three regions were observed in the human antrum and the same

FIGURE 20. Spatial relationship between GRP-IR nerve fibers and gastrin-IR cells in the human antrum. A maximum intensity projection created from 39 z plane images demonstrating gastrin-IR cells (Alexa 488, green) within the mucosa in close spatial proximity to GRP-IR nerve fibers (Cy3, red). A subset of gastrin-IR cells were not surrounded by GRP-IR fibers (arrowheads). Yellow areas (arrows) represent GRP-IR nerve fibers surrounding the gastrin-IR cells and are created when the red and green two-dimensional figures are merged.


100 μm

nomenclature has been used. As in the submucosal plexus of the human ileum, the majority of neurons were localized to zone 2 within the submucosal region.

Colchicine treatment provided a more effective method for viewing neuronal cell bodies. As the tissue sections are rather thick, the higher level of immunostaining observed in these sections allowed for easier identification of cell bodies below the tissue surface. Direct comparisons between treated tissue and non-treated tissue were not made. The antral tissue block treated with colchicine was removed prior to fixation and, subsequently, fixed the following day. While the sections for both colchicine treated and non-colchicine treated tissue were taken from the same region the orientation of the final tissue sections were different. As a result, only qualitative analysis of this treatment could be determined. Previous studies have shown colchicine treatment to be effective in increasing the ability to visualize cell bodies (Ekblad *et al.* 1996; Boyer *et al.* 1994).

The present study has shown that VIP- and NOS-IR neurons in both plexuses were co-labeled and that co-labeled nerve fibers are evident in the mucosal region. While recent studies have shown a co-localization of VIP with NOS, in myenteric inhibitory neurons and that both are co-released resulting in smooth muscle relaxation (Krowicki & Hornby, 1996; Murthy *et al.* 1995; Keef *et al.* 1994; Baccari *et al.* 1994; Gershon *et al.* 1994), the precise contribution of either modulator depends on the target muscle (Willis *et al.* 1996; Murthy *et al.* 1995; Keef *et al.* 1994). Although VIP and NO were known to be co-localized in the innervation of the muscle, secretomotor neurons supplying the mucosa were considered to be VIP-IR only.

This is the first time that NOS-IR has been demonstrated in the mucosa in such abundance. A previous study reports a few NADPH-diaphorase (NADPH-d) labeled fibers are in the mucosa of the human stomach (Manneschi *et al.* 1998) but not to the same extent as observed in the present study. While NADPH-d has been used to localize NOS containing nerve fibers in the rat and primate brain (Bredt *et al.* 1991), the human ileum (Dhatt & Buchan, 1994) and the rat larynx (Hisa *et al.* 1996), the lack of intense fiber staining in the mucosa of the human stomach may due to the sensitivity of the NADPH-d method for detecting fine NOS containing structures. The functional significance of NOS in the mucosa has yet to be determined, however, as both VIP and NOS are co-labeled throughout the rest of the antrum, it is possible that both substances, together, play a role in epithelial function. Both NOS- and VIP-IR have been reported, either together or in separate neurons, in the submucous and myenteric plexuses as well as the muscular layers of the pig gastric fundus (Lefebvre *et al.* 1995), human colon (Matini *et al.* 1995) and canine colon (Keef *et al.* 1994).

The requirement for antigen retrieval methods prior to incubation of antral sections with the NOS antibody to restore immunostaining of the fine varicose fibers in the mucosal region was not clear upon initial trial. Further investigation into the switch from abundant labeling to no labeling of mucosal fibers in sections from the 20th donor determined that the only change to the protocol was the use of a new batch of PFA which coincided with the manufacturer reducing the grade and purity of the compound. The lack of mucosal staining observed without antigen retrieval most probably results from increased cross linking during the fixation protocol preventing

access of the antibody to the antigen. A paper in 1996, outlined the antigen retrieval process in cryostat tissue sections and cultured cells (Brown *et al.* 1996) based on the findings that many antibodies recognize denatured proteins on Western blots but are poorly immunoreactive in tissue sections. Placing tissue sections in 1%SDS for 5 min prior to incubation with the primary antibody allows for the denaturing of proteins and, consequently, unmasking of antigenic sites. As was shown in the present study, the NOS antibody detected a protein of 155 kDa, the correct size for the NOS protein, demonstrating that the antibody does detect the protein when it has been denatured. In light of the finding that which mucosal immunolabeling was present prior to the change in PFA and antigen retrieval restored the same pattern of immunolabeling, the use of this retrieval technique can be considered reliable and useful in future investigations.

CGRP-IR staining was most abundant in the muscularis mucosa and in the underlying muscle layers as well as in the myenteric plexus. Staining observed in the mucosal region was expected due to the fact that CGRP is contained in capsaicin-sensitive primary sensory afferent nerve fibers and these nerves lie in close proximity to the gastric vasculature (Stroff *et al.* 1995; Kinoshita *et al.* 1993; Vanner, 1994). Gastric acid secretion has also been shown to be mediated by the release of CGRP (Manela *et al.* 1995; Tache, 1992; Inui *et al.* 1991). The results of this study demonstrated innervation of the vasculature by both CGRP- and SP-IR nerve fibers. These results are supported by a number of studies in different species (see Sternini, 1992 for references). Of the capsaicin sensitive sensory nerves, a large network of

CGRP-IR fibers has been located in the myenteric and submucous plexuses (Manela *et al.* 1995), while substance P (SP) has been shown to be co-released with CGRP such that both neuropeptides are required to stimulate vasodilation (Vanner, 1994). Both CGRP and SP have also been implicated in the regulation of gastric motility (Raybould, 1992; Holzer & Holzer-Petsche, 1997a; Dockray, 1987), therefore, the presence of CGRP-IR and SP-IR nerve fibers within the smooth muscle layers provides neuroanatomical support for these physiological functions.

Although there are regional and species differences in the density of SP innervation (Sundler *et al.* 1991), the overall distribution is very similar in most species (Holzer & Holzer-Petsche, 1997a). It has been shown in the mouse, rat, guinea pig and dog stomach, that myenteric neurons expressing SP innervate the two muscle layers as well as the muscularis mucosa and that the submucous plexus is virtually absent (Holzer & Holzer-Petsche, 1997a). Our data indicates that not only is there a defined submucous plexus in the human antrum, there are SP-IR neurons within the plexus. Our results do not show co-localization of SP with VIP- nor NOS-IR neurons which is also supported by studies in the mouse, guinea pig and dog gut (see Holzer & Holzer-Petsche, 1997a for references).

In view of the interest of our laboratory in endocrine secretions, we considered it to be important to investigate the distribution of GRP-IR nerves and its interaction with gastrin and somatostatin. This neuropeptide has been located in the submucous and myenteric plexuses, the mucosa and in the external muscle layers of several species (Berthoud, 1996). In the present study, GRP-IR was most abundant in the

mucosal and submucosal regions of the antrum. As GRP is a potent stimulator of gastrin release (Koop *et al.* 1997; Shimoda *et al.* 1996; Schubert, 1995; Buchan & Meloche, 1994; Schubert *et al.* 1982; Bunnett, 1994; Sawada & Dickinson, 1997; Sachs *et al.* 1997; Galan *et al.* 1996; Takehara *et al.* 1996) the presence of abundant GRP-IR in the mucosal region anatomically supports this physiological function.

Gastrin releasing cells, or G cells, are located in the lower portion of the antral glands in close spatial proximity to GRP-IR fibers (Dockray *et al.* 1996; Mccoll & Elomar, 1995; Sawada & Dickinson, 1997; Galan *et al.* 1996). It has previously been reported that GRP-IR nerve fibers were at least 2 μm away from their target G cells (Miller *et al.* 1989). No co-labeling of GRP-IR neurons with any of the other substances tested in this study was observed. However, in the rat stomach a subpopulation of myenteric GRP-IR neurons were co-localized with VIP-IR (Berthoud, 1996).

This study demonstrated the distribution of the predominant neuroactive substances within the human antrum. For the first time abundant NOS-IR fibers were observed in the lamina propria of the mucosal layer. This is also the first time VIP and NOS have been reported to be co-labeling nerve fibers outside of the myenteric and external muscle layer. It has been demonstrated that all of the neuroactive substances studied are present in all layers of the antral wall. This study demonstrates a unique chemical coding of the human antrum that provides a neuroanatomical basis for future physiological studies and support for physiological functions previously reported.

4. THE NEUROKININ 1 RECEPTOR

4.1 Introduction

In recent years cloning of receptors has made available the peptide sequence data required to provide antigens for the production of receptor subtype specific antibodies. These antisera can be used to localize the precise cellular distribution of receptors thereby providing detailed information that compliments autoradiographic studies. Moreover, when a peptide has more than one receptor subtype, specific antibodies can be used to define the location of the individual subtypes.

Substance P (SP) is a member of the tachykinin family of peptides which, in mammals, also includes neurokinin A (NKA) and neurokinin B (NKB) (Holzer & Holzer-Petsche, 1997a; Regoli *et al.* 1994). There are three distinct receptors for mammalian tachykinins (Grady *et al.* 1996a; Holzer & Holzer-Petsche, 1997a; Regoli *et al.* 1994). SP has been shown to have a higher affinity for the neurokinin-1 receptor (NK-1r) compared to the NK-2 or NK-3 receptors (Gerard *et al.* 1991; Grady *et al.* 1996a; Holzer & Holzer-Petsche, 1997a; Huang *et al.* 1995; Maggi & Schwartz, 1997; Regoli *et al.* 1994). The human substance P receptor is a 407 amino acid protein (Gerard *et al.* 1991; Takeda *et al.* 1991) with a molecular weight of 46 kD (Regoli *et al.* 1994). In order to determine which cell types in the human antrum and duodenum are capable of responding to SP, the purpose of the present study was to investigate the distribution of the NK-1r in relation to SP-immunoreactive (IR) fibers.

Immunocytochemical studies of the distribution of the NK-1 receptor have been

limited to rat (Grady *et al.* 1996a; Ladic & Buchan, 1996; Sternini *et al.* 1995; Vannucchi *et al.* 1997) and guinea pig (Grady *et al.* 1996b; Moore *et al.* 1997; Portbury *et al.* 1996; Southwell *et al.* 1996), due to the species-specificity of the available NK-1r antibodies which were raised to peptide sequences from the cloned rat NK-1r. Due to inter-species variation, these antibodies are unable to detect the receptor in human tissues. To overcome this problem, a monoclonal antibody (mAb12) against a peptide complementary to SP has been produced and shown, by competition binding experiments with SP, to be directed to the SP binding-site on the NK-1r (Dery *et al.* 1997). This antibody has been used to immunolocalize the NK-1r in various regions of the rat nervous system, both at the light and electron microscope levels (Zerari *et al.* 1995; Zerari *et al.* 1998). Since the ligand-binding domain has a high probability of being a well-conserved region among NK-1r from different species, the mAb12 antibody was considered likely to recognize receptors in other species. Indeed, it has been demonstrated previously that mAb12 recognized recombinant human NK-1r expressed in Chinese hamster ovary (CHO) cells (Dery *et al.* 1997). In the present study we demonstrate that this monoclonal antibody is capable of detecting NK-1r in human tissues.

4.2 Materials and Methods

Human antrum and attached duodenum tissue was obtained from 11 multiple organ donors (5 females, average age of 39 yrs and 6 males, average age of 32 yrs) in association with the Pacific Organ Retrieval for Transplantation Program, with ethical

approval from the Clinical Screening Committee of The University of British Columbia. Tissue samples were processed for immunocytochemical studies and Western Blot analysis according to the protocol outlined in Chapter 2.

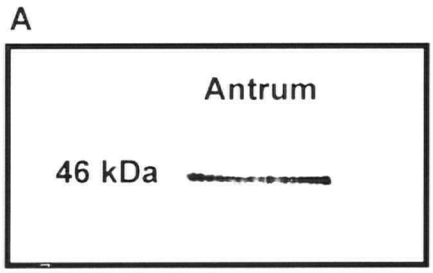
A guinea pig antiserum to SP (#6, a gift from Dr. Kwok, Department of Physiology, U.B.C.) at a dilution of 1:250 and a murine monoclonal antibody to the NK-1r (Dery *et al.* 1997; Zerari *et al.* 1995) at a dilution of 1:100 were incubated with the tissue sections for 48 hours at 4 °C. Control experiments included preabsorbtion of the antibody overnight at 4 °C with 100 nM of the immunogen peptide, PS5 (Zerari *et al.* 1995), prior to incubation with the tissue. The primary antibodies were localized using cyanine-3 (Cy3) conjugated goat anti-mouse IgG at a dilution of 1:2000 (Jackson Immunocytochemical Laboratories, West Grove, PA) or fluorescein isothiocyanate conjugated goat anti-guinea pig IgG at a dilution of 1:200 (Jackson Immunocytochemical Laboratories) for 90 min at RT.

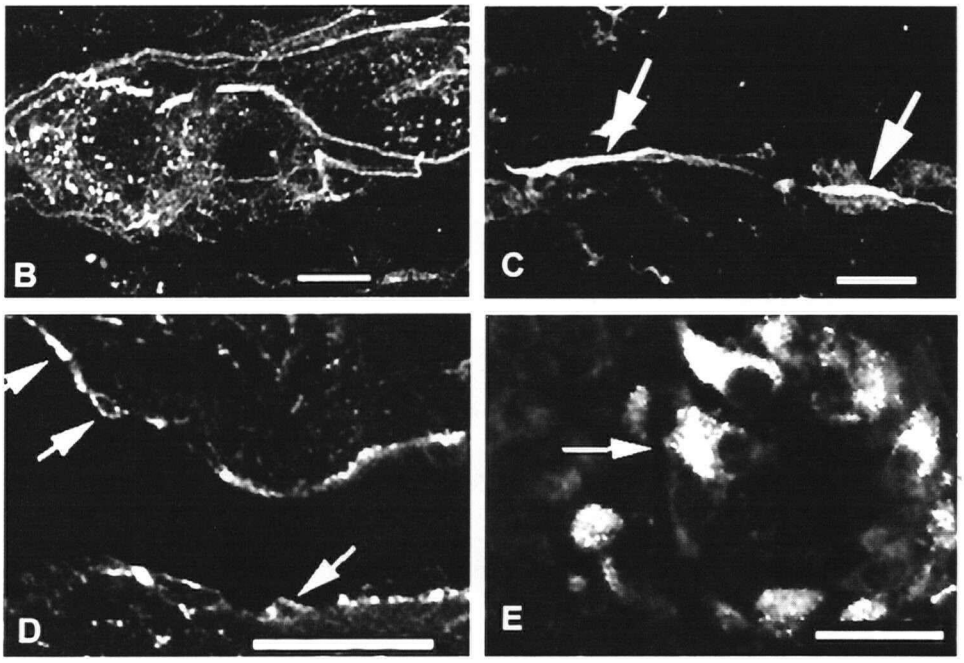
4.3 Results

Western blots demonstrated that the antibody detected a 46 kD protein, which is the appropriate molecular size for the NK-1r (Figure 21a). Preabsorption of the antibody with the immunogen completely removed the immunostaining (data not shown). These data confirmed the specificity of the antibody for the NK-1r in human tissues.

Single immunostains of the antrum and duodenum with the NK-1r antibody demonstrated expression of the receptor on neurons and fibers in the myenteric and

FIGURE 21. Detection and distribution of the NK-1r in the human antrum. A) a Western blot using the NK-1r antibody, mAb12, showing a positive band at 46 kDa, B) a submucosal ganglion in the Brunner's gland region, C) spindle-shaped cells in the circular muscle region (arrows), D) endothelial cells in a gastric blood vessel (arrows) and E) epithelial cells in the antral mucosa (arrows). Scale bar, 25 μm.


A
Antrum
46 kDa


B
C
D
E

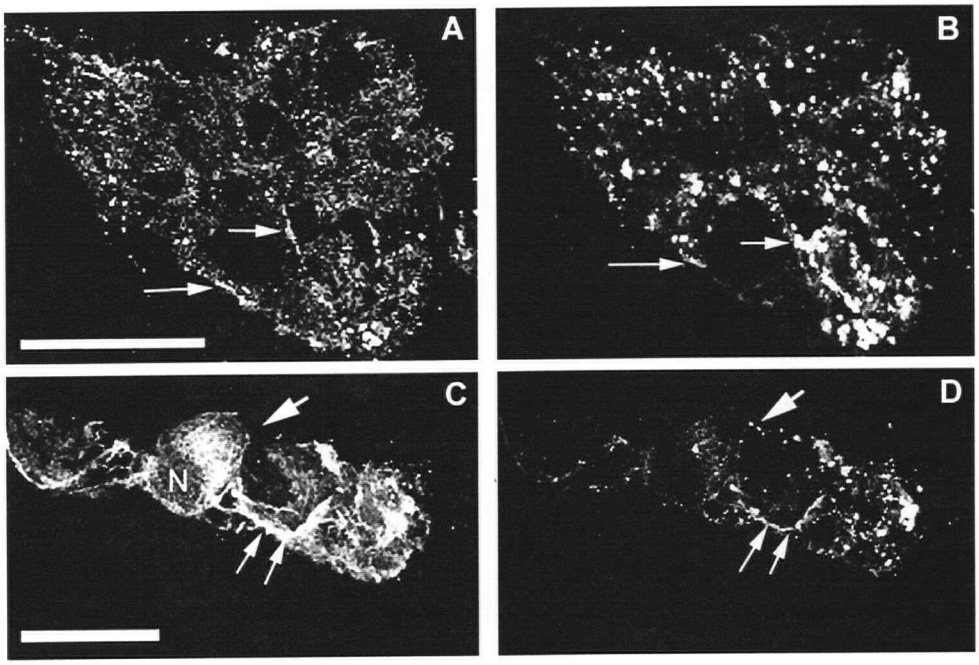
submucosal plexuses. Underlying the Brunner's glands, discrete ganglia containing NK-1r-IR neurons were noted (Figure 21b) and immunoreactive fibers were observed surrounding the glands (data not shown). NK-1r-IR was present on spindle shaped cells (probably interstitial cells of Cajal) (Figure 21c), endothelial cells lining the blood vessels (Figure 21d), and a population of mucosal cells (Figure 21e). No immunoreactivity was observed on smooth muscle cells. Double immunohistochemical labeling demonstrated that a proportion of NK-1r-IR neurons in both myenteric and submucosal ganglia were surrounded by SP-IR nerve fibers (Figure 22)

4.4 Discussion

The results indicated that the distribution of NK-1r-IR in the human antrum and duodenum was considerably greater than that previously reported for the corresponding region in either the rat or guinea pig. In the present study, NK-1r-IR was observed on spindle-shaped cells in the circular muscle layer. If these spindle-shaped cells are indeed the interstitial cells of Cajal, our results are in contrast to studies on the rat and the guinea pig where NK-1r-IR interstitial cells of Cajal were not observed in the stomach (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995).

Our results confirm the expression of the NK-1r on neurons within the myenteric plexus of the antrum and duodenum previously reported in the rat and guinea pig (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995). In all cases a proportion of the nerve fibers co-expressed SP-IR. Within the stomach of the rat or guinea pig, NK-1r-IR was confined to the myenteric plexus in the absence of a well defined

FIGURE 22. Examples of the double immunostaining with guinea pig anti-SP antibody and mAb12. A) NK-1r-IR in the myenteric plexus. Note the abundant punctate staining on the plasma membrane of neurons (small arrow), B) SP-IR in the same myenteric plexus. Note the presence of SP-IR varicose fibers in close association with the plasma membrane receptor shown in A (small arrows), C) NK-1r-IR cell body in the submucosal plexus shows NK-1r-IR in the cytoplasm and cell membrane (N, nucleus), and D) SP-IR in the same submucosal plexus. Note the presence of 2 varicosities adjacent to the NK-1r-IR neuron (large arrow) and the overlap with NK-1r-IR on nerve fibers (small arrows). Scale bars, 25 μm.


A
B
C
N
D

submucosal plexus. However, in human antrum directly underlying the muscularis mucosa and in the duodenum under the Brunner's glands was a recognizable submucosal plexus comprised of small ganglia containing 3-4 neurons and also individual neurons. Both neurons and fibers in the submucosal plexus demonstrated NK-1r-IR.

In neither the rat nor the guinea pig was NK-1r-IR reported on the endothelial cells lining the blood vessels (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995). In human tissue NK-1r-IR was clearly observed on the surface of the endothelial cells. This localization is consistent with the vascular effects of SP (Figini *et al.* 1997; Lu *et al.* 1997; Emanueli *et al.* 1998; McDonald *et al.* 1996; Moore *et al.* 1997; Vanner, 1994). Finally, NK-1r-IR was observed on a subpopulation of antral epithelial cells, possibly endocrine cells which had not previously been reported.

In summary, the present study demonstrates that the newly available antibody for the NK-1r was capable of detecting the receptor in human tissue. Although the distribution of the NK-1r is similar to the rat and guinea pig in the myenteric plexus, expression of receptor immunoreactivity on spindle-shaped cells, endothelial cells, submucosal neurons and mucosal cells has not been reported previously.

5. CELLULAR EXPRESSION OF THE NK-1r IN THE HUMAN ANTRUM

5.1 Introduction

The main members of the mammalian tachykinin family are substance P (SP) neurokinin A (NKA) and neurokinin B (NKB) (Holzer & Holzer-Petsche, 1997a). There are three primary tachykinin receptor subtypes which have been identified as the primary tachykinin receptors, neurokinin 1 (NK1), NK2 and NK3. Of the three receptors, NK1 preferentially binds SP while NK2 has a high affinity for NKA and NK3 selectively binds NKB (Maggi, 1995).

In the peripheral nervous system neural transmission is predominantly achieved by a non-synaptic mechanism. The critical element in the neural pathways is the location of the neuropeptide and their respective receptors and degredative enzymes. Although the localization of the NK receptors in the rat and guinea pig gastrointestinal tract has been extensively studied (Legat *et al.* 1996; Zagorodnyuk *et al.* 1997; Zagorodnyuk & Maggi, 1997) there is relatively little known about their distribution in humans.

In rat and guinea pig stomach the NK-1r has been localized primarily on neurons in the myenteric plexus (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995). In these species the stomach lacks a submucosal plexus, therefore, no NK-1r-IR has been reported in this region. In addition to the presence of the receptor on neuronal elements, NK-1r-IR has also been detected on chief cells in the corpus of the rat (Kitsukawa *et al.* 1996). In contrast, in the small intestine the NK-1r has been reported

to be expressed on both myenteric and submucosal neurons, the interstitial cells of Cajal and enterochromaffin cells (Southwell *et al.* 1996; Moore *et al.* 1997; Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995; Ginap & Kilbinger, 1997).

Although, in the stomach, there is no evidence for NK-1r-IR on smooth muscle cells within the mucosa or in the submucous plexus, SP-IR nerve fibers are present in all layers and SP is known to affect both motility and gastric secretion. These effects, while predominantly mediated by activation of the NK-1r, can also result from activation of the NK-2r and NK-3r. In both species NK-2r-IR has been observed on the smooth muscle cells (Zagorodnyuk & Maggi, 1997; Grady *et al.* 1996a). The most intense staining for the NK-2r in rat stomach was observed in the antral circular muscle layer (Grady *et al.* 1996a). In this study NK-3r-IR was localized to neurons in both the submucous and myenteric plexuses of the antrum and fundus.

The immunocytochemical data suggested that in rats and guinea pigs SP acts primarily on the NK-1r in the myenteric plexus to alter activity of intrinsic gastric neurons. The absence of NK-1r-IR in the smooth muscle layer, where abundant SP-IR occurs, indicated either a receptor/ligand mismatch or that SP in this region activates an alternative receptor subtype such as the NK-2r. Preliminary studies outlined in Chapter 4 indicated that the cellular expression of the NK-1r was more extensive in the human antrum in comparison to the reported localization in other animals (Smith *et al.* 1998). These data indicated that SP and NK-1r interactions in human antrum may not be limited to the myenteric plexus. The purpose of the present study was to establish the specific cell types expressing the NK-1r and the relationship of NK-1r expressing

cells to SP-IR fibers in human antrum.

5.2 Materials and Methods

Human antral tissue was obtained from 23 multiple organ donors (N=23, 12 females, 11 males, ranging in age from 13-59) in association with the Pacific Organ Retrieval for Transplantation Program with ethical approval from the Clinical Research Ethics Board of the University of British Columbia. The tissue was processed for immunocytochemical studies and Western blot analysis as outlined in Chapter 2.

The immunostaining protocol used a combination of chicken, rabbit or guinea pig antisera with murine monoclonal antibodies. In all cases, the primary antisera/antibodies and secondary antisera were prescreened to eliminate any possible cross reactivity. The details of the antisera/antibodies used are given in Table 4.

We obtained two antibodies to the NK-1r. The first was a monoclonal anti-complementary peptide antibody, mAb12, which was a kind gift from Dr. J-Y Couraud (Dery *et al.* 1997; Zerari *et al.* 1995). The second, a chicken anti-NK-1r antibody, was a gift from Dr. John Walsh (#19, provided by Center for Ulcer Research and Education/Gastroenteric Biology Center Antibody/RIA Core, National Institute of Diabetes and Digestive and Kidney Diseases Grant DK 41301). Antibodies to von Willebrand's factor were used to identify endothelial cells (Wall *et al.* 1980). C-kit-IR was used to locate the interstitial cells of Cajal and mast cells (Shimizu *et al.* 1996; Tsuura *et al.* 1994). To identify Schwann (glial) cells an antibody to S-100 was used and fibroblasts were identified using an antibody to fibronectin (Hassall *et al.* 1990).

TABLE 4. DETAILS OF ANTISERA/ANTIBODIES

Antigen	Species	Source[a]	Specificity	Dilution
mAb12	Mouse Monoclonal	J-YC	SP binding site	1:100
NK-1r	Chicken	CURE	C-terminal	1:5000
SP	Guinea Pig	KK	N-terminal	1:500
c-kit	Rabbit	Calbiochem	C-terminal	1:100
Fibronectin	Rabbit	Sigma		1:100
S-100	Rabbit	Sigma		1:5000
VIP	Rabbit	Milab	C-terminal	1:1000
CGRP	Rabbit	Polak		1:1000
Von Willebrand's factor	Rabbit	Sigma		1:1000
Gastrin	Rabbit	Dako		1:500
Somatostatin	Rabbit	Dako		1:500
5-HT	Rabbit	Sigma		1:30,000
Guinea Pig IgG	Donkey Texas Red	Jackson		1:1000
Mouse IgG	Donkey Cy3	Jackson		1:2000
Rabbit IgG	Donkey FITC	Jackson		1:500
Rabbit Alexa	Donkey HCA 488	MP		1:2000
Chicken IgG	Donkey Cy3	Jackson		1:1000

IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; Cy3, cyanine dye 3; ND, not determined; HCA, highly cross adsorbed.

[a]J-YC, Dr. Jean-Yves Couraud (CEA, Servce de Pharmacologie et d'Immunologie, DSV/DRM, CE-Saclay, France); CURE, Center for Ulcer Research and Education, (VA Wadsworth, Los Angeles, CA); KK, Dr. Yin Nam Kwok (Department of Physiology, University of British Columbia, Vancouver, Canada); Calbiochem, Calbiochem (Cambridge MA); Sigma, Sigma Immunocytochemicals (St. Louis, MO); Milab, Milab (Sweden); Polak, Dr J Polak (Hammersmith hospital, London, England); DAKO, DAKO Diagnostics Canada (Missisauga, ON); Jackson, Jackson Immunocytochemical Laboratories (West Grove, PA); MP, Molecular Probes (Eugene, OR).

5.3 Results

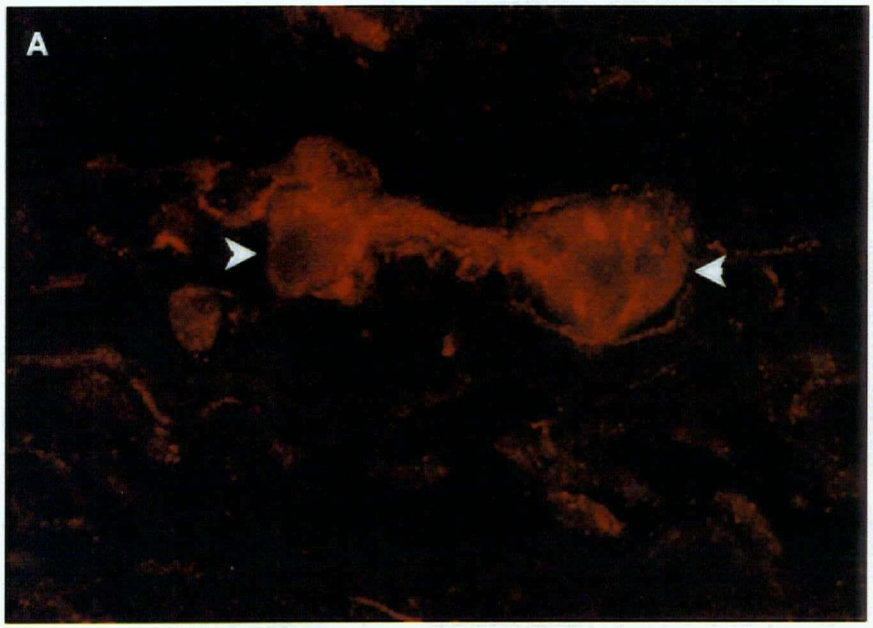
As previously reported the monoclonal NK-1r antibody labeled numerous cell types throughout all layers of the human antrum. However, the chicken antibody predominantly labeled neurons in the myenteric plexus and endothelial cells lining small blood vessels. We have previously completed Western blots using the monoclonal antibody, therefore, in the present study the ability of the chicken antibody to detect a protein of the relevant size (46 kDa) in Western blots of human antral tissue was determined. These experiments demonstrated that the chicken antisera detected both a 46 kDa protein and a larger molecular weight band of approximately 110 kDa (data not shown). Each cell type expressing the NK-1r will be discussed in more detail with respect to the corresponding region of the antrum.

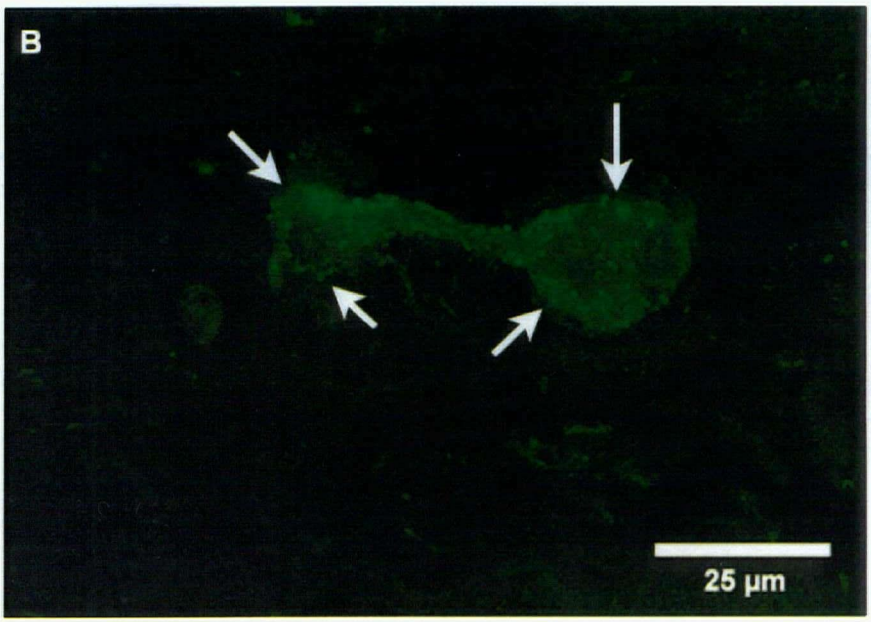
Myenteric Plexus

The myenteric plexus contained numerous NK-1r positive cell bodies, clearly identified by both NK-1r antibodies. A majority of NK-1r-IR cell bodies were surrounded by SP positive nerve fibers (Figure 23) observed to be running throughout the plexus. A small percentage of these NK-1r-IR neurons were not surrounded by SP-IR fibers, however, these neurons were in close spatial proximity to SP-IR nerve fibers.

Major blood vessels running between and underneath the myenteric plexus displayed von Willebrand's factor immunostained endothelial cells. These blood vessels appeared to form a network surrounding the nerve plexus. Von Willebrand's factor-IR endothelial cells lining the major blood vessels were co-immunostained with

FIGURE 23. Examples of double immunostaining of human antral sections for SP and mAb12. A) NK-1r-IR on myenteric cell bodies (arrowheads) and B) maximum intensity projection of SP-IR nerve fibers in the same myenteric plexus, surrounding the NK-1r-IR cell bodies (arrows). Maximum intensity projections of 6 z plane images (0.6 µm between images).


A


B
25 µm

both NK-1r antibodies. Interestingly, the endothelial cells lining small capillaries were only co-immunostained with the chicken antiserum. There was no evidence of NK-1r-IR on the adventitia or vascular smooth muscle surrounding the blood vessels.

Within this region, S-100-IR Schwann cell processes formed a meshwork around the neurons and nerve fibers but, no co-localization with NK-1r-IR was detected.

Circular and Longitudinal Muscle

Within the circular muscle layer NK-1r-IR was observed on spindle-shaped cells predominantly localized to the outer edges of the major muscle blocks. C-kit positive interstitial cells of Cajal were also localized to the edges of the circular muscle blocks. These cells were observed to run both parallel and perpendicular to the direction of the muscle fibers. The c-kit-IR cells were co-immunostained with the NK-1r-IR (Figure 24) and NK-1r-IR was also observed on c-kit positive cells lying just above the longitudinal muscle layer. The NK-1r-IR interstitial cells of Cajal were found to be in close proximity to SP-IR nerve fibers.

Von Willebrand's factor immunostained endothelial cells, observed in both muscle layers, were found to be NK-1r-IR. The smaller capillaries, predominantly localized to the circular muscle layer, were immunostained with the chicken NK-1r antibody while larger blood vessels were labeled by both NK-1r antibodies. Although the S-100 positive Schwann cells and fibronectin-IR fibroblasts could be seen in both muscle layers they were not NK-1r-IR (Figure 25).

FIGURE 24. C-kit and mAb12 immunoreactivity in the circular muscle layer of the human antrum. A) NK-1r-IR spindle-shaped cells within the circular muscle layer, B) c-kit-IR interstitial cells of Cajal in the same circular muscle section and C) overlay of the NK-1r-IR and c-kit-IR images in (A) and (B). A maximum intensity projection of 23 z plane images (1.8 μm between images). The yellowish-orange cells represent co-labeling of interstitial cells of Cajal with the NK-1r (arrow) which was confirmed by stepping through individual z plane images.

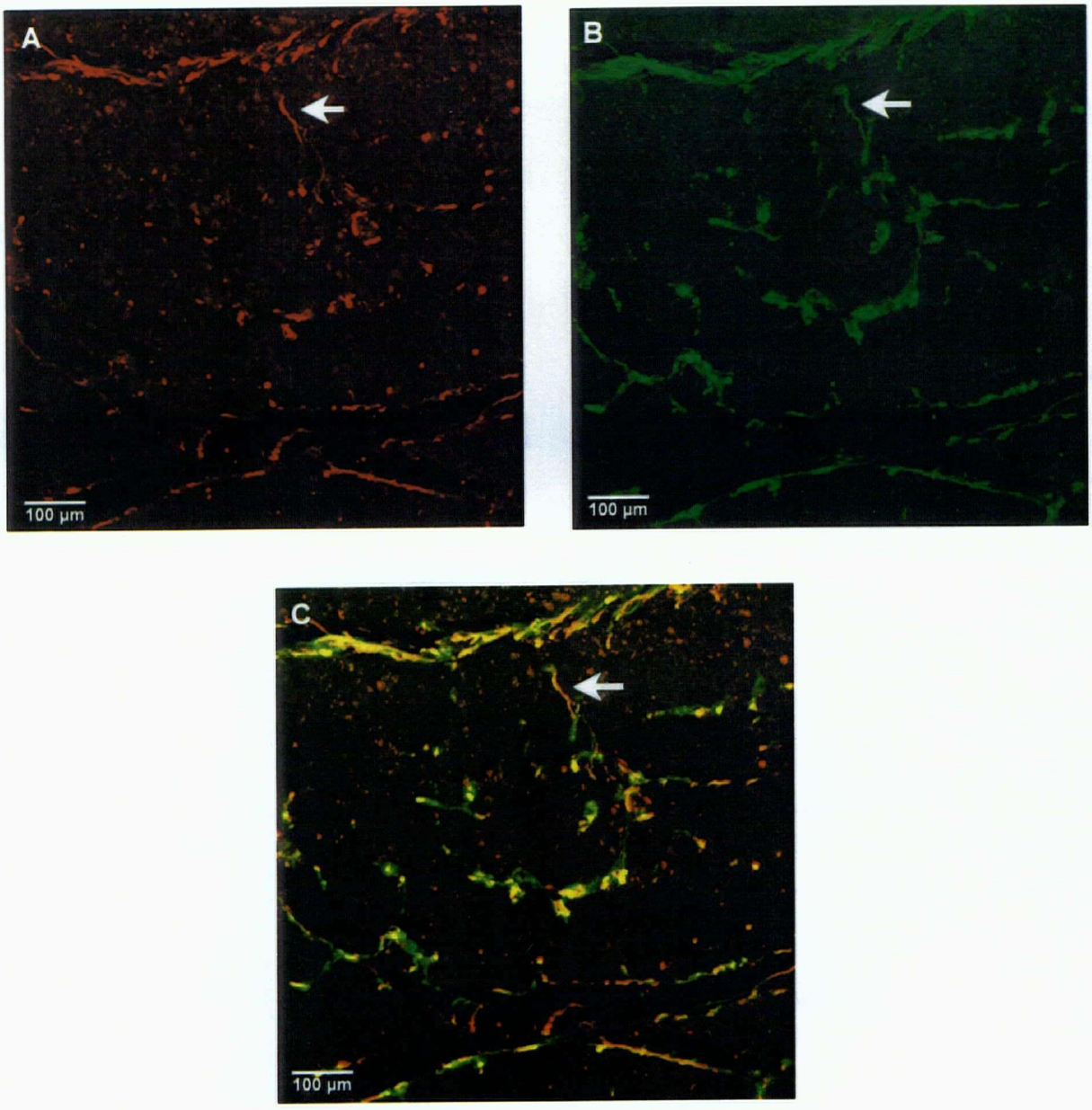
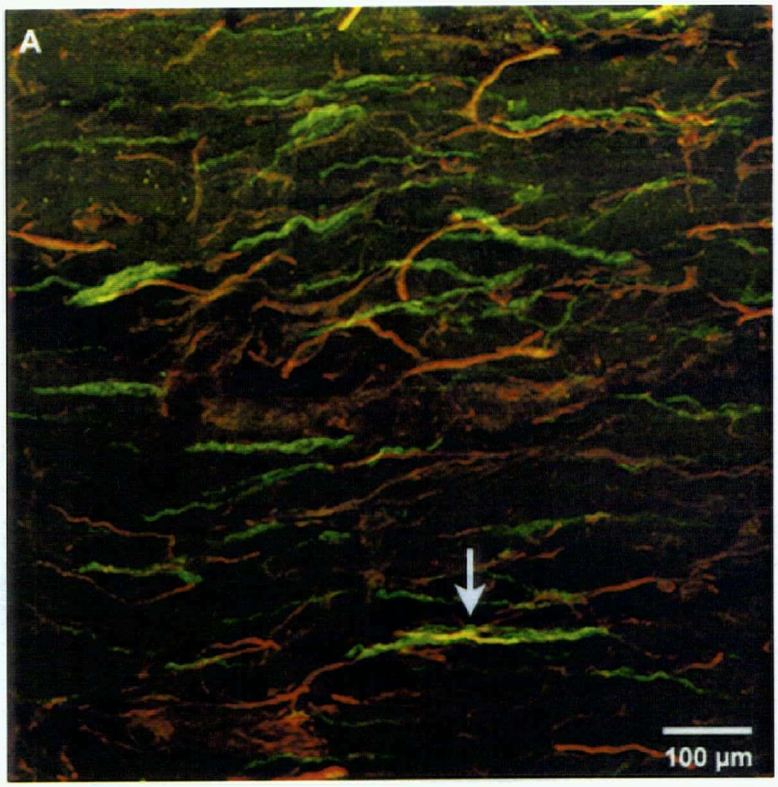
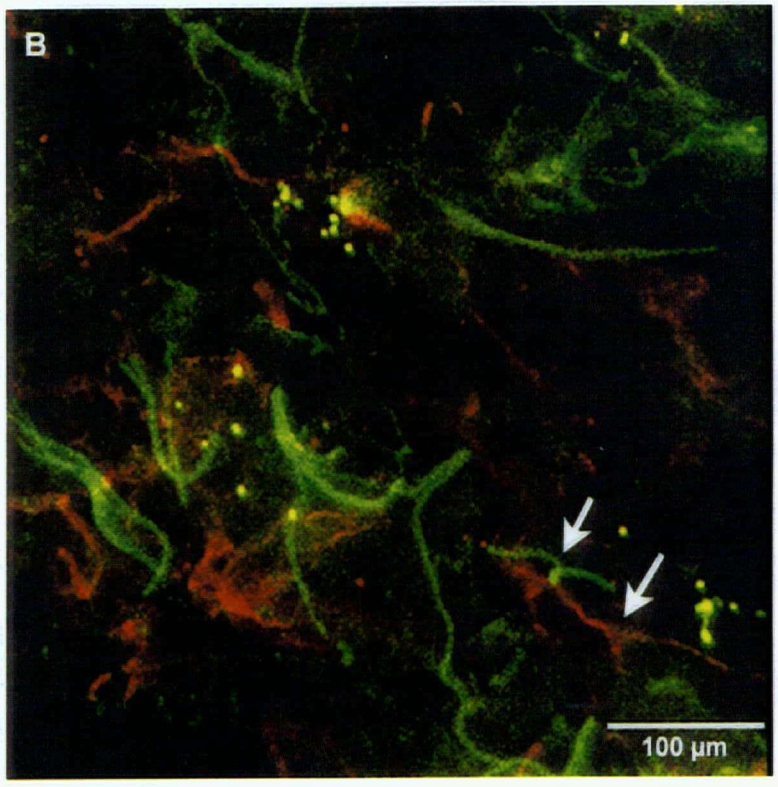

A
100 µm
B
100 µm
C
100 µm

FIGURE 25. Double immunostains for mAb12 with either s100-IR Schwann cells or fibronectin-IR fibroblasts in the human antrum. A) Schwann cells which were s100-IR were not found to be co-labeled with NK-1r-IR, although the two cells types have a similar morphology (arrow indicates an artifact of the maximum intensity projection and does not represent co-labeling) and B) fibronectin-IR fibroblast also have a similar morphology to NK-1r-IR cells (arrows), however the two cells types were not co-labeled.


A
100 µm


B
100 µm

Submucosa and Muscularis Mucosa

Blood vessels penetrating through the muscularis mucosa from the submucosa were co-labeled by von Willebrand's factor and both NK-1r antibodies (Figure 26). In addition, small submucosal ganglia underlying the muscularis mucosa were also found to contain NK-1r positive neurons. These NK-1r positive cell bodies were found to be encircled by SP-IR nerve fibers as shown within the myenteric plexus. None of these cell bodies were SP-IR nor did they exhibit VIP- or CGRP-IR. Schwann cells and c-kit positive mast cells were found to be abundant throughout both layers but neither cell type was co-labeled with the NK-1r.

Mucosa

Within the mucosa NK-1r-IR was observed on epithelial cells, endothelial cells and nerve fibers. It was found that NK-1r-IR was co-immunostained with SP-IR nerve fibers but was not observed to be co-localized with either CGRP- or VIP-IR fibers. The receptor was found on gastrin cells (Figure 27) but not on either somatostatin or serotonin producing cells (data not shown). Schwann cells immunoreactive with S100 were found lining the fiber tracts leading up through the lamina propria towards the epithelium but were not co-labeled with the NK-1r. C-kit positive mast cells could be seen throughout the mucosa, however, as in the submucosa, none of the mast cells showed NK-1r-IR.

FIGURE 26. Examples of NK-1r immunostaining of endothelial cells in the human antrum. A) NK-1r-IR on endothelial cells (arrows) lining the major blood vessels of the submucosa, B) von Willebrand's factor-IR on the same endothelial cells (arrows), C) endothelial cells, lining a major blood vessel in the submucosa, immunostained for the NK-1r with the chicken NK-1r antisera (arrows) and D) NK-1r-IR capillaries in the circular muscle layer immunostained only with the chicken NK-1r antisera (arrows).

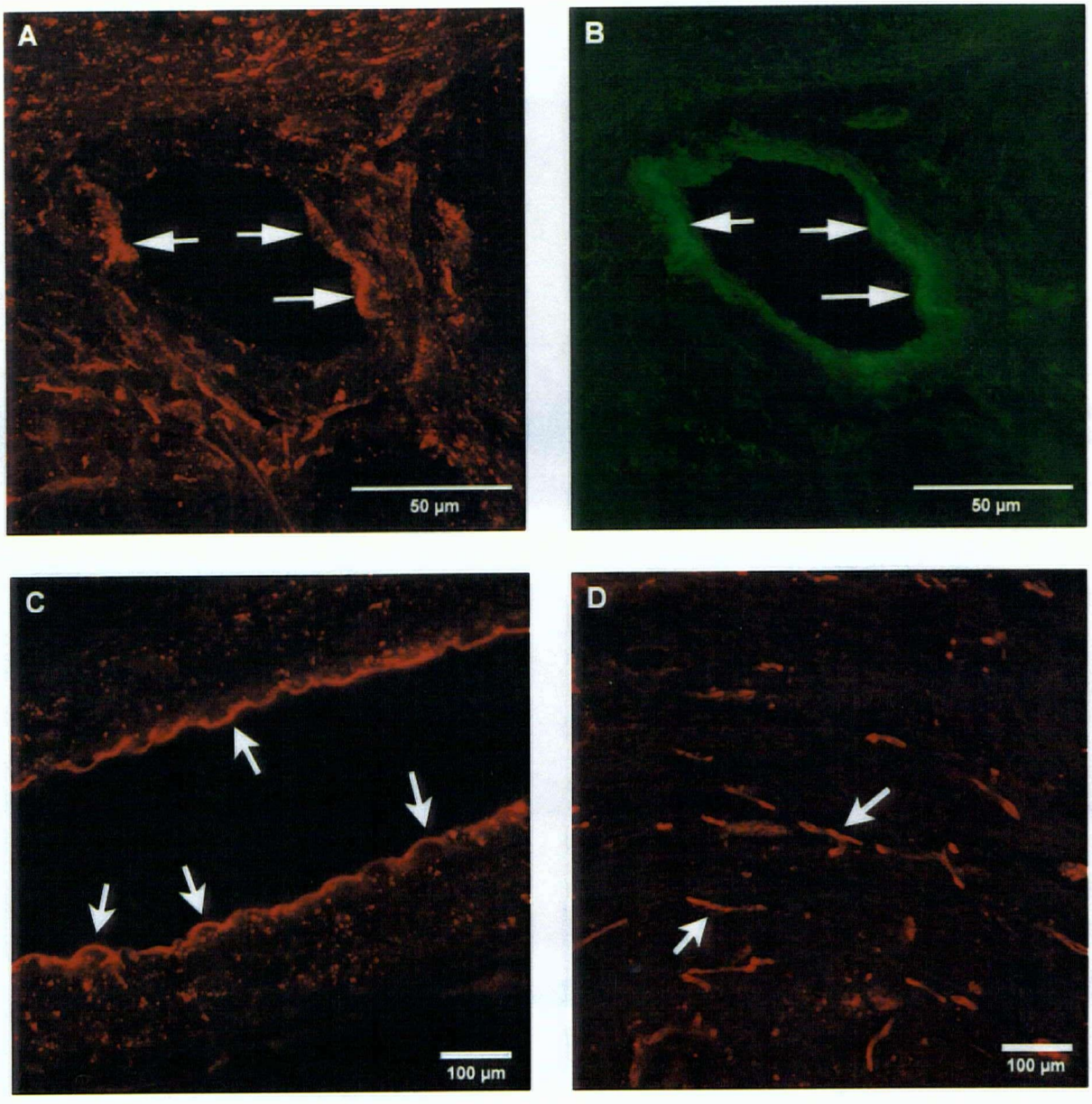
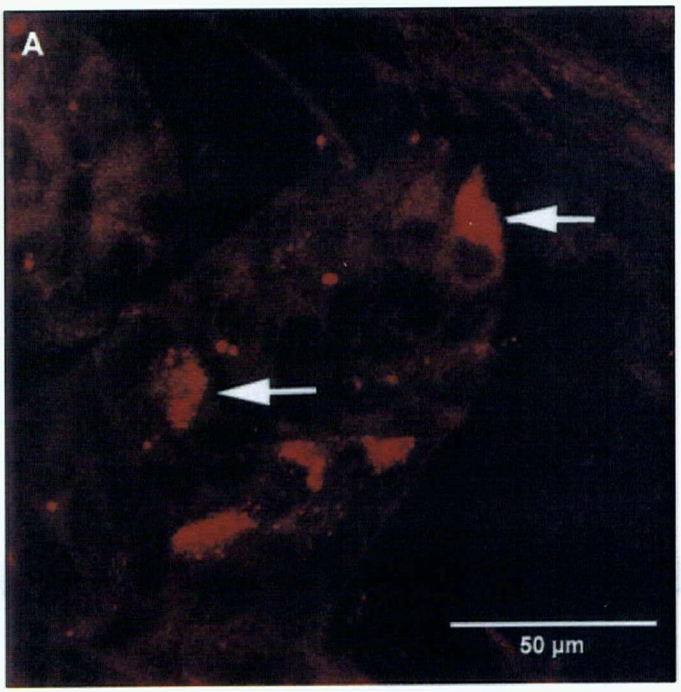
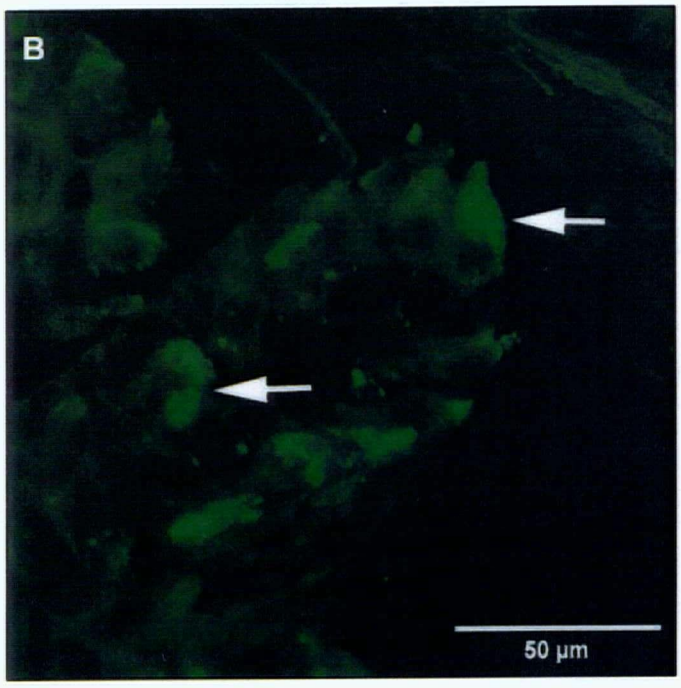

A
50 µm
B
50 µm
C
100 µm
D
100 µm

FIGURE 27. Epithelial endocrine cells co-labeled with mAb12 in the human antrum. A) NK-1r-IR epithelial cells in the mucosal layer and B) the same mucosal section showing gastrin-IR. Note the NK-1r-IR epithelial cells are co-labeled with gastrin-IR (arrows). A maximum intensity projection of 10 z plane images (1.2 μm between images).


A
50 µm


B
50 µm

5.4 Discussion

This study demonstrates that the cellular expression of the NK-1r in the human antrum is much more diverse than expected based on studies carried out in guinea pig and rat stomach. Although both NK-1r antibodies clearly label endothelial cells and neuronal cell bodies, the chicken antibody did not detect the NK-1r in as many cell types as did mAb12. Western blots demonstrated that the chicken antisera detected a 46 kDa protein and a higher molecular weight form most probably representing glycosylated receptor. In Chapter 4, mAb12 was also shown to detect a protein of 46 kDa, however, the two antibodies were raised to two distinct antigens. The monoclonal antibody was raised using the complementary or antisense peptide method (Blalock *et al.* 1989) and, using this approach, mAb12 has been described as the first monoclonal antibody directed against the SP binding domain of the NK-1r (Dery *et al.* 1997). The chicken antisera was raised against the final 15 amino acids at the C-terminus of the rat NK-1r. There is a large degree of homology (~92% identical) between the rat and human NK-1r (Gerard *et al.* 1991), however, within this C-terminal region there are four amino acid substitutes, two of which are non-conservative. As the two NK-1r antibodies detect different regions of the NK-1r it is possible that this may account for the discrepancy in immunostaining observed in the present study.

Previously, in antral tissue, SP and NK-1r interactions had been limited to myenteric neurons indicating that the actions of SP on gastric motility were confined to modulation of intrinsic neural pathways. In the present study, while the interaction on myenteric neurons, as previously reported in animal models (Grady *et al.* 1996a;

Sternini *et al.* 1995) was present, this did not represent the only pathway by which SP could act to influence gastric motility.

The presence of the NK-1r on antral interstitial cells of Cajal (suggested to function as "pacemaker" cells for gastric motility (Huizinga *et al.* 1997)) in close association with SP-IR fibers indicates that SP can modulate gastric motility by a non-neuronal mechanism. Interestingly, co-localization of the NK-1r with c-kit positive interstitial cells of Cajal has not previously been reported in rodent stomachs. This may be due to conflicting evidence concerning whether the interstitial cells of Cajal are even present in rodent stomach (Grady *et al.* 1996a; Sternini *et al.* 1995; Ishikawa *et al.* 1997). In the human stomach, interstitial cells of Cajal have previously been demonstrated in both muscle layers as well as the myenteric plexus using ultrastructural (Faussone-Pellegrini *et al.* 1989) and immunocytochemical techniques (He *et al.* 1998a; He *et al.* 1998b).

Our results showed that the NK-1r-IR was localized to the surface of endothelial cells lining the blood vessels in all layers of the antrum and that there was no evidence for the NK-1r on vascular smooth muscle cells. These data indicated that the vascular effects of SP in humans are mediated by modulation of endothelial cell function. The co-localization of the NK-1r to vascular endothelium in human antrum differed from that reported in rodents (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995). However, autoradiographic studies have demonstrated the presence of SP binding sites on endothelial cells and vascular smooth muscle in submucosal arterioles and venules in the canine and human gastrointestinal tract (Holzer & Holzer-Petsche,

1997b; Gates *et al.* 1988).

Localization of the NK-1r on the endothelial cells is consistent with the proposed mechanisms of action for SP on both vasodilation and plasma extravasation. Studies have shown that SP acts via the NK-1r located on endothelial cells to increase the formation of nitric oxide (NO) which then acts as the vasodilator (Holzer & Holzer-Petsche, 1997b). Substance P has also been proposed to stimulate an increase in vascular permeability through activation of the NK-1r expressed on the endothelial cells (Bowden *et al.* 1996). The results of the present study localize the NK-1r to the endothelial cells of the vasculature and not to the surrounding smooth muscle. Our findings in the human antrum support the concept that plasma extravasation and vasodilation are due to SP activation of the NK-1r localized to endothelial cells.

A novel finding in the human antrum was the presence of a clearly defined submucous plexus directly underlying the muscularis mucosa. Cell bodies were not observed in association with submucosal blood vessels nor was there an extensive deep muscular plexus overlying the circular muscle layer. In the submucous plexus, a minor population of neurons expressing the NK-1r were surrounded by SP-IR nerve fibers. Although we were unable to identify the type of neuron, they were possibly intrinsic cholinergic neurons. This possibility could not be examined in the present study due to the lack of a suitable antibody. The presence of NK-1r-IR in the submucous plexus neurons suggested that SP could alter blood vessel activity by a neuronal mechanism. Previous studies have determined that submucosal neurons

innervated mucosal blood vessels (Vanner & Surprenant, 1996). However, in the present study, although extensive peptidergic innervation of blood vessels was observed (especially with CGRP and VIP), we had no evidence for expression of the NK-1r on these vascular fibers. The presence of NK-1r-IR nerve fibers in the mucosa suggested that the NK-1r positive neurons in the submucous plexus innervate this region.

In the mucosal region the NK-1r was localized to a sub-population of mucosal endocrine cells. Gastrin cells, but not somatostatin or enterochromaffin cells, expressed the receptor and were in close spatial proximity to SP-IR fibers. In isolated canine G cells SP lacked an effect on bombesin-stimulated gastrin release (Giraud *et al.* 1987). Studies of the perfused rat stomach showed that SP had a small inhibitory effect on the release of somatostatin-like immunoreactivity, however this effect was mediated by the NK-2r localized to the somatostatin producing cells (Kwok *et al.* 1988). Our data suggest SP may have a direct effect on gastrin release from human G cells.

The previous chapter, in which only the modified anti-idiotypic antibody was used, demonstrated the presence of numerous spindle-shaped cells concentrated to the muscle layers of the antral tissue. In addition to the interstitial cells of Cajal, it was possible that these were either Schwann cells or fibroblasts and both of these cell types have been reported to express the NK-1r (Mantyh *et al.* 1989; Ziche *et al.* 1990). In the human antrum, S-100-IR Schwann cells were present in both neural plexuses with processes extending through the muscle and mucosal layers. Although these cells were in close spatial proximity to NK-1r positive cells, on no occasion were double

labeled cells observed. In addition spindle-shaped fibronectin-IR cells were concentrated to the edges of the muscle cell layers, serosa and submucosal regions. Once again there was no overlap between the NK-1r-IR and fibronectin stained cells. These data suggest that modulation of these two cell types by SP in the human antrum is unlikely to involve the NK-1r.

This study clearly demonstrated a widespread cellular expression of the NK-1r in the human antrum compared to that of the guinea pig and rat. This study supports findings in animal models for the presence of SP-IR fibers surrounding NK-1r positive cells bodies. In the human antrum the lack of NK-1r-IR on the smooth muscle cells and the localization of the receptor on both myenteric neurons and the interstitial cells of Cajal indicated that SP affects gastric motility by two distinct mechanisms. Endothelial cells of the blood vessels in all layers of the antrum expressed the NK-1r indicating that the ability of SP to elicit both vasodilation and plasma extravasation is due to alterations in endothelial cell function. In conclusion the cellular expression of the NK-1r suggests that the mechanisms of action of SP is not limited to the modulation of myenteric neuronal pathways in human antrum.

6. General Discussion

6.1 Peptidergic and Nitrergic Innervation of the Human Antrum

In Chapter 3, it was clearly demonstrated that within the human antrum there is an abundant innervation in all layers. The prevalence of peptidergic fibers in the mucosa is far greater than that reported in the same region of animal models. The presence of NOS-IR nerve fibers in the mucosa opens up an entirely new area of research. The physiological function of NO on gastric mucosal functions has not been examined in detail probably due to the lack of NOS containing fibers reported there.

Animal models have demonstrated a small submucosal plexus in the stomach of some species, including the rat and mouse (Shimoda *et al.* 1996; Berthoud, 1996; Sharkey *et al.* 1984; Ekblad *et al.* 1985; Dockray *et al.* 1979). While this plexus is made up of small ganglia interconnected by intrinsic nerve fibers, it has not been well defined. However, the results of the present study have revealed a prominent, clearly defined submucosal plexus in the human antrum. The thickness of the submucosal region allowed division into three regions, zone 1 directly underneath the muscularis mucosa, zone 2 in the central region of the plexus and zone 3 directly above the circular muscle layer.

The presence of a well defined submucosal plexus containing cell bodies for every neuromodulator examined in this thesis most probably accounts for the abundant innervation of the mucosa. Animal studies have shown that peptidergic innervation of the gastric mucosa is present (Sundler *et al.* 1991; Dockray *et al.* 1979; Ekblad *et al.*

1985) however, they have not reported such an extensive innervation as observed in the present studies. Similarly, a study of the human stomach reported a few NADPH-diaphorase positive fibers in the mucosa (Manneschi *et al.* 1998), whereas the present study demonstrates an abundant nitrergic innervation of this antral layer. Studies have shown that in animal models a large percentage of the mucosal innervation is of myenteric origin (Furness *et al.* 1991; Pfannkuche *et al.* 1998; Reiche & Schemann, 1998) and that may account for the lack of submucosal ganglia. While it was believed that a majority of neural input to the stomach mucosa was either extrinsic or derived from the myenteric plexus, the presence of numerous neurons for every neuromodulator studied and the magnitude of the submucosal plexus in the human suggests that it has a significant role in regulating gastric mucosal functions.

The requirement for antigen retrieval methods prior to incubation of antral sections with the NOS antibody to restore immunostaining of the fine varicose fibers in the mucosal region was not expected. However, it has been demonstrated that the only alteration to the immunostaining protocol was the use of a new batch of PFA with a reduced purity. The lack of mucosal staining observed without antigen retrieval most probably results from increased cross linking during the fixation protocol preventing access of the antibody to the antigen. The use of 1% SDS as a denaturing agent restored immunostaining of the fine varicose mucosal fibers. The use of this retrieval technique can be considered reliable and useful in future investigations as it restored the same pattern of innervation observed prior to the switch in PFA. The observation that mucosal staining could be restored following antigen retrieval indicates that the

use of this simple and quick method can help to crosscheck that the results of immunohistochemical labeling are more complete. However, antigen retrieval can only allow for detection of tissue antigens present at concentrations above the limit of detection of the antibody and containing the relevant antigenic sites. In cases where low levels of antigen are present (or of course is if the antigen is absent) antigen retrieval will be ineffective.

6.2 Cellular Expression of the Neurokinin 1 Receptor

Chapters 4 and 5 were dedicated to characterizing the distribution and cellular expression of the NK-1r. The distribution of this receptor has been well characterized in the gastrointestinal system of the rat and the guinea pig (Southwell *et al.* 1996; Kitsukawa *et al.* 1996; Moore *et al.* 1997; Grady *et al.* 1996a; Grady *et al.* 1996a; Sternini *et al.* 1995; Ginap & Kilbinger, 1997). However, the receptor's distribution has not been determined in the human antrum. The studies described in Chapters 4 and 5 demonstrate that the cellular expression of the NK-1r in the human antrum is much more diverse than expected based on studies carried out in guinea pig and rat stomach. Although both NK-1r antibodies clearly label endothelial cells and neuronal cell bodies, the chicken antibody did not detect the NK-1r in as many cell types as did mAb12. Western blots demonstrated that the chicken antisera detected a 46 kDa protein and a higher molecular weight form most probably representing glycosylated receptor. In Chapter 4, the results showed that mAb12 was also capable of detecting a protein of 46 kDa, however, the two antibodies were raised to two distinct antigens.

The monoclonal antibody was raised using the complementary or antisense peptide has been described as the first monoclonal antibody directed against the SP binding domain of the NK-1r (Dery *et al.* 1997). The chicken antisera was raised against the final 15 amino acids at the C-terminus of the rat NK-1r. As the two NK-1r antibodies detect different regions of the NK-1r it is possible that this may account for the discrepancy in immunostaining observed in the present study.

These studies clearly demonstrated an expanded cellular expression of the NK-1r in the human antrum compared to that of the guinea pig and rat. In both plexuses NK-1r-IR neurons were observed, most of which were encircled by SP containing nerve fibers. While it has been reported in animal models that the NK-1r is present on myenteric neurons, the receptor had not been localized to neurons of the submucous plexus. This may be due the relative lack of a well defined submucosal plexus, discussed in the previous section.

The NK-1r was also localized to the interstitial cells of Cajal in the human antrum. While this co-localization has been observed in the small intestine of guinea pigs and rats (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995), it has not previously been reported in stomach. This is most likely the result of conflicting evidence as to the presence of the interstitial cells of Cajal in the rodent stomach (Grady *et al.* 1996a; Sternini *et al.* 1995; Ishikawa *et al.* 1997).

Endothelial cells of the blood vessels in all layers of the human antrum expressed the NK-1r indicating that the ability of SP to elicit both vasodilation and plasma extravasation is due to alterations in endothelial cell function. The presence

of NK-1r-IR on the vascular endothelium in human antrum differs from that reported in rodents (Grady *et al.* 1996a; Portbury *et al.* 1996; Sternini *et al.* 1995).

In the mucosal region the NK-1r was localized to gastrin cells, a sub-population of mucosal endocrine cells, suggesting that SP may have a direct effect on gastrin release from human G cells.

The expanded cellular expression of the NK-1r observed in the human antrum suggests that the mechanisms of action of SP, in this region, is not limited to the modulation of myenteric neuronal pathways.

7. Summary and Future Directions

7.1 Summary

In the human antrum the peptidergic and nitrergic innervation are considerably different from the reported distribution in animal models. The presence of abundant mucosal innervation along with a well defined large submucosal plexus suggests that in the human antrum gastric functions may be regulated by alternative mechanisms than those proposed from animal model investigations. Along with the differences in the innervation of the human antrum, studies reported in this thesis reveal that the NK-1r distribution is also differs significantly. The data presented herein indicate that the physiological regulation of the human antrum is likely to be species specific. Therefore, the use of animal models to predict human responses should be undertaken with extreme caution.

7.2 Future Directions

The work presented in this thesis gives rise to a number of future investigations. The dense innervation of the mucosal region by all neuroactive substances examined, prompts investigation into the regulation of mucosal functions. In the human, it has been demonstrated that the regulation of endocrine cell function is different from that reported in animal models. For example, it has been demonstrated that muscarinic agonists did not directly stimulate release of gastrin from human G cell cultures (Koop *et al.* 1997) and that these agonists actually had a direct stimulatory effect on

somatostatin release (Buchan *et al.* 1992). However, studies have shown that in the perfused rat stomach cholinergic agonists have an inhibitory effect on somatostatin secretion (Schubert *et al.* 1982; Koop *et al.* 1982; Saffouri *et al.* 1980) thereby increasing gastrin release. In canine antral gastrin cell (G cell) cultures, it has been demonstrated that cholinergic agonists directly stimulate gastrin release (Yokotani *et al.* 1995).

Many studies of gastric endocrine cell function have been carried out in animal models (Shimoda *et al.* 1996; Schubert & Makhlouf, 1982a; Schubert *et al.* 1982; Schubert, 1991; Vuyyuru *et al.* 1995; Manela *et al.* 1995; Weigert *et al.* 1997; Schubert & Makhlouf, 1982b; Koop *et al.* 1987; Campos *et al.* 1989; Kwok *et al.* 1988; Holst *et al.* 1987; Giraud *et al.* 1987; McIntosh *et al.* 1987; Short *et al.* 1985; DuVal *et al.* 1981; Koop *et al.* 1982) however, there are not as many investigations into their regulation in humans (Koop *et al.* 1997; Buchan & Meloche, 1994; Buchan *et al.* 1992; Buchan, 1991; Buchan *et al.* 1990; Campos *et al.* 1990). The data presented in this thesis suggest studies on the release of gastrin by VIP and NOS be carried out on isolated human antral G cells due to the close spatial arrangement of VIP- and NOS-IR nerve fibers to these cells. These studies could include the use of VIP analogues and antagonists, NO donors (spermine-NO (Maragos *et al.* 1991)), or NOS inhibitors, such as L-NAME or L-NMMA (for review see Moore & Handy, 1997), to determine the effect on gastrin release. The data in this thesis also suggests that the effect of SP on gastrin release should be determined in light of the results localizing the NK-1r to the G cells.

The NK-1r was localized using two different antibodies. One was created using the complementary or antisense peptide method, while the other was raised against the final 15 amino acids at the C-terminal tail of the rat NK-1r. As discussed previously there is a high degree of homology between the rat and human NK-1r (Gerard *et al.* 1991), however within the final 15 amino acids of the C-terminus there are four amino acid substitutions. The significance of these substitutions should be investigated. Western blot analysis of the chicken antisera demonstrated that the antibody detected two molecular weight bands, one at 46 kDA, the expected molecular weight of the NK-1r and another at approximately 110 kDA. Further studies must be carried out to determine the exact molecular weight of the higher band. This higher molecular weight band may represent the glycosylated receptor, however, deglycosylation studies should be carried out to confirm this possibility. Studies have shown that the use of endoglycosidase F, is effective at deglycosylating the NK-1r (Li *et al.* 1997; Kage *et al.* 1993), therefore the protocol should be tested on human antral tissue prior to fixation.

The reason for the differential distribution of the NK-1r observed with the two antibodies remains to be answered. Studies have shown that there is a C-terminally truncated form of the receptor that is 311 amino acids long as opposed to the 407 amino acids of the full length receptor (Li *et al.* 1997; Mantyh *et al.* 1996; Kage *et al.* 1993; Quartara & Maggi, 1997). Further investigations into the truncated receptor indicate that it may be resilient to desensitization and cells expressing the receptor would, therefore, undergo a prolonged responsiveness (Li *et al.* 1997). An autoradiographic study in 1996, which compared the distribution of the two receptor

isoforms *in vivo*, concluded that there is differential expression of the NK-1r isoforms in the rat (Mantyh *et al.* 1996). They found that in the rat brain the full length receptor isoform dominated.

The results in chapter 5 indicate that the chicken antisera did not detect the receptor in as many cell types as did the monoclonal antibody. As the chicken antisera is raised against the final 15 amino acids of the C-terminal tail, it is possible that this antibody is only detecting the full length receptor isoform. The monoclonal antibody is not dependent on the length of the C- terminus and may therefore be detecting the short isoform. Molecular biological techniques could be used to determine if the two receptor isoforms are present in the human antrum. The presence of the C-terminally truncated receptor would generate numerous studies into its physiological significance.

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