STUDIES ON RAT GASTROINTESTINAL NEUROPEPTIDE Y

By

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Date October 12, 1989
ABSTRACT

A sensitive and specific radioimmunoassay (RIA) for Neuropeptide Y (NPY) was developed and quantitation, characterization and release studies were performed.

The development of the RIA required the purification of the NPY tracer due to both multiple iodinated products resulting from the five tyrosine residues in its amino acid sequence, and the presence of unlabelled NPY. Ion-exchange and reverse phase high performance chromatography (HPLC) purification of $^{125}$I-NPY were performed. Optimal purification of $^{125}$I-NPY was achieved using a HPLC with a $\mu$Bondapak C$_8$ column and a 45-50% acetonitrile concentration gradient. A polyethylene glycol separation technique was used in conjunction with the HPLC purified tracer to improve assay conditions.

Although many studies aimed at elucidating the actions of NPY have been performed, little information is available on the distribution of gastrointestinal (GI) NPY in the rat. Therefore the NPY-immunoreactivity (IR) in extracts of the various regions of the GI tract were determined using the developed RIA. The tissue content of NPY was found to be highest in the various segments of the rat stomach, with a decreasing trend in NPY-IR down the GI tract until the level of the ascending colon where an increase was detected. Characterization studies on the tissue extracts were performed using gel filtration chromatography and HPLC. One immunoreactive species was detected in the corpus and ileum extracts using gel filtration chromatography, and in the corpus and colon extracts using HPLC. This immunoreactive species eluted in a position similar to synthetic porcine NPY and later than peptide YY (PYY).

In the physiological investigation of the the role of neuropeptides in the regulation of GI functions, release studies are crucial. The presence of high levels of NPY-IR in the stomach allowed the investigation of the release mechanisms of NPY in the perfused isolated rat stomach. However, due to the low basal secreted levels of NPY in comparison with other gastric peptides, as well as the enzymatic degradation and/or peptide uptake that occurs in the stomach vasculature, certain steps had to be taken to allow for the detection of
the endogenously released peptide. Sep Pak extraction was found to be required to concentrate the endogenously released peptide. Proteolytic inhibitors were also added to the perfusate to reduce enzymatic degradation. A low basal level of NPY was detected which ranged from 98 to 147 fmole/min. Neuropeptide Y was found to be released into the gastric vasculature in response to high potassium depolarization.

A few studies have been performed on cholinergic effects on NPY release, however no direct release studies have been performed on NPY-containing neurones innervating the stomach. Therefore, the actions of cholinergic agonists and antagonists on NPY secretion in the isolated perfused rat stomach were investigated. Acetylcholine and the nicotinic ganglionic agonist dimethyl-phenyl-piperazinium (DMPP) stimulated NPY secretion. The acetylcholine-stimulated secretion was not blocked by the cholinergic muscarinic antagonist atropine and was partially blocked by the ganglionic cholinergic antagonist hexamethonium.

The effects of α- and β-adrenergic agonists and antagonists on NPY secretion into the stomach vasculature were investigated in order to elucidate possible adrenergic release mechanisms. There were conflicting results on the α-adrenergic release of NPY. Both the α-adrenergic antagonist phentolamine and the agonist phenylephrine had a stimulatory effect on NPY secretion in the stomach. The β-adrenergic agonist isoproterenol had a stimulatory effect on NPY release. The β-adrenergic antagonist propranolol caused an initial small increase in mean NPY levels followed by a decrease, but this was not found to be statistically significant.

These studies demonstrated the presence of NPY in the GI tract of the rat with the highest content being in the rat stomach. There are cholinergic stimulatory mechanisms involved in the secretion of NPY which are partially ganglionically mediated. The results did not conclusively demonstrate as to whether there is an α-adrenergic stimulatory or inhibitory action on NPY-containing neurones in the gut, however preliminary release studies suggest there is a β-adrenergic stimulatory mechanism involved in NPY secretion.
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<tr>
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<tr>
<td>Acetonitrile</td>
<td>ACN</td>
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<tr>
<td>Acetylcholine</td>
<td>ACh</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>BSA</td>
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<td>C-Flanking Peptide Of Neuropeptide Y</td>
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<td>Cholecystokinin Octapeptide</td>
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<td>1, 1-Dimethyl-4-phenyl-piperazinium iodide</td>
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<td>High Performance Liquid Chromatography</td>
<td>HPLC</td>
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</tr>
<tr>
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<td>TFA</td>
</tr>
<tr>
<td>Vasoactive Intestinal Peptide</td>
<td>VIP</td>
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DEDICATED TO MY PARENTS: RAHIM AND JOHANNA
INTRODUCTION

General Information

Neuropeptide-Y (NPY) was isolated from extracts of pig brain using a chemical assay for C-terminal amides (Tatemoto et al., 1980; 1982a). NPY is a 36 amino acid peptide which shares considerable sequence homology with pancreatic polypeptide (50%) and peptide YY (70%) (Fig.1) (Thackery et al., 1986). The peptides were named YY and Y because of their terminal tyrosines (Y=tyrosine). The genes that encode pancreatic polypeptide (PP) and NPY are localized on separate chromosomes: PP is encoded on chromosome 17 and NPY on chromosome 7 (Adrian et al., 1983a). PP is localized in pancreatic islets (Tatemoto, 1984), PYY is predominately found in endocrine cells in the distal small intestine and colon (Tatemoto, 1982c), and NPY is neuronally located, except for a sub-population of adrenal medullary cells (Sundler et al., 1986).

Figure 1: The amino acid sequence of the pancreatic polypeptide family. Regions of homology are underlined. pPP represents porcine pancreatic peptide and pPYY represents porcine peptide YY.

<table>
<thead>
<tr>
<th></th>
<th>NPY</th>
<th>pPYY</th>
<th>pPP</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-</td>
<td>Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Glx-Asx-Ala-Ser-Pro-</td>
<td>Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro-</td>
</tr>
<tr>
<td>5</td>
<td>Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-</td>
<td>Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Glx-Asx-Ala-Ser-Pro-</td>
<td>Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro-</td>
</tr>
<tr>
<td>15</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr-NH2</td>
</tr>
<tr>
<td>20</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr-NH2</td>
</tr>
<tr>
<td>25</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Leu-Ile-Thr-Arg-Glu-Arg-Tyr-NH2</td>
<td>Asn-Met-Leu-Thr-Arg-Pro-Arg-Tyr-NH2</td>
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Early studies suggested that PP is also present in the mammalian nervous system (Hunt et al., 1984; Lundberg et al., 1980). However, attempts to characterize PP in the central nervous system with specifically directed radioimmunoassays (RIA) resulted in only trace amounts of immunoreactivity being detected despite strong immunoflorescent staining of PP immunoreactive cells. Following the discovery of NPY, further characterization of PP-like immunoreactivity in rat brain extracts by chromatography and RIA showed that this immunoreactivity coeluted with synthetic porcine NPY (Dimaggio et al., 1985). Pancreatic polypeptide immunoreactivity could not be detected in neural tissue following preabsorption of avian PP and bovine PP antisera with porcine NPY (Dimaggio et al., 1985). As a result of these studies and others a consensus was developed that previous accounts of PP-IR in the brain were due to cross-reactivity with endogenous NPY.

Sequence and Structure

Both NPY and its C-flanking peptide are highly conserved in sequence in various species. Human NPY has been shown to be identical to rabbit, rat, and guinea pig NPY (O'Hare et al., 1988). In contrast, the PP sequence of rat has eight or nine differences from the PP sequence of human, dog, pig, cow, and sheep (Yamamoto et al., 1986). With the exception of porcine NPY, which has a leucine residue in position 17, all mammalian NPYs characterized to date have a methionine residue in position 17 (O'Hare et al., 1988). This methionine residue is prone to oxidation by hydrogen peroxide, however the NPY released from nerve endings is likely to be in the non-oxidized form (O'Hare et al., 1988). The human C-flanking peptide (CPON) was shown to differ from the rat at only two sites (Larhammar et al., 1987), suggesting a possible functional importance. Since CPON has an identical distribution to NPY, CPON has been used as a marker for NPY localization (Gulbenkian et al., 1985).

NPY appears to be conserved in evolution with structurally related peptides being found in the anglerfish (Andrews et al., 1985) and the Pacific salmon (Kimmel et al., 1986). The peptide isolated from the endocrine pancreas of the anglerfish shares marked
sequence homology with NPY (Andrews et al., 1985). This peptide was named anglerfish peptide YG to designate the amino terminal tyrosine and carboxyl terminal glycine. The amino acid sequence of a 36 residue peptide isolated from the endocrine pancreas of the Pacific salmon (sPP) was found to be identical to porcine NPY at 30 of 36 positions (Kimmel et al., 1986). Such conservation of NPY structure is consistent with some role for this peptide as a physiological regulator.

The genetic sequence of the NPY precursor has been determined by in vitro translation of RNA isolated from a human phaeochromocytoma. The coding sequence consists of 291 bases (Larhammar et al., 1987). The deduced amino acid sequence of the precursor had two proteolytic cleavage sites suggesting the presence of a 28 amino acid signal peptide, the 36 amino acid NPY and a 30 amino acid CPON. Five of the 36 amino acids of NPY were shown to be tyrosine residues located at the N- and C-termini, and the remaining three in the middle portion of the molecule (Larhammar et al., 1987).

Allen and coworkers have recently derived a hypothetical tertiary structure for NPY by application of computer modeling (Allen et al., 1987b) to the X-ray crystal structure of the related avian PP previously solved by Glover and coworkers (Glover et al., 1983). The derived structure consists of an N-terminal polyproline II helix (residues 1-8) and an antiparallel α-helix (residues 14-32) connected by a tight band. Residues 32-36 form a flexible tail. This structure is highly compact and is stabilized by intramolecular hydrophobic interactions involving, in particular, the proline residues at position 2,5 and 8 (Allen et al., 1987b). Unlike many other peptides, the pancreatic polypeptide family appear to retain the folded structure even in dilute aqueous solutions (Glover et al., 1983). The tertiary structure of NPY allows for the formation of dimers in aqueous solution (Minakata et al., 1989).
Localization of NPY

A RIA for the predicted sequence of CPON has shown NPY to be one of the most widespread neuronal peptides. In the central nervous system (CNS) the peptide is found in neurones from the cerebral cortex to the spinal cord, occurring in the cerebral cortical and basal ganglia interneurones, hypothalamic arcuate neurones, and catecholamine-containing neurones of the central adrenergic cell groups. Outside the CNS, NPY-containing neurones constitute a distinct group in the enteric nervous system, and throughout the periphery NPY is found in adrenergic nerves innervating both non-vascular and vascular smooth muscle (Tatemoto, 1984). NPY-IR has been shown to be distributed at the subcellular level in the cytoplasmic matrix, in the secretory granules and in the nucleus (Chabot et al., 1988).

(1) Localization of NPY in the Gastrointestinal (GI) Tract

NPY-immunoreactive nerve fibers are abundant in the GI tract, occurring all along the gut and in all layers (Sundler et al., 1983; Furness et al., 1983a). The largest number of NPY-IR neurones are found in the duodenum, while the majority of the NPY-IR fibers are found in the circular muscle layer of the rat using immunoflorescence histochemistry (Wang et al., 1987). Extrinsic sympathetic denervation or treatment with the neurotoxin 6-hydroxydopamine (6-OHDA) caused perivascular NPY fibers to disappear from the gut wall. These fibers are therefore adrenergic. After elimination of extrinsic (adrenergic) NPY fibers, there remains a prominent population of NPY-IR fibers of apparently intrinsic origin (Sundler et al., 1983). Such an origin for the nonadrenergic NPY fibers is compatible with the finding of NPY-IR nerve cell bodies in intramural ganglia. In the guinea pig small intestine, approximately 5% of the nerve cell bodies in the myenteric ganglia and approximately 26% of those in the submucous ganglia store NPY (Furness et al., 1983a). In contrast in the rat, following colchicine treatment, which inhibits axonal flow and therefore increases the cell body content of neurotransmitters, 30% of the ganglion cells in the myenteric plexus stained for NPY using immunoflorescence (Lee et al., 1985). None of the NPY-IR cell bodies in the submucosal or myenteric plexi and few of the NPY-
containing nerve fibers located in the smooth muscle layer contain noradrenaline (NA) (Wang et al., 1987). Vagotomy does not visibly affect the density or distribution of NPY-IR fibers in the gut (Lee et al., 1985; Sundler et al., 1983). Therefore, extrinsic parasympathetic nerves do not contribute to the NPY-IR observed in the GI tract.

The NPY-IR in the GI tract has also been investigated using specific RIAs. In agreement with immunohistochemical studies, the highest concentration of NPY-IR was found to be in the proximal gut. The highest NPY content was in the lower oesophageal sphincter in rat and porcine gut and in the pylorus in guinea pig gut (Allen et al., 1987a). By microdissection, the NPY-IR in the mucosa-submucosa and muscularis externa layers of the GI tract of the human gut have also been determined separately (Koch et al., 1988). NPY-IR was present in highest concentration in the muscularis externa of the stomach and in lowest concentration in the muscularis externa of the ileum and descending colon. The concentrations in the mucosa-submucosa layers were similar throughout the human gut. NPY in the stomach was present in higher concentrations in the muscularis externa than in the mucosa-submucosa, but in the descending colon the reverse was found (Koch et al., 1988).

The projections of the myenteric NPY fibers have been studied in the small intestine of the guinea pig (Furness et al., 1983a). By analysing the changes in the distribution of NPY-IR nerve fibers after microsurgical lesions of neuronal pathways, it was concluded that myenteric NPY nerve fibers project to the underlying circular smooth muscle and to myenteric ganglia up to approximately 2 mm anally (Furness et al., 1983b). Submucous NPY nerve fibers seemed to project mainly to the mucosa (Furness et al., 1983b). The majority of the densely arranged NPY-IR fibers in the myenteric plexus (Feher et al., 1986), and the NPY-IR fibers along the blood vessels in the muscle layers originate from NPY-IR cell bodies located in the coeliac sympathetic ganglion which also contains NA (Lee et al., 1985). Approximately 65% of the neuronal cell bodies in the guinea pig coeliac-superior mesenteric ganglion contain NPY-IR. Considerably more NPY-IR fibers than NA
fibers are seen in the GI tract from the oesophagus to the anal sphincter (Lundberg et al., 1983). Mucosal NPY-IR fibers are more numerous in the small intestine than any other region of the gut. They form a dense subepithelial network extending to the tips of the villi (Furness et al., 1983b). The rich supply of NPY nerve fibers in the gut wall places NPY among the major gut neuropeptides (Sundler et al., 1983).

(2) Innervation of Blood Vessels of the Digestive Tract

NPY-containing nerve fibers can be seen around most blood vessels, both main trunks and smaller arterioles and veins (Ekbald et al., 1984a). Generally veins are less densely innervated by NPY fibers than the corresponding arteries (Sundler et al., 1986; Uddman et al., 1985). Small arteries, such as the submucous arterioles, are particularly richly supplied by NPY fibers. Large elastic arteries and veins have a sparse innervation compared to large muscular arteries, such as the the inferior and superior mesenterics (Uddman et al., 1985). The fibers run longitudinally in the adventia-media border (Ekbald et al., 1984a). The superior mesenteric artery possesses prominent bundles of preterminal axons with NPY-IR arranged in two layers: an outer layer of non-varicose axons which run diagonally or longitudinally along the vessel and an inner layer of smaller axon bundles containing both varicose and non-varicose axons which have a distinct circular arrangement (Morris et al., 1986).

Perivascular NPY fibers have been shown to be sympathetic and adrenergic as indicated by the following studies:

1. Surgical sympathectomy eliminates perivascular NPY fibers (Sundler et al., 1983).
2. Treatment with 6-OHDA eliminated both NA and NPY from perivascular nerve fibers (Uddman et al., 1985).
3. Enzymes involved in NA formation (tyrosine hydroxylase, dopamine-β-hydroxylase) coexist with NPY in nerve cell bodies of sympathetic ganglia and in nerve fibers originating from these ganglia (Lundberg et al., 1982a; Lundberg et al., 1983).
4. NPY is released upon stimulation of sympathetic nerves (Allen et al., 1984a).
(3) Coexistence of NPY with Neurotransmitters and Neuropeptides

Neurones in the submucosal and myenteric plexus which display NPY-IR are also labelled with antisera to somatostatin (Furness et al., 1984), cholecystokinin (Furness et al., 1985), vasoactive intestinal peptide (VIP) (Buchan et al., 1988), peptide histidine isoleucine (PHI) (Ekblad et al., 1984b), calbindin D28K (Buchan et al., 1988), or calcitonin gene-related peptide (Furness et al., 1985). The NPY intrinsic neurones of the rat and mouse gut are identical to those containing VIP (Ekblad et al., 1984b), while in the guinea pig submucous ganglia NPY neurones contain choline acetyltransferase but not VIP (Furness et al., 1984). Thus species differences in colocalization exist. NPY in nerves innervating the hypogastric artery and the inferior mesenteric artery of the male guinea pig is also colocalized with VIP and dynorphin (Morris et al., 1985). In the coeliac ganglion NPY is costored with enkephalins (Fried et al., 1986), and with VIP and PHI both of which are derived from the same precursor (Lindh et al., 1986).

NPY has been found to coexist with the classical neurotransmitters: NA in perivascular sympathetic nerves (Lundberg et al., 1982a; Lundberg et al., 1983) and acetylcholine (ACh) in the submucosal ganglia of the GI tract (Furness et al., 1984). The observed molar ratios between NA and NPY vary considerably in ganglia with ratios as low as 10:1 (NA/NPY) in the cat coeliac ganglion (Fried et al., 1985a), and as high as 160:1 in the superior mesenteric ganglion (Lundberg et al., 1983).
Release of NPY

Numerous studies have been performed on the release of NPY upon sympathetic nerve activation, however, only a limited amount of information is available on cholinergically stimulated NPY release.

(i) Release of NPY from the Sympathetic Nervous System

Plasma NPY levels have been shown to increase following haemorrhage in rats (Morris et al., 1987). To determine the source of the increase in the circulating NPY after haemorrhage, rats were subjected to adrenalectomy or to chemical sympathectomy with intravenous 6-OHDA administration (Morris et al., 1987). Following treatment with 6-OHDA, there was no significant increase in plasma NPY after haemorrhage, whereas adrenalectomized rats had an enhanced NPY response. Allen and coworkers (1983b) have shown that the adrenal gland in the conscious calf contributes to a small but significant extent to increases in response to splanchnic nerve stimulation. These results suggest that the sympathetic nerves make the major contribution to the increase in NPY concentration after activation of the sympathoadrenal system by haemorrhagic stress (Morris et al., 1987). The increase in circulating NPY in adrenalectomized rats both at rest and after haemorrhage may reflect a compensatory increase in activity of sympathetic nerves (Morris et al., 1987).

Increases in plasma NPY concentrations have been observed during activation of the sympathetic nervous system under a variety of stressful conditions, such as strenuous exercise (Lundberg et al., 1985c). The increase in NPY upon sympathetic stimulation is smaller compared to NA due to NPY representing only 5% of the vesicles in the sympathetic terminals (Morris et al., 1987).

There are conflicting reports as to whether NPY and NA are released by the same mechanism. During strenuous exercise in the human (Pernow et al., 1986a) the plasma NPY-IR correlated well with the plasma concentration of NA. This corelease of NPY and NA has been shown to be abolished by pretreatment with the adrenergic neuron-blocking
agent guanethidine (Lundberg et al., 1985a). These studies indicate that similar mechanisms of release may be involved. However, the rate of increase and decrease of NPY compared with NA upon activation of the nervous system was found by Howe and coworkers (1986) to be slower. This may be related to a slower diffusion of NPY into the systemic circulation after release, the result of the longer half-life of NPY compared to NA in plasma (approximately 5 minutes compared to 1-2 minutes for NA), or due to differential release mechanisms (Pernow et al., 1986a). Evidence for a differential release mechanism came from a study that showed NPY is released together with NA during strong, but not during mild, sympathetic activation under physiological conditions (Pernow et al., 1986a). Experiments using the pig spleen also showed that sympathetic nerve stimulation released relatively more NPY-IR at high frequency irregular bursts than at low continuous frequency, when compared to the release of NA (Lundberg et al., 1986b).

Further evidence for differential release mechanisms comes from studies using pharmacological agents, such as reserpine, 6-OHDA and clonidine. Reserpine impairs amine uptake systems and activates sympathetic neurones and clonidine is an \( \alpha_2 \)-agonist which also reduces sympathetic activity via a central action. These drugs have differential effects on the release and synthesis of NPY and NA. Reserpine and 6-OHDA pretreatment induced a tissue- and dose-dependent depletion of NPY-IR at certain nerve terminals (Lundberg et al., 1985d), which was prevented by concomitant clonidine treatment, while no effect was observed on NA depletion (Franco-Cereceda et al., 1987a). The varying tissue-specific depletion of NPY is thought to be reflective of either the extent of sympathetic activity of that particular tissue or the presence of nonadrenergic NPY containing fibers. In contrast to NPY, NA was markedly depleted in all tissues following 6-OHDA and reserpine pretreatment. The depletion of NA was more extensive, and occurred more rapidly at much lower doses as compared to the effects on NPY-IR (Lundberg et al., 1985d). There is a requirement for neurogenic activation for the depletion of NPY as illustrated by experiments in which denervation and ganglionic blockade were
shown to inhibit the reserpine-induced depletion of NPY-IR, but not NA content (Lundberg et al., 1987).

The reason for the apparent differential release of NPY and NA may be related to the different mechanisms of resupply (Fried et al., 1985a), or different vesicular storage sites (Fried et al., 1985b). The molar ratio between vesicular NA and NPY is high in terminal regions (150 to 1) and much lower in axons and cell bodies (10 to 1), thus reflecting the different mechanisms of resupply for classical transmitter and peptide (Fried et al., 1985a). In contrast to NA, which has a rapid local synthesis, it is likely that NPY has a limited resupply capacity by axonal transport from the cell body (Lundberg et al., 1985d). This is consistent with the fact that long-term stimulation in the pig spleen in vivo resulted in approximately 60% depletion of the total splenic content of NPY-IR, while the corresponding NA content remained unchanged (Lundberg et al., 1989). The calculated total turnover time for splenic NPY content considering resupply by axonal transport is around 11 days compared to about 5 days for the life span of adrenergic granules. The elimination half-life for infused NPY in man is 20 minutes (Pernow et al., 1987). NPY is stored in large granular synaptic vesicles (Fried et al., 1985b). Noradrenaline and NPY are co-stored in large dense vesicles, whereas NA but not NPY occurs in the population of small "classical" vesicles (Fried et al., 1985b). In conclusion, the main resupply of NPY to terminals is, in contrast to NA, most likely by axonal transport, which implicates differences in storage, turnover, and release of these co-existing substances in the sympathoadrenal system.

The presynaptic actions of NPY and NA may also contribute to the observed differential release. Noradrenaline presynaptically reduces NPY release (Wahlestedt et al., 1986; Lundberg et al., 1989). In the pithed guinea pig the $\alpha_2$-adrenoceptor agonist clonidine reduced by almost 50% the increase in plasma NPY-IR induced by preganglionic nerve stimulation. This effect was reversed by the $\alpha_2$-antagonist yohimbine (Wahlestedt et al., 1986). Pharmacological drugs which inhibit NA uptake have also been shown to
reduce NPY overflow at low and moderate frequency stimulation (Lundberg et al., 1989). Therefore, NA presynaptically reduces NPY release via an $\alpha_2$-adrenoceptor mediated mechanism at low and moderate frequency stimulation.

There is also evidence that NPY influences NA release. NPY had a stimulatory effect on NA release in the myenteric plexus of the colon (Wiley et al., 1987) and an inhibitory effect on NA release in the mesenteric vein (Pernow et al., 1987) and superior mesenteric artery of the rat (Pernow et al., 1986b). The inhibitory action of NPY on NA release is not mediated via $\alpha_2$-adrenergic receptors and is inversely related to the stimulation frequency (Serfozo et al., 1986). The interaction between NA and NPY is thought to largely depend on whether they are colocalized or distributed in separate neurones (Wiley et al., 1987). While exogenous NA inhibited the release of NPY by 90%, the maximum inhibition of NA by NPY was not higher than 60%, indicating that either the intrinsic activity of NPY is lower, or fewer axon terminals possess NPY receptors (Serfozo et al., 1986). The $\alpha_2$-mediated inhibition of NPY release could be important during conditions of low sympathetic activity, whereas the NPY-induced inhibition of NA release may be of relevance at high nervous activity (Dahlof et al., 1986). In contrast to the above studies, NPY was found to have no effect on the electrically evoked release of NA in the gastroepiploic artery of the rat (Ekbald et al., 1984a), or the mesenteric artery of the rabbit (Oshita et al., 1989).

There is also evidence for a reciprocal receptor-receptor interaction between the $\alpha_2$-adrenoceptor and NPY receptors, that takes place in the nucleus tractus solitarius (Fuxe et al., 1984) which may modulate the differential release of NPY and NA.

(ii) Cholinergic Stimulation of NPY Release

A number of studies have demonstrated that cholinergic stimulatory pathways are involved in the regulation of NPY secretion. The bovine adrenal medulla has been shown to release NPY upon nicotinic receptor stimulation (Hexum et al., 1986). A similar response was obtained in the isolated perfused guinea pig heart in which nicotine exposure
dose-dependently resulted in a seven fold increase in NPY-IR overflow (Franco-Cereceda et al., 1987b). The cholinergically-stimulated release of NPY was shown to be suppressed by ganglionic blockade with hexamethonium (Richardt et al., 1988). In addition, ganglionic blockade by agents such as chlorisondamine inhibited the splanchnic nerve stimulated NPY depletion (Lundberg et al., 1987). Thus nicotinic receptor activation resulting from ACh release from preganglionic nerves seem to underly the release of NPY-IR from both the adrenal and the postganglionic, sympathetic nerve terminals (Lundberg et al., 1987).

Stimulation of the vagal nerve supply of the isolated perfused pancreas caused a seven fold larger release of NPY-IR than splanchnic stimulation without affecting NA release (Sheikh et al., 1988). However in the guinea pig heart, electrical stimulation of the vagus did not release NPY in to the perfused coronary vessels (Archelos et al., 1987). Further studies are required to explain the differential tissue responsiveness to vagal stimulation.

Gastrointestinal Actions of NPY

A physiological function for NPY in the GI tract has not been established but it has been demonstrated to exert a number of effects.

1. Regulation of Blood Flow

Although few studies have been performed on the effect of NPY on GI blood flow it is likely that it has similar effects as in other vascular beds. NPY has been shown to have both a direct and an indirect vasoconstrictive action on blood vessels, thus reducing blood flow. Direct, dose-dependent vasoconstrictive effects of NPY have been demonstrated both in vivo (Lundberg et al., 1982a; Pernow et al., 1986a) and in vitro (Ekbald et al., 1984a). Lundberg and Tatemoto showed the direct vasoconstrictive action of NPY to have a response threshold of 10nM which is the same order of potency as angiotensin II (Lundberg et al., 1982b) and 50 times greater potency than NA (Lundberg et al., 1986b). However, even though the threshold dose is lower, the maximum vasoconstriction
produced by NPY is less than the maximum effect of NA (Lundberg et al., 1986b). There is considerable evidence that NPY mediates the nonadrenergically mediated contractions upon sympathetic stimulation: (1) NPY is contained in noradrenergic axons; (2) stimulation of the nerve fibers supplying the uterine artery produces a biphasic contraction. The fast phase is blocked by α-adrenoceptor blockade and the slow phase is mimicked by NPY; (3) guanethidine blocks both components of neurogenic activation; (4) trypsin and NPY desensitization selectively block the slow neurogenic contraction and the contraction produced by exogenous NPY (Morris et al., 1988); (5) unlike NA, NPY results in a slowly developing, prolonged contraction which is not followed by a vascular escape in the cat colon (Hellstrom et al., 1985), and submandibular gland (Lundberg and Tattemoto, 1982b); (6) only combined infusion of NA and NPY caused a vascular response which was similar to that seen upon sympathetic nerve stimulation (Lundberg and Tattemoto, 1982b); (7) the vasoconstriction is not affected by α- or β-adrenoceptor blockade (Rudehill et al., 1986), confirming a direct action of NPY, not mediated by adrenergic mechanisms.

NPY also shifts adenosine and ACh relaxation dose-response curves to the right and potently inhibits the relaxation of rabbit coronary vessels caused by stimulation of β-receptors with NA (Han and Abel, 1987). Han and Abel (1987) have suggested that the inhibition of vasodilation of coronary vessels by NPY may be more important than its vasoconstrictive effects in the regulation of blood flow.

Systemic administration of NPY induces a long lasting increase in arterial blood pressure, indicating a general vasoconstrictive effect (Lundberg and Tatemoto, 1982b). However, whether NPY is acting on veins or arteries to induce this general vasoconstrictive effect has been disputed. Several studies have shown that certain peripheral veins seem to respond more readily, while most peripheral arteries seem to be less responsive (Sundler et al., 1986; Pernow et al., 1987). NPY was shown to cause a dose-dependent contraction of the mesenteric vein, but not of the mesenteric arteries (Pernow et al., 1987). In contrast, other studies have shown that NPY produced
contraction of arteries and arterioles but had no effect on veins, which is also in agreement with the distribution of NPY-IR around the vasculature (Tatemoto et al., 1982b).

The indirect action of NPY on blood flow is through the sensitization of blood vessels to other vasoconstrictors (Wahlestedt et al., 1986). Evidence for this comes from a study where elevated levels of NPY in the cerebrospinal fluid following subarachnoid haemorrhage potentiated the NA-induced contractions of the middle cerebral artery; this activity being abolished after immunoprecipitation of NPY (Abel et al., 1988). The fairly long lasting potentiating effect of NPY on the vasomotor response to electrical stimulation is probably not presynaptic, since NPY does not generally stimulate either spontaneous or electrically evoked release of radiolabelled NA from perivascular nerve fibers (Ekblad et al., 1984a). The potentiation of the action of NA by NPY is more potent on arteries (Edvinsson et al., 1984). Interestingly, the potentiation of responses to NA was found to require lower concentrations of NPY (10^{-10}M) than the direct vasoconstrictor effect (10^{-7}M) (Pernow et al., 1986b). Similarly, in the pithed rat, the \( \alpha_2 \)-adrenoceptor-stimulated blood pressure increase was enhanced by NPY at doses lower than those required for a direct pressor effect of NPY (Dahlof et al., 1985). Therefore, the potentiative action of NPY is thought to be more physiologically relevant than its direct vasoconstrictive action.

In summary, NPY has three sites of action at the level of the sympathetic neuroeffector junction which may contribute to the regulation of blood flow. Firstly, it has a direct postjunctional effect which is thought to involve specific NPY receptors, leading to the constriction of certain blood vessels. Secondly, NPY potentiate the response to various vasoconstrictors; here the mechanism involves some kind of sensitization of the particular blood vessels. Thirdly, NPY acts prejunctionally to modulate synaptic release of NA, as discussed previously. The combination of pre- and postjunctional effects of NPY on NA action may be desirable, leading to a rapid turn-off of NA release and a more prolonged effector response, respectively.
The firing pattern of sympathetic nerves upon physiological activation is highly irregular with high frequency bursts of impulses. Larger vasoconstrictor responses and a several fold larger release of NPY-IR relative to NA are also obtained in isolated mesenteric arteries in vitro, upon stimulation with irregular frequencies at a high rate compared to a low continuous frequency (Lundberg et al., 1986b). However, in intact mammals it is unlikely that NPY levels circulating in plasma released under physiological conditions are sufficient to cause a general vasoconstriction. The systemic plasma concentrations of NPY (100pM) observed during physical exercise in humans is shown to be about one tenth of the concentrations required to cause a slight vasoconstriction and prejunctional inhibition of NA overflow in human blood vessels in vitro (Pernow et al., 1986a). NPY-IR found in the plasma, however, probably reflects spillover from the neuroeffector junction. Thus, the concentrations of NPY may be considerably higher in the vicinity of the nerve terminals close to the vascular smooth muscle cells. Also in patients with elevated levels of NPY, e.g. phaeochromocytoma or ganglioneuroblastoma patients (Adrian et al., 1983b; Allen et al., 1986), it may well contribute to the hypertension characteristic of these condition.

NPY also indirectly regulates blood flow in the GI tract via its effect on the heart. NPY has a positive chronotropic and inotropic action on the heart in vitro (Potter et al., 1985; Lundberg et al., 1984a). NPY, released from sympathetic nerve fibers, results in a prolonged inhibition of the negative chronotropic action of the cardiac vagus by a nonadrenergic mechanism in the dog (Potter et al., 1985) and guinea pig (Lundberg et al., 1984a). In contrast to the action of NA on the cardiac vagus, NPYs action is long lasting and does not involve β-adrenoceptors (Potter et al., 1985). In the isolated atrial preparation the presynaptic inhibitory action of NPY has been shown to be directed specifically on postganglionic vagal fibers, since the effect is not suppressed by vagal transmission blockade at the ganglionic level by hexamethonium (Potter et al., 1987). NPY reduces the field stimulatory responses of both the noradrenergic (β-receptor mediated) and cholinergic (muscaranic receptor mediated) systems in the right guinea pig atrium. Since the effects of
exogenous NA and ACh were not affected, NPY most likely exerts its effect on endogenous NA and ACh release presynaptically (Lundberg et al., 1984a). NPY's hypertensive action has been shown to be accompanied by a reflex bradycardia (Corder et al., 1986a); probably adrenergically mediated by a reflexogenic inhibition of the sympathetic tone (Lundberg et al., 1984b). In agreement with this possibility, Allen and coworkers (1983a) found that NPY produces a reduction in myocardial perfusion and inhibits cardiac contractivity in the isolated perfused rabbit heart. In conclusion, NPY has been demonstrated to have three separate indirect mechanisms of action on the heart, which are likely to affect blood flow in the GI tract: (1) in vivo NPY, as a result of its vasoconstrictive action, has a more pronounced inhibitory reflex action on the beating rate than on tension in the heart; (2) in vitro NPY presynaptically inhibits ACh release from postganglionic vagal nerves resulting in positive chronotropic and inotropic effects, and (3) upon field stimulation NPY also presynaptically decreases NA release which results in a decreased chronotropic and inotropic effect.

2. Neuromodulation

(i) Influence of NPY on NA Release

NPY has been shown to act prejunctionally to modulate NA release (Pernow et al., 1987; 1986b; Wiley et al., 1987). NPY has been demonstrated to have a stimulatory effect on NA release in the myenteric plexus of the colon (Wiley et al., 1987) and an inhibitory effect on NA release in the mesenteric vein (Pernow et al., 1986b) and superior mesenteric artery (Pernow et al., 1987). The interaction between NA and NPY is thought to largely depend on whether they are colocalized or distributed in separate neurones (Wiley et al., 1987). Under conditions of prolonged and intense sympathetic activity, transmitter stores will be conserved by the prejunctional inhibitory action of NPY without compromising the magnitude of the postjunctional response, which would be enhanced by NPY.
(ii) **Influence of NPY on Cholinergically Mediated Secretion**

NPY has been shown to augment the cholinergic receptor mediated secretion of enkephalin-IR and catecholamines. NPY (1x10^{-8}M), alone, had no effect on secretion in the adrenal gland, but in the presence of the cholinergic agonist DMPP, nicotine or ACh, produced a dose-dependent increase in the secretion of enkephalin-IR and catecholamines. This was not mimicked by the structurally related peptides PYY or PP (Hexum et al., 1989). In contrast to the potentiation observed in the perfused adrenal gland, NPY inhibited the cholinergically mediated secretion of enkephalin-IR and catecholamines from cultured bovine chromaffin cells (Hexum et al., 1989). Thus the cholinergic stimulation of secretion varies depending upon whether the cells are in culture or in situ; cell culturing being reflective of denervated tissue.

3. **Motility**

NPY has been shown to inhibit smooth muscle contraction resulting from electrical stimulation (Holzer et al., 1987; Allen et al., 1987a; Lundberg et al., 1982a). NPY has also been shown to inhibit the ascending enteric reflex contraction of the circular muscle in the guinea pig small intestine; both the cholinergic and non-cholinergic components of the ascending enteric reflex were suppressed (Holzer et al., 1987). Like NA, NPY does not act directly on intestinal muscles but rather interrupts nonadrenergic excitatory pathways of the enteric nervous system (Holzer et al., 1987). NPY in a dose-dependent manner also reduced the neurally-mediated excitatory effect of cholecystokinin octapeptide (CCK8) on the guinea pig ileum (Garzon et al., 1986) and the potentiation of the twitch response induced by neurotensin and substance P in the mouse vas deferens (Allen et al., 1982). CCK8 normally exerts its excitatory action by modulating the release of ACh and substance P from the myenteric plexus (Garzon et al., 1986). NPY does not modify the effect of exogenous ACh, therefore confirming that NPY must be acting indirectly by inhibiting the release of ACh from the myenteric plexus (Garzon et al., 1986). Studies have also shown that NPY reduced the resting tension of the myenteric plexus-longitudinal muscle
preparation of the guinea pig ileum (Garzon et al., 1986) and colon (Wiley et al., 1987). In agreement with the previous studies, intra-arterial infusion of NPY in the anaesthetized rat inhibited colonic motility (Hellstrom et al., 1985). Studies on the guinea pig colon have demonstrated that NPY modulates the release of NA which, in turn, is thought to inhibit the release of ACh via receptors located on postganglionic nerves (Wiley et al., 1987). In contrast, the indirect inhibitory effect of NPY on smooth muscle contraction of the guinea pig small intestine (Holzer et al., 1987) and cat colon (Hellstrom et al., 1985) does not seem to require activation of the α-adrenergic receptors upon electrical stimulation. In addition, other studies have shown that NPY does not influence the release of \[^3\]H-ACh from the longitudinal muscle strips of the guinea pig ileum (Serfozo et al., 1986). Therefore, NPY seems to exert its actions on some but not all types of autonomic transmission. There is also evidence to suggest that the inhibitory action of NPY on colonic motility may actually be secondary to its potent vasoconstrictive action (Hellstrom et al., 1985).

NPY caused a reversible inhibition of the contractile response to field stimulation in the guinea pig urinary bladder which is thought to occur via ATP release. NPY also more effectively blocked the twitch response, which is purinergically mediated, than the tonic contraction in the vas deferens stimulated contractile response. These studies provide evidence for a possible nonadrenergic, noncholinergic mechanism of action of NPY (Lundberg et al., 1984a).

Postganglionic sympathetic neurones containing NPY have been shown to project to the pyloric sphincter in the guinea pig (Lindh et al., 1986), suggesting a role for NPY in the regulation of gastric emptying. However, infusion of NPY had no significant effect on the rate of gastric emptying in man (Allen et al., 1984b).

In vivo, NPY produced a biphasic effect on the lower esophageal sphincter (LES) of the anaesthetized cat, with an initial contraction followed by a relaxation. This response is thought to occur via non-vagal cholinergic neurones acting on muscarinic receptors.
NPY also augments the contractile effect on the LES to phenylephrine by a nonadrenergic mechanism, but does not potentiate its maximum response (Parkman et al., 1989).

Recently NPY has been shown to significantly increase the wave frequency, amplitude and motility index of the sphincter of Oddi and gall bladder pressure upon intravenous administration in the prairie dog (Hellstrom et al., 1989). Upon intravenous NPY administration, there is also an increase in rectal tone and anal canal pressure. This distal segment of the GI tract is innervated by the sympathetic lumbar colonic and hypogastric nerves which emerge from the inferior mesenteric ganglia. NPY, on a molar basis, was 25 times more potent than NA but four times less potent than PYY (Hellstrom et al., 1989).

4. Electrolyte Transport

Another possible function of gastrointestinal NPY is the regulation of fluid and electrolyte transport (McFadyen et al., 1986; Hubel et al., 1985; Friel et al., 1986). NPY has been shown to be one of the most potent neuronal peptides so far described in its ability to attenuate electrogenic transport in the small intestine of the rabbit and guinea pig (Friel et al., 1986). In the rabbit ileum NPY \(1 \times 10^{-7} M\) enhanced mucosal-to-serosal sodium and chloride fluxes and reduced serosal-to-mucosal chloride flux (Friel et al., 1986). Two other studies obtained similar results in terms of chloride fluxes, but in one study it was found that NPY actively diminished net sodium uptake (Hubel et al., 1985) and in the second study no significant change in sodium movement was detected (Cox et al., 1988a). NPY produced a slow and prolonged inhibition of the potential difference in the distal ileum (Friel et al., 1986) which was due to the net absorption of chloride (Cox et al., 1988a). The rat jejunum was more sensitive to NPY than the descending colon, while fundic stomach preparations were insensitive (Cox et al., 1988b).

Intravenous infusion of NPY in the rat was shown to cause net absorption in the proximal duodenum from its net secretory state in the control situation (McFadyen et al., 1986). In addition, NPY was found to inhibit the net secretory effect of simultaneously
infused VIP in the proximal duodenum and jejunum (McFadyen et al., 1986). The effect of NPY may have been mediated by local vascular changes in this study (McFadyen et al., 1986). However, in another study intra-arterial infusion of NPY, like sympathetic stimulation, reduced VIP release and this was correlated to a decrease in fluid secretion (Sjoqvist et al., 1988). Therefore, the inhibitory effect of NPY on secretion may be exerted by presynaptic inhibition of VIP release (Sjoqvist et al., 1988). NPY has also been shown to inhibit prostaglandin-E\(_{2\alpha}\) (PGE\(_{2\alpha}\))-induced intestinal fluid and electrolyte secretion in the tied-off rat jejunum preparation in vivo (Saria and Beubler, 1985). A marked inhibition of PGE\(_{2\alpha}\)-induced secretion was seen with concentrations as low as 10\(^{-10}\) M NPY, which is close to the concentration of NPY in the blood of normal rats (Lundberg et al., 1986a). In contrast, NPY alone was without any effect on fluid and electrolyte absorption (Saria and Beubler, 1985). Although these studies are difficult to compare since different preparations and animals were used, it seems these effects of NPY, which are quite potent on a molar basis, strongly implicate NPY as a major physiological regulator of fluid and electrolyte transport in the small intestine.

Central Functions of NPY Related to Gastrointestinal Action

One of the proposed functions of NPY in the paraventricular nucleus of the hypothalamus is to stimulate feeding and drinking behavior (Morley et al., 1987). Direct injection of NPY into the nucleus enhanced ingestion of carbohydrates and induced hyperphagia and obesity via a NA-independent mechanism (Morley et al., 1987). NPY produced a far greater and more prolonged feeding effect than the opioid peptides but was less potent than PYY (Wahlestedt et al., 1989). Tolerance to the sustained, though episodic effects of NPY on feeding does not appear to occur. The C-terminus of NPY is thought to be crucial for its action. These feeding effects of NPY may be related to the stimulation of insulin secretion which remains significantly elevated two hours after third cerebroventriculat injection of NPY (Moltz et al., 1985), or the evoked increment in the
circulating glucocorticoids (Wahlestedt et al., 1989). In conclusion, NPY is capable of overriding mechanisms of satiety and body weight control.

NPY also evoked a rapid, dose-dependent and prolonged decrease in interdigestive gastric acid output in the anesthetized rat when injected directly into the paraventricular nucleus or immediately ventral to it. The paraventricular provides direct neural input to the dorsal motor nucleus of the vagus which contains gastric vagal cell bodies (Humphrey et al., 1988). Water immersion stress-induced gastric erosion was reduced by intracerebroventricular administration of NPY. The plasma corticosterone levels were not affected (Heilig et al., 1987).
Rationale for the Present Studies

The objective of the present studies was to develop a sensitive and specific RIA for NPY to enable quantitative studies to be performed. The development of the RIA would require the purification of the NPY tracer due to both multiple iodinated products resulting from the five tyrosine residues in its amino acid sequence, and the presence of unlabelled NPY. Ion-exchange and reverse phase HPLC purification of $^{125}$I-NPY would be performed. The use of different separation techniques would also be tested to improve assay conditions.

Although many studies aimed at elucidating the actions of NPY have been performed, little information is available on the distribution of gastrointestinal NPY in the rat. Therefore the NPY-IR in extracts of the various regions of the GI tract would be determined using the developed RIA. The authenticity of the NPY-IR detected in these tissue extracts would then be established using gel filtration chromatography and HPLC.

In the physiological investigation of the role of neuropeptides in the regulation of GI functions, release studies are crucial. The isolated perfused rat stomach preparation has been employed successfully to measure the release of peptides such as somatostatin (McIntosh et al., 1981). The presence of high levels of NPY-IR in the stomach and the fact that NPY-containing neurones constitute a distinct non-adrenergic intrinsic innervation in the gut, as well as a dense extrinsic adrenergic innervation of its vasculature (Sundler et al., 1983), allows the investigation of the release mechanisms of NPY in the rat stomach. However, due to the low basal secreted levels of NPY in comparison with other gastric peptides, as well as the enzymatic degradation and/or peptide uptake that occurs in the stomach vasculature, certain steps have to be taken to allow for the detection of the endogenously released peptide. Extraction steps would be required to concentrate the endogenously released peptide and to remove the possible interfering perfusate constituents in the assay. Proteolytic inhibitors would also have to be added to the perfusate to reduce enzymatic degradation.
A few studies have been performed on the cholinergic influence on NPY release. Nicotinic receptor stimulation has been shown to release NPY in the bovine adrenal medulla (Hexum et al., 1986) and the isolated perfused guinea pig heart (Franco-Cereceda et al., 1987b). However, no direct release studies have been performed on the cholinergic actions on NPY-containing neurons innervating the stomach. Therefore, the actions of cholinergic agonists and antagonists on NPY secretion in the isolated perfused rat stomach will be investigated.

Indirect adrenergic release studies on NPY have also been attempted. NPY has been shown to be coreleased with NA upon the activation of the sympathetic nervous system (Lundberg et al., 1985c), and its release to be influenced by a presynaptic α₂-adrenergic receptor-mediated action of NA (Wahlestedt et al., 1986). The effects of α- and β-adrenergic agonists and antagonists in NPY secretion into the stomach vasculature would further elucidate the possible adrenergic release mechanisms.
METHODS

I. Extraction

A. Tissue Extraction

Male Wistar rats weighing 250-350g were used. Animals were housed in a light-controlled room with free access to laboratory diet and water. Animals were fasted overnight (15-18 hours). The rats were then anaesthetized (65mg/kg sodium pentobarbital, Somnotol®, MTC Pharmaceuticals) and various segments of their GI tract were rapidly removed, weighed and boiled in 2M acetic acid for 30 minutes to inactivate degradative enzymes at a 1:10 tissue/volume ratio. The extracts were then homogenized using a Tekmar tissumizer and centrifuged to precipitate undissolved large particles. The supernatants were then divided into aliquots and frozen at -20°C for NPY quantitation and characterization. The regions taken for the study were the fundus, corpus and antrum of the stomach plus segments of the intestine as outlined in Table I.

Table I: Location of the various segments of the intestine used for quantitation study.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Distance with reference to the pyloric sphincter (cm)</th>
<th>Segment length (cm)</th>
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<tr>
<td>Duodenum</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>Jejunum</td>
<td>16-18</td>
<td>5</td>
</tr>
<tr>
<td>Ileum</td>
<td>36-43</td>
<td>5</td>
</tr>
<tr>
<td>Colon</td>
<td>4cm distal from the caecum</td>
<td>5</td>
</tr>
</tbody>
</table>
B. Perfusate Extraction

Initial attempts at measuring NPY-IR in the unextracted perfusate samples collected from the isolated stomach preparation of the rat were unsuccessful. This was probably due to interference of the perfusate constituents in the assay and also the presence of very low levels of basal NPY secretion. Therefore, both Sep Pak and amberlite extraction were used in an attempt to overcome these problems.

(i) Sep Pak Extraction

Sep Pak C<sub>18</sub> cartridges (Waters Inc) remove interfering compounds based on their polarity. The cartridges contain octadecylsilane which binds compounds through hydrophobic interaction. By choosing the appropriate solvents for washing the cartridge the compound of interest can be preferentially eluted.

Procedure

The solvents used for the extractions were water (H<sub>2</sub>O) and 100% acetonitrile (ACN), each containing 0.1% trifluoroacetic acid (TFA). ACN was used as the nonpolar organic solvent for eluting the peptides, and TFA was added to decrease non-specific binding to the cartridge. Each of the cartridges was used for 10-12 perfusate samples of a particular rat. The cartridges were primed prior to each run as outlined in Table II. To test for peptide recovery, unpurified <sup>125</sup>I-NPY (5000cpm) or standard porcine NPY (1nM or 500pM) were loaded onto primed cartridges and eluted with 20%, 40%, 60%, 80% and 100% ACN containing 0.1% TFA. The effluent was collected and counted in a gamma spectrometer and/or analysed using RIA. The overall scheme utilized is outlined in Table II.
Table II: Outline for the extraction procedure of NPY on the Sep Pak C18 cartridge.

<table>
<thead>
<tr>
<th>Cartridge treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prime:</td>
</tr>
<tr>
<td>1. Wash with 6ml 100% ACN &amp; 0.1% TFA</td>
</tr>
<tr>
<td>2. Rinse with 6ml H2O &amp; 0.1% TFA</td>
</tr>
<tr>
<td>3. Apply 6ml air</td>
</tr>
<tr>
<td>Procedure:</td>
</tr>
<tr>
<td>4. Apply 1-2ml of sample</td>
</tr>
<tr>
<td>5. Rinse with 0.5ml H2O &amp; 0.1% TFA</td>
</tr>
<tr>
<td>6. Elute peptide with 1.0ml 100% ACN &amp; 0.1% TFA</td>
</tr>
<tr>
<td>7. Re-prime.</td>
</tr>
</tbody>
</table>

The extracts were then concentrated using a Speed Vac Concentrator (Savant Instruments, Inc). The Speed Vac Concentrator consists of a centrifuge, refrigeration trap, volatile gas trap, and a vacuum pump. The centrifuge produces a centrifugal force which results in particle migration to the bottom of the test tube while the volatile solvent is removed from the surface. The lyophilized samples were then reconstituted in assay buffer for RIA quantitation.

(ii) Amberlite Extraction

The neutral polystyrene resin, Amberlite XAD-2 (BDH Chemicals) separates on the basis of differential hydrophobic interactions.

Procedure

The resin was purified by washing three times with methanol (10ml/g) followed by several rinses with distilled water to remove all traces of alcohol. The resin was poured into columns (0.7 x 9cm). The samples were absorbed onto the column and washed with 15ml 0.1M hydrochloric acid and 30ml distilled water to remove hydrophilic compounds. NPY and other hydrophobic compounds were eluted with 20ml of 90% ethanol. To test for
peptide recovery, 4500cpm of unpurified NPY tracer or standard NPY (1nM or 500pM) was loaded onto the column and eluted. The effluent was collected and counted in a gamma spectrometer and/or analysed using RIA.

II. Radioimmunoassay (RIA)

Rationale

Radioimmunoassay was first introduced by Berson and Yalow (1960) to measure minute levels of peptide hormones. RIA is based on the production in the experimental animal of an antibody by the injection of either an antigenic protein, e.g. a polypeptide hormone, or a low-molecular-weight substance, in itself nonantigenic but converted to a hapten by covalent linkage to a protein carrier. The antibody formed is then used in a competitive binding assay with radioactively labelled and unlabelled antigen. The amount of isotope bound is estimated by reference to a calibration curve obtained utilizing pure antigen in varying concentrations. In order for the RIA to be sensitive and specific, the antiserum, labelled peptide and assay conditions must be carefully selected.

A. NPY Radioimmunoassay

(a) Iodination of NPY

The chloramine-T method was used for the iodination of NPY. This method involved dissolving 5-10µg of NPY in 10µl of 0.5M phosphate buffer and reacting it with 10µl of Na\textsuperscript{125}I (1mCi) in the presence of an oxidative solution (20mg chloramine-T in 10ml of 0.05M phosphate buffer) for 30 seconds. The reaction was terminated with 10µl of reducing agent (50mg sodium metabisulphite in 10ml of 0.05M phosphate buffer). One ml of hormone free plasma was added, followed by microfine silica (20mg QUSO G32, Philadelphia Quartz Co) to bind the iodinated NPY. This was followed by successive centrifugation and resuspension in distilled water. Upon removal of the free \textsuperscript{125}I, the iodinated NPY (\textsuperscript{125}I-NPY) was separated from the QUSO G32 using 1ml acetic
acid/acetone/water solution (100μl acetic acid + 3.9ml acetone + 4ml distilled water). After mixing, the supernatant containing the iodinated NPY was stored frozen (-20°C).

(b) Purification of $^{125}$I-NPY by Column Chromatography

In initial studies a very low binding (11%) was obtained in the RIA using the unpurified $^{125}$I-NPY. This may have been due to both the multiple iodinated products, resulting from the five tyrosine residues in the molecule, and the presence of unlabelled NPY. Therefore purification was necessary.

1. Ion-Exchange Chromatography

In ion-exchange chromatography separation is achieved based on ionic interactions (retarding forces) between the peptide and the charged matrix column. A suitably charged insoluble matrix column is equilibrated with an aqueous buffer prior to loading of sample. The migration down the column of the ionic peptide would then depend on the nature of the ionic matrix, the ionic properties of the peptide, and the solvent.

Procedure

A cation exchange column, Whatman carboxymethyl cellulose 52 (pre-swollen, microgranular cation exchange (12 x 170mm)), with a 0.2M to 2M ammonium acetate gradient, pH 4.7, was used at a flow rate of 1ml/min. Two milliliter fractions were then collected and counted in a gamma spectrometer.

2. Reversed Phase High Performance Liquid Chromatography

Reverse phase HPLC separates peptides according to hydrophobicity. The stationary, hydrophobic matrix consists of silica particles coated with chemically bonded hydrocarbon chains. As the solute passes down the column, depending on the hydrophobicity of the eluent relative to the stationary phase, the sample will be separated into its constituents. An increasing concentration gradient of a hydrophobic, organic solvent then elutes the compounds according to their polarity. The unique feature of the HPLC is that the sample can be passed through an extremely tightly packed matrix by means of very high pressures, resulting in high resolution separations which can not be
achieved using other methods. Peptides differing by one amino acid can be readily resolved.

**Apparatus**

HPLC was performed using a μBondapak C₁₈ or C₈ reverse phase column (4.6 x 250mm). An Altex injection port was used for loading the label onto the column. A Beckman 421A system controller and two 110B solvent delivery module were used to deliver a linear concentration gradient of ACN in water containing 0.1% trifluoroacetic acid over a 10 minute period to the column. The flow rate was set at 1ml/min. A model #170 radioisotope detector measured the radioactivity of the effluent. The elution profiles were printed and plotted out using an Epson MX-82111 printer and Fisher 5000 pen recorder. Fractions were collected at 1 minute intervals using a Gilson Model #203 microfraction collector. There was 83-95% recovery of the approximately 1x10⁶ cpm of ¹²⁵I-NPY applied to the column.

**Procedure**

Before use, HPLC grade water and ACN were filtered and degassed using a Waters millipore filtration system and 0.1% TFA was added to each. The solvents were stored in sealed bottles. The system controller was programmed to deliver the gradient over a ten minute time interval and maintain the peak ACN concentration for 5min prior to a decrease in the gradient during a 1min interval. There was a delay time of approximately 8 minutes between the injection and the start of the gradient at the level of the column. In order to determine suitable conditions whereby labelled peptides could be optimally separated, several runs were made, manipulating the initial and final ACN concentrations. Different columns (C₁₈ or C₈ reverse phase column) were also used in the tracer purification procedure.

With standard iodination procedures unlabelled peptide is present in the product of the QUSO purification. This would decrease the sensitivity of the assay as it would compete with the tracer in binding to the antibody. In order to determine whether HPLC
purification of the tracer had adequately separated labelled from unlabelled peptide, 3.2μg of standard porcine NPY (Peninsula Laboratories) was applied to the C8 HPLC column and effluent collected for RIA analysis.

(c) NPY Antisera

Lyophilized rabbit anti-NPY serum (Peninsula Laboratories, catalog #RAS-7172) was rehydrated with 50ml of 0.1% Triton X-100 in distilled water. Upon reconstitution, the solution contained the antibody in 0.1M sodium phosphate buffer (pH 7.4), 0.05M NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% NaN3. The antiserum solution was then divided into 8ml aliquots and frozen at -20°C.

For comparative specificity and selectivity studies a second rabbit antiserum YN7 was used (Allen et al., 1984). YN7 is specific for the N-terminal region of NPY. The N-terminal region of PYY and NPY contain the greatest variability and therefore minimal antiserum cross-reactivity has been obtained (Allen et al., 1984). The YN7 antiserum was provided in lyophilized form. It was reconstituted in 0.05M phosphate buffer and 0.1% bovine serum albumin and stored in aliquots at -20°C. On the day of the assay the antiserum was further diluted with the appropriate amount of NPY buffer to give a final dilution of 1 : 400.

Cross-reactivity studies were performed using human PP (Peninsula Laboratories, catalog #7198), bovine PP (gift from Dr. R.E. Chance) and human PYY (Peninsula Laboratories, catalog #7650). Concentrations of 10nM, 1nM and 100pM were used as standards in the NPY RIA.

(d) Assay Buffer

RIA's were performed in an assay buffer consisting of 19mM monobasic and 81mM dibasic sodium phosphate (BDH)[pH 7.4], 0.05M NaCl, 0.1% Triton X-100 (B grade, Calbiochem), 0.1% NaN3 (BDH) and 0.1% bovine serum albumin (BSA, Pentex®, insulin RIA grade, Miles). The BSA and Triton X-100 were added on the day of the assay.
(e) **NPY Standards**

Different concentrations of standards (9.8pM to 2.5nM) were prepared by serial dilution using 83% pure synthetic porcine NPY (Peninsula Laboratories, catalog #731) in the RIA buffer.

(f) **Assay Protocol**

One hundred microliters of standard or unknown, 100μl of Peninsula or YN7 antiserum, and 100μl of HPLC purified label (14,000cpm) were added to 12 x 75mm polystyrene or glass culture tubes. The mixture was then vortexed and incubated overnight at 4°C.

(g) **Assay Separation Technique**

Separation of antibody bound and free $^{125}$I-NPY was performed by either a dextran coated charcoal or a polyethylene glycol procedure.

(i) **Charcoal Separation**

The dextran coated charcoal was prepared by dissolving 0.25% dextran T-70 (Pharmacia) and 1.25% activated charcoal (Fisher) in 0.05M phosphate buffer (pH 7.5). After stirring for 30 minutes at 4°C, 100μl hormone-free human plasma was added and stirring was continued for another hour. One milliliter of this mixture was added to each of the assay tubes. The tubes were vortex mixed, left at 4°C for 15-20 minutes and centrifuged at 1800g for 30 minutes. The tubes were then drained overnight and the free $^{125}$I-NPY in the pellet counted in a gamma spectrometer.

(ii) **Polyethylene Glycol Separation**

Separate solutions of 0.5% γ-globulin (Nutritional Biochemical Co) and 30% polyethylene glycol 8000 (PEG; Baker Co) were prepared in 0.05M phosphate buffer (pH 7.5). To each RIA tube a 100μl aliquot of 0.5% γ-globulin was added. After mixing, 500μl of 30% PEG was added, the tubes re-mixed and left at 4°C for at least 20 minutes until a precipitate formed. The tubes were then centrifuged at 1800g for 45 minutes and drained
for no longer than 2 hours since the pellet would otherwise tend to slide down the sides of the tube. Bound $^{125}$I-NPY was then counted in a γ-spectrometer for 3 minutes.

B. Somatostatin Radioimmunoassay

The somatostatin-IR was measured using a specific RIA (McIntosh et al., 1987).

III. Characterization of NPY from Gastrointestinal Extracts

1. Gel Filtration Chromatography

Gel filtration separates proteins according to molecular size. The column is filled with a matrix which consists of material with small pores. As the solute is passed down this gel matrix, movement of the molecules is dependent on their ability to pass through the pores. Large molecules which can not enter into the gel pores move rapidly down the column with the liquid phase, therefore eluting first. The passage of small molecules which can enter the gel pores is impeded by the stationary phase and these elute according to their size. Since NPY is a low molecular weight peptide, gels with a low molecular size fractionation range of up to 25,000 daltons were used (Sephadex G-25, Pharmacia Fine Chemicals).

Procedure

Lyophilized GI extracts were reconstituted in 1ml of 0.2M acetic acid, diluted and centrifuged. The extracts of rat ileum (160mg) and corpus (130mg) were then applied to a 0.2M acetic acid equilibrated G-25 column (95 x 1cm). The flow rate was 0.4ml/min. The void volume (26ml) was discarded and subsequent 1ml effluent fractions were collected and lyophilized for RIA analysis.

2. Reverse Phase High Performance Liquid Chromatography

Characterization studies were performed on a μBondapak C$_{18}$ column. The apparatus used was similar to that used in $^{125}$I-NPY purification except for the use of a V6K injector, two 114 solvent delivery modules and a programmable multi-wavelength
detector (Waters 490) which was used, in place of a radioisotope detector, for detecting amide peptide bonds at a set wavelength of 225nm.

**Procedure**

Standard peptides, NPY and peptide YY (Peninsula Laboratories, Inc) were dissolved in HPLC water containing 0.1% TFA to give a concentration of 5µg/10µl. Single peptide injections of 5µl, or a mixture of several peptides were injected into the mixing chamber such that 2.5µg of each peptide was applied to the column. Lyophilized colon and corpus extracts were reconstituted with 100µl of 0.2M acetic acid and diluted with HPLC water containing 0.1% TFA. The extracts were then centrifuged and applied (6mg) to the column. A 20-50% concentration gradient of ACN was used with a gradient duration of 10 minutes. The effluent was collected and lyophilized for RIA analysis.

**IV. Isolated Perfused Rat Stomach**

In isolated organ systems, the effect of substances on peptide secretion can be examined in the absence of complicating central effects. The gastric circulation is completely isolated in the perfused rat stomach preparation, allowing the control of the composition of the perfusate and the collection of effluent for RIA analysis.

**(a) Perfusate Preparation**

The Krebs' concentrate was prepared by mixing 285ml of a 154mM solution of potassium chloride (KCl, BDH), 243ml of a 102.7mM solution of calcium chloride (CaCl₂, BDH), 78ml of 154mM hydrated magnesium sulphate (MgSO₄.7H₂O, Fisher Scientific Co) and 97ml of 154mM potassium phosphate (KH₂PO₄, Baker Chemical Co). Dextran (clinical grade, Sigma Chemical Co), bovine serum albumin (BSA, Pentex®, insulin RIA grade, Miles) and dextrose, 0.8g/L, (Fisher Scientific) were dissolved overnight in distilled water containing 0.9% sodium chloride. On the morning of the experiment, 70.3ml of Krebs' concentrate and 162.5ml of a 154mM sodium bicarbonate (Caledon Laboratories LTD) solution were added to the mixture and made up to one liter using distilled water containing 0.9% sodium chloride to give the final concentrations
shown in Table III. Protease inhibitors were added to the modified perfusate to prevent NPY degradation by enzymes in the stomach vasculature. The protease inhibitors used were 0.1μM thiorphan (Peninsula Laboratories), 2μM bestatin (Sigma) and 1μM captopril (Nishimura, 1981). Bestatin is a general aminopeptidase inhibitor, thiorphan is an inhibitor of enkephalinase and captopril is known to inhibit angiotensin-converting enzyme.

Table III: Composition of modified Krebs' bicarbonate solution.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>120.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>4.4 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Dextrose</td>
<td>4.4 mM</td>
</tr>
<tr>
<td>Dextran</td>
<td>3.0 %</td>
</tr>
<tr>
<td>BSA</td>
<td>0.2 %</td>
</tr>
</tbody>
</table>

(b) Surgical Procedure

Male Wistar rats (250-350g) were housed in a light controlled room and given free access to laboratory rat food and water. The rats were fasted overnight (16-18h) and anaesthetized with an intraperitoneal injection of 65mg/kg sodium pentobarbital (Somnootol®, MTC Pharmaceuticals). The surgical procedure of Kwok and coworkers (1985) was followed. A midline incision was made to expose the viscera and the abdominal aorta was located. The left renal artery and vein, and the left adrenal gland were sectioned before preparing the aorta below the area of the coeliac branch for the insertion of an arterial cannula using a polyethylene tubing (PE 160). The colon was sectioned at the rectum which allowed for the removal of the small and large intestines, the pancreas, and spleen. The mesenteric artery was ligated and cut, and the portal vein prepared for cannulation (PE 160). The spleen and the head of the pancreas were isolated from the greater curvature of the stomach. To allow drainage, a cannula was inserted through the duodenum and into the lumen of the stomach. After the right renal artery and vein were sectioned between double
ligatures, the arterial cannula was inserted adjacent to the coeliac artery through which a single dose of heparinized saline (Sigma, 800U) was administered. The vena cava was then tied off and the rat was guillotined below the level of the diaphragm. A second cannula was placed into the portal vein in order to collect the venous effluent. The stomach was perfused with a modified Krebs' bicarbonate solution for an equilibration period of 20 minutes. It has been demonstrated (McIntosh et al., 1981) that following the removal of the pancreas, the small remaining portion attached to the portal vein and bile duct gives only 1% of the insulin response of an intact, isolated pancreas to a stimulus of 300mg% of glucose. Therefore, this remaining pancreas is assumed to contribute negligibly to the peptide secretion into the portal effluent.

(c) **Perfusion Procedure**

The perfusate was continuously gassed with a water saturated mixture of 95% oxygen and 5% carbon dioxide and pumped at a set flow rate of 2.5ml/min through a heating chamber to a bubble trap in which a temperature probe was inserted. The temperature of the perfusate entering the stomach was maintained at 37°C. The perfusate flowed via the gastric circulation through the aortic cannula and the venous effluent was collected. The perfusion pressure was monitored by means of a transducer converter (SE laboratories) and in some experiments plotted on a Gould chart recorder, model #2200. The perfusion pressure was between 40 and 70 mmHg. Stimulation with high potassium was achieved by exchanging at the level of the bubble trap, regular perfusate with perfusate in which the potassium chloride concentration was increased to 50mM and the sodium chloride reduced appropriately to maintain a constant osmolality. Peptides and other chemicals were introduced into the gastric circulation through a side arm infusion entering the aortic cannula. This was accomplished using a Harvard infusion pump (model #600-910/920). The rate of the side arm infusion was calculated to give the following final concentrations: acetylcholine (1μM), 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) (10μM), atropine (3μM), hexamethonium (400μM), isoproterenol (15μM), phentolamine
(4μM), phenylephrine (7.5μM) and propranolol (4μM) (Sigma Chemical Co.). Following a 30 minute stabilization period, the perfusate from the portal vein cannula was collected for 3 minute intervals in chilled 10ml test tubes each containing 1ml aprotinin (Trasylo®), 1000 Kallikrein inhibitor units KIU/ml aprotinin, Miles, Canada). The perfusate samples were then stored at -20°C. The samples were then Sep Pak extracted and lyophilized for RIA analysis.

The concentrations of the drugs used in the current studies were chosen from literature values. Fifty millimolar potassium chloride and 10μM DMPP concentrations were used since they have been established to significantly increase gastrin-IR and leu-enkephalin-IR secretion in the isolated perfused rat stomach (Nishimura, 1981). Acetylcholine in the low μM range has been shown to inhibit GIP-stimulated somatostatin release from the isolated perfused rat stomach (McIntosh et al., 1981) and atropine (3μM) blocked the vagally induced inhibition of somatostatin release in the same model (McIntosh et al., 1989). Hexamethonium in the hundred μM concentration range has been demonstrated to block the nicotinic stimulation of NPY and NA release in the isolated perfused guinea pig heart (Richardt et al., 1988). Propranolol (4μM) has been shown to inhibit the release of somatostatin by NA (1.5 x 10^{-7}M) in the isolated perfused rat stomach preparation (Koop et al., 1983). Local infusion of phentolamine (at an estimated plasma concentration of 10μM) increased the release of NA and NPY upon 2 Hz stimulation of the splenic nerve in the blood perfused pig spleen in vivo (Lundberg et al., 1986b), and isoproterenol has been demonstrated to stimulate gastric somatostatin secretion with a maximum stimulation observed at 15μM (Koop et al., 1983). Upon infusion of 15μM of phenylephrine in the isolated perfused rat stomach preparation major perfusion pressure changes were detected in our studies, therefore 7.5μM phenylephrine was infused in the release studies that followed.
Fig. 2  Schematic outline of the apparatus for in vitro perfusion of the rat stomach vasculature (Nishimura, 1981). The perfusate was pumped at a set flow rate of 2ml/min through a heating chamber to a bubble trap in which a temperature probe was inserted. The perfusate flowed via the gastric circulation through the aortic cannula and the venous effluent was collected. The perfusion pressure was monitored by means of a transducer converter.
Data Expression and Analysis

The minimal detection limit (i.e. sensitivity) of the NPY standard curves was taken as the concentration of peptide producing 10% reduction in binding of $^{125}$I-NPY to antibody. The coefficient of variance (standard deviation/mean x 100) of the ED$_{50}$ of the standard curve was used as an estimate of inter-assay variability. Data in the representative standard curve are expressed as mean of triplicate values ± standard error.

Quantitative data of NPY-IR in the GI extracts is expressed as the mean of 'n' rats ± standard error of the mean.

The perfusion data is expressed as follows:

$$\text{Percentage of basal NPY-IR} = \frac{\text{rate of secretion during a 3-min period}}{\text{rate of secretion during period 1}} \times 100$$

The mean and the standard error of the mean for each period were calculated for the different stimuli and significant changes in NPY release were determined using the raw data (fmole/min) in comparison to pre-test periods by one-way analysis of variance and Newman-Keuls multiple comparison test (* for p < 0.05). In some perfusion experiments pressure (mmHg) was monitored and the difference between the mean pre-test level and the mean post-infusion level calculated. The range of pressure changes for the rats tested is quoted in the text. The increase in flow rate (0.2ml/min) through the vasculature of the stomach upon infusion via the side-arm contributed to these differences in pressure. There was 3-5mmHg increase in pressure compared to pre-test periods upon infusion of the buffer alone.
RESULTS

I. NPY Radioimmunoassay

In order to measure the very low levels of peptide in the perfusate samples, a sensitive and specific RIA was required. To develop such a RIA tracer purification studies were performed and different assay separation techniques were attempted.

(a) Purification of $^{125}\text{I}-\text{NPY}$

(i) Whatman Carboxymethyl Cellulose (CM-52) Chromatography

The $^{125}\text{I}-\text{NPY}$ eluted in a broad peak with the highest radioactivity in fraction 19 at approximately 1.5M ammonium acetate (Fig.3). Upon a subsequent RIA the percentage of binding of CM-52 purified $^{125}\text{I}-\text{NPY}$ was found to increase by two-fold in comparison to the unpurified tracer (22% vs 11%) (Fig.3). There was also an increase in the sensitivity of the CM-52 purified tracer compared to unpurified $^{125}\text{I}-\text{NPY}$ (570pM vs 700pM). Since the sensitivity and binding of $^{125}\text{I}-\text{NPY}$ was not adequately improved using the CM-52 chromatography, HPLC purification of the tracer was then performed.
Fig. 3 Whatman carboxymethyl cellulose (CM-52) chromatography of $^{125}$I-NPY. The chromatography of the $^{125}$I-NPY is illustrated in the upper panel and the standard curve obtained using the purified tracer shown in the lower panel. The NPY tracer was applied to the cation exchange column (12 x 170mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was ammonium acetate which was applied over a linear concentration gradient of 0.2 to 2M (→). Two minute fractions were collected and counted in a gamma spectrometer. The purified tracer was then used in the standard curve illustrated above. Data in the representative standard curve is expressed as mean of triplicate values ± standard error of mean.
(ii) Reverse Phase High Performance Liquid Chromatography

The $^{125}$I-NPY was initially purified using a C$_{18}$ column and a 35-50% linear acetonitrile concentration gradient. The tracer eluted from the column as a single peak with an elution time of 19 minutes and at approximately a 50% acetonitrile concentration. A shoulder on the descending limb indicates the presence of a second peak with an elution time of 19.6 minutes which overlapped with the first peak (Fig.4). Upon a subsequent RIA using the effluent from the peak fraction, there was a two-fold increase in binding over the unpurified NPY (25% vs 11%) (Fig.5), but the sensitivity of the assay was not significantly enhanced in comparison with the CM-52 purified tracer (520pM vs 570pM). The acetonitrile concentration gradient was then manipulated to separate the two overlapping peaks. After various trials, a 45-50% linear concentration gradient was found to partially resolve the single peak into two separate peaks with elution times of 15 and 16 minutes (Fig.6). The two peaks, which probably represent different iodinated products of NPY, were collected for RIA analysis. The NPY standard curve for peak 1 had a 5% higher maximum binding (Fig.7), however the overall sensitivity of the standard curves for peak 1 and 2 were not significantly different (510pM and 600pM for peak 1 and 2 respectively).

The NPY tracer which eluted from the C$_{18}$ column had a long retention time and a broad peak span and it seemed likely that the tracer was adhering too strongly to the hydrophobic column for adequate resolution to occur. Therefore, to obtain a more satisfactory purification of the NPY tracer the HPLC column was changed from a C$_{18}$ to a less hydrophobic C$_{8}$ column. This resolved the two peaks of NPY tracer into three separate peaks with elution times of 12, 14 and 15 minutes (Fig.8). The first peak was collected and used as the purified NPY tracer in the studies that followed.
Fig. 4 Reverse phase HPLC elution profile of $^{125}$I-NPY. The tracer was applied to the 
µBondapak C$_{18}$ reverse phase column (4.6 x 250mm) and the chromatography performed 
at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient 
(35-50%) containing 0.1% TFA. The gradient was delivered over a 10 minute time 
interval. One minute fractions of the effluent were collected and the peak fractions diluted in 
the appropriate assay buffer for RIA.
Fig. 5 NPY standard curve for the μBondapak C₁₈ reverse phase HPLC purified tracer. A 35-50% acetonitrile concentration gradient was used to purify the tracer. Data in the representative standard curve is expressed as mean of triplicate values ± standard error of mean.
Fig. 6 Reverse phase HPLC profile of $^{125}$I-NPY. The tracer was applied to the µBondapak C$_{18}$ reverse phase column (4.6 x 250mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient (45-50%) containing 0.1% TFA. The gradient was delivered over a 10 minute time interval. One minute fractions of the effluent were collected and the peak fractions diluted in the appropriate assay buffer for RIA.
Fig. 7 NPY standard curves for the two peaks of NPY tracer (Peak 1: ← and Peak 2: →) purified using the μBondapak C18 reverse phase HPLC column and a 45-50% acetonitrile concentration gradient. Data in the representative standard curve is expressed as mean of triplicate values ± standard error of mean.
Fig. 8 Reverse phase HPLC profile of $^{125}$I-NPY. The tracer was applied to the μBondapak C$_8$ reverse phase column (4.6 x 250mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient (45-50%) containing 0.1% TFA. The gradient was delivered over a 10 minute time interval. One minute fractions of the effluent were collected and the peak fractions diluted in the appropriate assay buffer for RIA.
The iodination of peptides is usually incomplete and trace amounts of unlabelled peptide are present. This would decrease the sensitivity of the assay as the unlabelled peptide would compete with the tracer for the antibody in the assay. In order to show that the HPLC purification adequately separated the labelled from unlabelled peptide, the elution profiles of standard and tracer NPY were compared. The purified tracer was shown to be adequately separated from unlabelled NPY. Standard NPY eluted in fraction number 22 which was 4 fractions prior to the elution of $^{125}$I-NPY (Fig.9). A second minor peak of NPY-IR eluted at fraction number 34.

![Graph](image-url)

**Fig. 9** Elution profile of standard porcine NPY (•) and NPY tracer (□). The tracer or standard NPY (0.75nM) were applied to the μBondapak C8 reverse phase column (4.6 x 250mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient (45-50%) containing 0.1% TFA. The gradient was delivered over a 10 minute time interval. Half minute fractions of the effluent were collected for analysis.
In an attempt to further increase the sensitivity of the RIA, a different separation technique was attempted. Instead of the dextran coated charcoal separation, a PEG separation was used. The PEG separation traps the bound $^{125}$I-NPY, while charcoal separation precipitates the unbound NPY tracer. Upon using the PEG separation technique with the purified tracer eluted from the $\mu$Bondapak C$_8$ column there was a further 2-fold increase in the maximum binding (52% vs 25%) (Fig. 10) and the sensitivity of the assay (290pM vs 600pM) when compared to the charcoal separation technique with the tracer purified using the C$_{18}$ column (peak 2). The only disadvantage with the developed RIA was found to be the higher non-specific binding compared with the charcoal separation (9.7% vs 1.5%). The inter-assay variability for the standard curve was found to be 10.8% [i.e. the coefficient of variance of the ED$_{50}$ value (1665 ± 180pM) for 6 representative standard curves]. The $\mu$Bondapak C$_8$ column purified $^{125}$I-NPY and the PEG separation technique were used to quantitate NPY in the studies that followed.
Fig. 10 Comparison of the NPY standard curves: μBondapak C8 column purified tracer with the PEG separation technique (––) vs C18 column purified tracer with the charcoal separation technique (•). A 45-50% acetonitrile concentration gradient was used to purify the NPY tracer for both standard curves. Data in the representative standard curve is expressed as mean of triplicate values ± standard error of mean.
(c) **Characterization of NPY Antisera**

The Peninsula reconstituted NPY antiserum was equally stable with only a 4% reduction in zero binding after three months of storage at -20°C. Both the Peninsula antiserum and the YN7 antiserum, which was provided by Allen and coworkers (1984), had minimal cross reactivity with human pancreatic polypeptide, bovine pancreatic polypeptide and human peptide YY as shown in Table IV.

**Table IV:** Cross-reactivity studies using YN7 and Peninsula antisera.

<table>
<thead>
<tr>
<th></th>
<th>Peninsula Antiserum</th>
<th>YN7 Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Pancreatic Polypeptide</td>
<td>0.26%</td>
<td>0.17%</td>
</tr>
<tr>
<td>Bovine Pancreatic Polypeptide</td>
<td>0%</td>
<td>0.23%</td>
</tr>
<tr>
<td>Human Peptide YY</td>
<td>0.17%</td>
<td>0.10%</td>
</tr>
</tbody>
</table>
II. NPY-Immunoreactivity in the Gastrointestinal Tract

The tissue content of NPY-IR was found to be highest in the various segments of the rat stomach [fundus: 76.3 ± 5.6 pmol/g (n=18), corpus: 67.1 ± 9.8 pmol/g (n=15), antrum: 67.2 ± 2.0 pmol/g (n=15)]. There was a greater variability in the corpus compared to other regions of the GI tract. A significant (p < 0.05) decreasing trend in NPY-IR was detected from the stomach to the ileum [duodenum: 41.8 ± 2.4 pmol/g (n=27), jejunum: 31.7 ± 2.0 (n=29), ileum: 15.2 ± 1.0 pmol/g (n=8)]. Levels in the colon [31.3 ± 5.0 pmol/g (n=10)] were higher than the ileum and similar to those found in the jejunum (Fig.11).

![NPY-IR Levels](image)

**Fig. 11** Quantitation of NPY-IR (pmol/g wet weight) in the various segments of the gastrointestinal tract of the rat (n ≥ 8). Duoden and Jejun represent the duodenum and the jejunum intestinal regions. Asterisks represent the statistically significant differences (p < 0.05) between the stomach regions (fundus, corpus and antrum) and the more distally located gastrointestinal regions.
III. Characterization of NPY in Gastrointestinal Extracts

In order to characterize the NPY-IR detected in the tissue extracts chromatography was performed and the elution characteristics compared with the standard peptide.

(a) G-25 Sephadex Chromatography

The NPY-IR in the ileum and corpus (Fig.12) eluted in fraction number 38 from the G-25 column. There was over 90% recovery of the NPY-IR loaded onto the column. The standard $^{125}$I-NPY eluted in fraction number 39. Approximately 70% of the tracer was recovered.

(b) Reverse Phase High Performance Liquid Chromatography

The standard NPY eluted from the C$_{18}$ HPLC column as a single, sharp peak at 15.9 minutes and at a 45% acetonitrile concentration, while PYY eluted 2.6 minutes earlier at a 30% acetonitrile concentration (Fig.13). The colon and corpus extracts were then applied to the column. The U.V. detector, which was set at a wavelength of 225nm, detected a number of peaks (Fig.14). After collecting and concentrating the HPLC effluent, RIA analysis revealed a single peak of NPY-IR eluting at the position corresponding to standard NPY (Fig.15).
Fig. 12 Sephadex G-25 chromatography of the extracts of rat corpus (upper panel) and ileum (lower panel). The lyophilized extracts were reconstituted in 1 ml of 0.2 M acetic acid and centrifuged. The extracts of rat corpus (130 mg) and ileum (160 mg) were applied to a G-25 column (95 x 1 cm) equilibrated in 0.2 M acetic acid. The flow rate was 0.4 ml/min. The void volume (26 ml) was discarded and subsequent 1 ml effluent fractions were collected and lyophilized for RIA analysis. $^{125}$I-NPY (5000 cpm) was then applied to the column. One milliliter effluent fractions were collected and counted in a gamma spectrometer.
Fig. 13 Reverse phase HPLC elution profile of NPY and PYY standards. The standard peptides were dissolved in HPLC water containing 0.1% TFA to give a final concentration of 5μg/μl. Single peptide injections of 5μl, or a mixture of both peptides were injected into the mixing chamber such that 2.5μg of each peptide was applied to the column. The standards were applied to the μBondapak C_{18} reverse phase HPLC column (4.6 x 250mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient (20-55%) containing 0.1% TFA which was delivered over a 10 minute time interval. One minute fractions of the effluent were collected and lyophilized for NPY RIA analysis. The programmable multi-wavelength detector was set at a wavelength of 225nm. The change in base-line represents the change in acetonitrile concentration (20-55%).
Fig. 14 Reverse phase HPLC elution profile of the rat colon. The extract (6mg) was applied to the µBondapak C₁₈ reverse phase HPLC column (4.6 x 250mm) and the chromatography performed at a flow rate of 1ml/min. The eluent was an acetonitrile in water concentration gradient (20-55%) containing 0.1% TFA which was delivered over a 10 minute time interval. One minute fractions of the effluent were collected and lyophilized for RIA analysis. The programmable multi-wavelength detector was set at a wavelength of 225nm. The change in base-line represents the change in acetonitrile concentration (20-55%). The arrows indicate the elution positions of standard peptides NPY and PYY determined in the previous study.
Fig. 15 The HPLC elution profiles of the extracts of the rat colon (upper panel) and corpus (lower panel) as determined by the NPY RIA analysis.
IV. Perfusate Extraction

Sep Pak and amberlite extraction of the perfusate samples collected from the isolated perfused rat stomach were performed to remove possible interference between the perfusate constituents and the RIA, and also to concentrate the secreted levels of endogenous peptide.

Comparison of Sep Pak and Amberlite Extraction Methods

Both methods of extraction allowed for 2 to 10-fold concentration of the samples, thereby allowing the analysis of very low levels of the peptide. However, Sep Pak extraction was found to be more simple, rapid and efficient than amberlite extraction. The recovery of unpurified $^{125}$I-NPY obtained by Sep Pak extraction was over 90%, while there was only approximately 70% recovery upon amberlite extraction. The peak elution of $^{125}$I-NPY from the Sep Pak cartridges occurred within the 40-60% acetonitrile concentration range. There was still over 90% recovery of $^{125}$I-NPY from the Sep Pak cartridge after the extraction of 10-12 perfusate samples. There was $45 \pm 5\% (n = 4)$ recovery of standard porcine NPY using amberlite extraction and $79 \pm 8\% (n = 6)$ recovery of the NPY-IR using Sep Pak extraction. The higher recovery obtained using the $^{125}$I-NPY is probably due to losses which occur during drying of samples prior to measurement by RIA and inherent inaccuracies of any RIA procedure. Sep Pak extraction was used in the release studies that followed.
V. Release Studies on the Isolated Perfused Stomach

Since high levels of NPY-IR were detected in extracts of the stomach (Fig. 11) release studies in the isolated perfused rat stomach were attempted.

In order to determine whether the constituents of the perfusate interfered with the assay, 100μl of unextracted or extracted perfusate was added to the assay tubes of the NPY standard curves. There was no significant change in either the sensitivity or maximum binding of $^{125}\text{I}$-NPY in the assay.

Enzymatic degradation and/or peptide uptake usually occurs in isolated perfused organ preparations. In order to investigate this possibility, standard porcine NPY was infused into the isolated perfused rat stomach at a rate to give a final concentration of 2nM. There was a 40 ± 4% recovery of the peptide. The recovery was significantly improved (55 ± 5%) by the addition of a cocktail of enzyme inhibitors to the perfusate.

(i) Basal Secretion of NPY

Upon perfusion of the stomach, a low level of basal NPY release was detected in the perfusate samples. The basal level varied between rats (98-147 fmole/min) and thus the perfusion data was expressed as a percentage of basal NPY-IR. The secretion rate of basal NPY was shown to be maintained during 7 periods of perfusate infusion (Fig. 16). The detected levels probably accounts for only a portion of the actual amount of NPY secreted into the stomach vasculature due to peptide degradation and/or uptake by the vasculature. Both intrinsic nonadrenergic neurones and extrinsic adrenergic nerve terminals innervating the stomach, which have been shown to contain NPY (Sundler et al., 1983), could be contributing to these basal levels of NPY-IR.
Fig. 16 Basal secretion of NPY-IR. The secretion was maintained throughout the 7 periods.
(ii) Potassium Infusion

In order to establish the physiological relevance of NPY as a regulator in the enteric nervous system of the stomach, it was necessary to determine whether levels of NPY could be stimulated above basal. Since NPY is a neuronal peptide a potassium stimulus was initially used. There was a NPY basal level of $127 \pm 5$ fmole/min in period 1. Raising the potassium chloride concentration of the perfusate from 4.4mM to 50mM resulted in a significant ($p < 0.05, n=6$) increase in NPY secretion above basal levels. This increase was sustained over the infusion periods with a maximum secretion in period 5 ($214 \pm 9$ fmole/min) (Fig.17). The NPY release returned immediately to basal upon removal of the high potassium stimulus. The secretion of somatostatin was also simultaneously analyzed since high potassium is known to stimulate its secretion (Nishimura, unpublished observation). There was a concomitant six-fold increase in somatostatin release upon infusion of the high potassium perfusate from a basal level of $109 \pm 14$ fmole/min in period 1 to a maximum secretion in period 4 of $747 \pm 96$ fmole/min (Fig.17). However unlike NPY release, the increase in somatostatin was not maintained across the infusion period and returned to basal levels prior to the termination of high potassium infusion in period 6.
Fig. 17 The effect of 50mM potassium on basal secretion of NPY (upper panel) and somatostatin (lower panel) in the isolated perfused stomach. Stimulation with high potassium was achieved by exchanging at the level of the bubble trap, regular perfusate with perfusate in which the potassium chloride concentration was increased to 50mM and the sodium chloride concentration reduced appropriately to maintain a constant osmolality. The high potassium stimulus increased the NPY release above basal levels during infusion periods 3-6. Somatostatin secretion was increased over basal levels during infusion periods 4 and 5. Asterisks represent a significant difference compared to pre-test periods (p < 0.05 or greater).
(iii) The Effects of Cholinergic Agonists and Antagonists on NPY Release

Nicotinic receptor stimulation has been shown to release NPY in the bovine adrenal medulla (Hexum et al., 1986) and the isolated perfused guinea pig heart (Franco-Cereceda et al., 1987b). However, the actions of cholinergic agonists and antagonists on NPY secretion in the isolated perfused rat stomach have not been investigated.

(a) Effect of Acetylcholine on Basal NPY Secretion

The effect of acetylcholine (ACh) (1μM) on the basal secretion of NPY was first investigated. The basal level was 117 ± 10 fmole/min in period 1. Following the infusion of 1μM ACh, there were an increase in perfusion pressure (6-8mmHg) when compared to the pre-test periods. The NPY secretion increased in period 4 (161 ± 12 fmole/min) and was sustained at this level throughout the ACh infusion periods (Fig.18). Upon termination of ACh infusion there was a decrease in NPY secretion to basal levels.

(b) Effect of Atropine on Acetylcholine-Stimulated NPY Secretion

The effect of the cholinergic muscarinic antagonist atropine (3μM) on NPY secretion is shown in Fig.19. There was a NPY basal level of 114 ± 7 fmole/min. Atropine (3μM) had no effect on basal NPY secretion during period 2. Upon the simultaneous infusion of ACh (1μM) there was an immediate rise in NPY secretion above basal with a maximum increase in period 3 (187 ± 14 fmole/min). Upon termination of infusion of both ACh and atropine the NPY secretion returned to basal levels.
**Fig. 18** The effect of ACh (1μM) on basal secretion of NPY. Following two 3-minute periods of perfusion of buffer alone, ACh (1μM) was infused during periods 3-6. NPY secretion increased significantly during periods 4-6 (n=4). Asterisks represent a significant difference compared to pre-test periods (p < 0.05 or greater).

**Fig. 19** The effect of atropine (3μM) on ACh-stimulated release of NPY. Atropine had no effect on the basal level of NPY in period 2. Acetylcholine still had a stimulatory effect on NPY release upon concomitant infusion with atropine during periods 3-5 (n=4). Asterisks represent a significant difference compared to pre-test period 1 (p < 0.05 or greater).
(c) **Effect of Hexamethonium on Basal and Acetylcholine-Stimulated NPY Secretion**

The effect of the ganglionic cholinergic antagonist hexamethonium on basal NPY release was studied in an attempt to elucidate the mechanism of cholinergic stimulation of NPY release. The basal level was $118 \pm 11$ fmole/min in period 1. Hexamethonium (400\textmu M) had an inhibitory effect on basal release of NPY into the stomach vasculature with a maximum decrease in period 6 ($77 \pm 15$ fmole/min) (Fig.20). The decrease in NPY secretion below basal levels was maintained throughout infusion periods 3-6 and returned to basal levels upon the termination of hexamethonium infusion.

The effect of hexamethonium (400\textmu M) on ACh-stimulated NPY release was then investigated. There was a basal level of $122 \pm 7$ fmole/min. Upon infusion of ACh (1\textmu M), there was an increase in NPY release in period 3 ($208 \pm 9$ fmole/min) which was maintained during the infusion periods 3-5. Upon concomitant infusion of 400\textmu M hexamethonium, there was a significant ($p < 0.05$, $n=5$) initial reduction in NPY secretion in infusion period 6 below stimulated levels ($169 \pm 12$ fmole/min), however the secretion remained above basal levels. This decrease in NPY release was found to be transient with an increase to the ACh-stimulated level occurring in period 7 (Fig.20).
Fig. 20 The effect of hexamethonium (400μM) on basal (upper panel) and ACh-stimulated NPY release (lower panel). Hexamethonium had a significant inhibitory effect on basal NPY secretion during periods 3-6 (n=4) and a significant inhibitory effect on ACh-stimulated NPY levels in period 6 (n=5). Asterisks represent a significant difference compared to pre-test period(s) (p < 0.05 or greater). Hexam represents hexamethonium.
(d) **Effect of DMPP on NPY Secretion**

There was a stimulatory effect on NPY secretion upon the infusion of the nicotinic ganglionic agonist DMPP (10μM) (Fig.21). There was a significant stimulation in period 4 (176 ± 16 fmole/min) and period 7 (189 ± 28 fmole/min) compared to the basal period (125 ± 5 fmole/min). Pressure changes (11-15 mmHg above pre-test levels) were observed at regular time intervals.

**Fig.21** The effect of DMPP (10μM) on the basal secretion of NPY. DMPP had a stimulatory effect on NPY release (n=6). Asterisks represent a significant difference compared to pre-test period 1 (p < 0.05 or greater).
Adrenergic Effects on NPY Release

NPY has been shown to be coreleased with noradrenaline (NA) upon the activation of the sympathetic nervous system (Lundberg et al., 1985c), and its release is influenced by a presynaptic α2-adrenergic receptor-mediated action of NA (Wahlestedt et al., 1986). The effects of α- and β-adrenergic agonists and antagonists on gastric NPY secretion in the stomach have not been studied. Therefore the possible adrenergic regulatory mechanisms involved in the regulation of NPY release into the stomach vasculature were investigated.

(a) Effect of Phenylephrine on Basal NPY Secretion

The α-adrenergic agonist phenylephrine (7.5μM) had a stimulatory effect on NPY release (Fig.22). NPY secretion was significantly increased in the infusion periods 4-7 compared to period 1 (125 ± 7 fmole/min). The maximum increase in secretion occurred in period 5 (183 ± 11 fmole/min). There was an immediate return to basal levels upon termination of infusion. There was a increase in perfusion pressure (+17-24mmHg) throughout the infusion periods.

(b) Effect of Phentolamine on Basal NPY Secretion

The α-adrenergic antagonist phentolamine (4μM) induced an increase in NPY release. The basal level of NPY was 123 ± 7 fmole/min. There was a latent period prior to the increase in NPY secretion above basal in periods 4 (162 ± 6 fmole/min) and 5 (148 ± 11 fmole/min) (Fig.22). There was an immediate return to basal levels upon termination of infusion. There were no significant perfusion pressure changes.
Fig. 22. The effect of phenylephrine (7.5µM) (upper panel) and phentolamine (4µM) (lower panel) on basal release of NPY. Phenylephrine had a stimulatory effect on basal NPY levels during periods 4-7. Phentolamine had a latent stimulatory effect on NPY secretion during periods 4 and 5 (n=6). Asterisks represent a significant difference compared to pre-test period(s) (p < 0.05 or greater).
(c) Effect of Propranolol on Basal NPY Secretion

The basal level of NPY was 124 ± 10 fmole/min. The β-adrenergic blocker propranolol (4μM) produced a small increase in mean basal NPY release in period 2 (151 ± 14 fmole/min) followed by a reduction in period 4 (105 ± 10 fmole/min), however these changes in secretion did not reach significance (Fig.23). There was a slight transient increase in the perfusion pressure (+8-10mmHg) compared to pre-test levels.

(d) Effect of Isoproterenol on the Basal Release of NPY

The effect of the β-adrenergic agonist isoproterenol (15μM) was investigated to further elucidate any β-adrenergic mediated effects on NPY release. There was a basal level of 129 ± 4 fmole/min in period 1. Isoproterenol had a stimulatory effect on NPY secretion throughout the infusion periods with a maximum stimulatory effect in period 5 (260 ± 13 fmole/min). Upon the termination of the isoproterenol infusion NPY release returned immediately to basal levels (Fig.24). There was a temporary reduction in perfusion pressure (-10-14mmHg) which lasted for the initial 1-3 minutes of isoproterenol infusion.
Fig. 23 The effect of propranolol (4 μM) on the release of NPY. The changes in NPY secretion were not statistically significant. Propranolol produced an initial small increase in mean basal NPY release during periods 2 and 3, which was followed by a reduction during periods 4-6 (n=5).

Fig. 24 The effect of isoproterenol (15 μM) on the basal secretion of NPY. Isoproterenol had a significant stimulatory effect on NPY release during periods 3-7 (n=5). Asterisks represent a significant difference compared to pre-test periods (p < 0.05 or greater).
DISCUSSION

NPY is one of the major neuropeptides in the GI tract. The distribution in intrinsic neurones and extrinsic sympathetic neurones innervating the GI tract suggests that it may serve more than one function in the regulation of gut functions. In addition, the complex neural pathways which exist in the GI tract indicate that multiple pathways are probably involved in the regulation of its secretion. In the current studies a RIA for NPY has been developed, the NPY-IR in the GI tract characterized and studies on the regulation of NPY secretion performed.

(i) Development of the NPY RIA

In order to accomplish any quantitative studies on NPY, a specific and sensitive RIA for this peptide was required. Five of the 36 amino acids of NPY are tyrosine residues located at the N- and C-termini, and the remaining three at positions 20, 21 and 27 of its amino acid sequence (Larhammar et al., 1987) (Fig.1). Upon chloramine-T iodination, which non-selectively iodinates tyrosine residues, a number of iodinated NPY products were formed. The iodine moiety can alter the secondary and tertiary structures of NPY and therefore affect its binding affinity to the antibody used in the assay. The objective of the tracer purification study was to separate and collect the iodinated product that had undergone the least amount of structural rearrangements upon iodination. The more structurally similar the iodinated NPY is to the unlabelled peptide the greater is its specificity for the antibody.

Tracer purification was initially performed using ion-exchange chromatography. In ion-exchange chromatography separation is achieved based on ionic interactions (retarding forces) between the $^{125}$I-NPY and the charged matrix column. A cation exchange column [carboxymethyl cellulose (CM-52)] was used in the purification of $^{125}$I-NPY. The CM-52 purification of the tracer increased the binding of $^{125}$I-NPY by two-fold and improved the sensitivity of the assay as indicated in Table V. Even though this method of purification has been shown to be suitable for other peptides, such as $^{125}$I-somatostatin (Nishimura, 1981),
was not found to be adequately purified to allow quantitative studies of very low levels of the peptide to be performed. Therefore reverse phase HPLC of $^{125}$I-NPY was next tested.

Reverse phase HPLC separates the $^{125}$I-NPY according to hydrophobicity. The unique feature of the HPLC is that the sample can be passed through an extremely tightly packed matrix by means of very high pressures, resulting in high resolution separations which can not be achieved using other methods. The HPLC conditions, such as the acetonitrile concentration gradient, can also be easily manipulated to allow for optimal purification of the $^{125}$I-NPY. The $^{125}$I-NPY was initially purified using a C$_{18}$ column and a 35-50% linear acetonitrile concentration gradient. The tracer eluted from the column as a single peak with a shoulder on the descending limb indicating heterogeneity (Fig.4). Upon a subsequent RIA using the effluent from the peak fraction, there was a two-fold increase in binding over the unpurified NPY, but the sensitivity of the assay was not significantly enhanced in comparison with the CM-52 purified tracer (Fig.5) (Table V). The acetonitrile concentration gradient was then manipulated to separate the overlapping peaks. After various trials, a 45-50% linear concentration gradient was found to partially resolve the single peak into two separate peaks (Fig.6). The two peaks, which probably represent different iodinated products of NPY, were collected for RIA analysis. The NPY standard curve for peak 1 had a higher binding, however the overall sensitivity of the standard curves of the two peaks were not significantly different (Fig.7) (Table V).

The NPY tracer which eluted from the C$_{18}$ column was found to have a long retention time and a broad peak span and it seemed likely that the tracer was adhering too strongly to the hydrophobic column for adequate resolution to occur. Therefore, to obtain a more satisfactory purification of the NPY tracer the HPLC column was changed from a C$_{18}$ to a less hydrophobic C$_8$ column. Three iodinated products of NPY, based on their different elution times, were detected using these HPLC conditions (Fig.8) (Table V). These HPLC studies
emphasise the importance of not only using the appropriate concentration gradient but also a suitable column to achieve optimal purification.

The iodination of peptides is usually incomplete and trace amounts of unlabelled peptide are present. This would decrease the sensitivity of the assay as the unlabelled peptide would compete with the tracer for the antibody in the assay. The optimized HPLC conditions using the C₈ column and a concentration gradient of 45-50% acetonitrile were shown to adequately separate the labelled from unlabelled peptide (Fig.9).

In conclusion, ^125^I-NPY purification was shown to be best achieved using HPLC which not only showed a better resolution of the tracer peaks compared to carboxymethyl cellulose chromatography, but also adequately separated the labelled from unlabelled peptide.

However, tracer purification by itself was not found to be sufficient for the development of the NPY RIA. A PEG separation technique had to be used in conjunction with the HPLC purified tracer to result in a sensitive enough RIA to allow for the quantitation of very low levels of the peptide. The PEG separation traps the bound ^125^I-NPY, while charcoal separation precipitates the unbound NPY tracer. Upon using the PEG separation technique with the purified tracer eluted from the ^μ^Bondapak C₈ column there was a further 2-fold increase in the maximum binding and the sensitivity of the assay when compared to the charcoal separation technique with the tracer purified using the C₁₈ column (peak 2) (Table V). The only disadvantage with the developed RIA was found to be the six-fold higher non-specific binding compared with the charcoal separation. The overall scheme of changes performed to improve the assay conditions has been summarized in Table V.
Table V: Summary of the data on the improvement of the NPY assay conditions.

<table>
<thead>
<tr>
<th>Assay Separation Technique</th>
<th>Conditions of $^{125}$I-NPY Purification</th>
<th>Resolution</th>
<th>Elution Times (min)</th>
<th>% Maximum Binding</th>
<th>Sensitivity (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charcoal</td>
<td>Unpurified</td>
<td>Single broad peak</td>
<td>38</td>
<td>22</td>
<td>570</td>
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<tr>
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<td>520</td>
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<td></td>
<td></td>
<td></td>
<td>Peak2: 25</td>
<td>Peak2: 600</td>
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<tr>
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<td>12, 14 and 15</td>
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<td>Peak1: 290</td>
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<tr>
<td>PEG</td>
<td>$C{\text{8}}$ HPLC column Gradient: 45-50% ACN</td>
<td>Three peaks</td>
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</table>

(ii) Tissue Content of NPY-IR in the Rat GI Tract

NPY was found to be present in all the regions of the rat GI tract tested. The distribution was in good agreement with the study of Koch and coworkers (1988) on the human gut. The highest content was found in the stomach with a decreasing trend in NPY-IR down the GI tract until the level of the ascending colon where an increase was detected (Fig. 11). Unlike the present study in which the NPY-IR was determined in homogenized whole extracts of the GI tract, Koch and coworkers (1988) microdissected the mucosa-submucosa and muscularis externa layers and determined the content of NPY in the two layers separately. It was shown that the overall trend in NPY content was determined by the muscularis externa layers, while the concentration of the peptide in the mucosa-submucosa layers was similar throughout the human gut. These studies are in agreement with the immunohistochemical localization of NPY-IR (Wang et al., 1987). The present study was also in partial agreement with the work of Allen and coworkers (1987a), which showed the highest concentration of NPY to be present in the proximal gut of the rat, guinea pig and porcine GI tract. In the latter study, the highest concentrations of NPY in the rat and porcine gut were shown to be in the lower oesophageal sphincter, while the highest concentration in the guinea...
pig gut was found in the pylorus (Allen et al., 1987a). However in contrast to our study, they found the content in all the other regions of the GI tract of the rat to be three-fold lower. They also found a slightly higher NPY content in the upper jejunum compared to the stomach. The differences between the results of Allen and coworkers study and our study may be due to differences in antibody characteristics. In order to investigate this possibility cross-reactivity studies were performed. Both their YN7 antiserum and the Peninsula antiserum used in our study showed no cross-reactivity to the structurally related peptide human PYY, which is predominately found in endocrine cells in the distal small intestine and colon (Tatemoto, 1982c), and human and bovine PP. The YN7 antiserum has been shown to be specific for the N-terminal region of NPY. The N-terminal region of PYY and NPY contain the greatest variability and therefore there is minimal antiserum cross-reactivity (Allen et al., 1984). It is not known what sequence of the NPY molecule the Peninsula antiserum is directed towards. The possibility does exist that the two antisera are directed towards different epitopes of NPY. Peptides undergo enzymatic breakdown in tissues and these degraded products may contain the amino acid sequence that the antibody is directed towards. In this situation there would be the false conception of there being a greater peptide content in the tissue extracts than there actually exists.

The content of NPY in other organs of the rat have also been studied by various investigators. The NPY content found in the adrenal gland (308.5 ± 27.9 pmol/g) (Allen et al., 1983b), hypothalamus (170 ± 40 pmol/g) and the olfactory tubercle (300 ± 25 pmol/g) (Tatemoto et al., 1984) were higher than the mean concentration in the GI tract, while the immunoreactivity in the renal artery (57.0 ± 9.2 pmol/g), superior vena cava (47.8 ± 16.1 pmol/g) (Allen et al., 1984d) and hippocampus (55 ± 10 pmol/g) (Tatemoto et al., 1984) were comparable. The concentration in the right ventricule (22.4 ± 3.3 pmol/g) and atria (18.4 ± 1.6 pmol/g) of the rat heart and superior mesenteric artery (31.4 ± 8.4 pmol/g) (Allen et al., 1984d) were found to be lower than the mean content in the gut.
In order to characterize the NPY-IR detected in the tissue extracts gel filtration chromatography was initially performed. Gel filtration separates peptides according to molecular size. Since NPY is a low molecular weight peptide, a gel with a low molecular size fractionation range was used (Sephadex G-25). There was one immunoreactive species detected in the corpus and ileum extracts (Fig. 12) using gel filtration chromatography. This immunoreactive species eluted in a similar position to $^{125}$I-NPY.

Characterization studies using HPLC were also performed using a $\mu$Bondapak C$_{18}$ column. There was one immunoreactive species detected in the corpus and colon extracts (Fig. 15). This immunoreactive species eluted in a position similar to synthetic porcine NPY and later than PYY. Upon applying synthetic porcine NPY to the C$_{8}$ HPLC column, a second minor peak of NPY-IR was detected which eluted much later than the standard peptide peak (Fig. 9). This could possibly represent a dimer of NPY. The tertiary structure of NPY has been shown to allow for the formation of dimers (Minakata et al., 1989).

(iii) Regulation of NPY Secretion in the Isolated Perfused Rat Stomach

Peptide secretion is often investigated using isolated organ systems. The effect of substances on peptide secretion can be examined in relative isolation in these systems without complication by central effects. The isolated perfused rat stomach preparation has been employed successfully to measure the release of peptides such as somatostatin (McIntosh et al., 1981).

In the physiological investigation of the role of neuropeptides in the regulation of GI functions, the regulatory pathways involved in their secretion must be determined. Although many studies on the actions of NPY have been performed, no information is available on the secretion of this peptide in the GI tract. Since there were high levels of NPY-IR in the stomach, the mechanisms involved in NPY secretion in the isolated perfused rat stomach were investigated.
In some of the release experiments performed pressure changes were monitored. It is not known whether vascular effects produced by the infused drugs had any effect on the secretion of the peptide. Therefore, further studies are required to determine the contribution of these pressure changes to the responses in secretion observed.

In order to investigate the release of the peptide in an isolated organ several criteria had to be met. There should be no interference between the constituents of the unextracted or extracted perfusate and the RIA, and also a relatively good recovery of the infused standard peptide. The latter study would demonstrate whether enzymatic degradation and/or peptide uptake by the vasculature is sufficiently low to allow for the detection of the endogenous peptide being released. Both these criteria were found to hold for NPY. There was no significant change in either the sensitivity or binding of $^{125}$I-NPY in the assay in the presence of the unextracted or extracted perfusate constituents. There was also a good recovery of the peptide which was significantly improved by the addition of a cocktail of enzyme inhibitors to the perfusate.

In initial perfusion studies basal secreted levels of NPY were undetectable using RIA. This was probably due to the low levels of NPY secreted compared to other gastric peptides and degradation processes which are active in the stomach vasculature. Therefore extraction steps were neccessary to concentrate the endogenously released NPY to allow for RIA detection. Amberlite and Sep Pak extractions were both performed. The neutral polystyrene resin, Amberlite and the octadecysilane Sep Pak cartridges separate based on hydrophobic interactions. Both methods of extraction allowed for considerable concentration of the samples, thereby allowing the analysis of very low levels of the peptide. However, Sep Pak extraction was found to be more simple, rapid and efficient than amberlite extraction. The recovery of $^{125}$I-NPY and standard porcine NPY was also higher using Sep Pak extraction compared to amberlite extraction. The Sep Pak cartridge was found to give high recoveries even after the extraction of up to twelve perfusate samples. Sep Pak extraction was used in the release studies that followed.
Low levels of basal NPY release were detected in the perfusate samples which varied between rats (98-147 fmole/min). The secretion rate of NPY was maintained throughout the infusion periods tested (Fig.16). The detected levels probably account for only a portion of the actual amount of NPY secreted into the stomach vasculature due to peptide degradation and/or uptake by the vasculature. NPY-IR secreted under basal conditions probably originated from both intrinsic nonadrenergic neurones and extrinsic adrenergic nerve terminals innervating the stomach, both of which have been shown to contain NPY (Sundler et al., 1983).

Secretion of NPY-IR was stimulated by perfusion of a high potassium solution. High potassium has a general stimulatory effect on neuronal peptide secretion via the depolarization of nerves. The non-specific nature of this stimulus was demonstrated by the increase in secretion of both NPY and somatostatin (Fig.17). The stimulated NPY release was reduced to basal levels upon removal of the high potassium stimulus, while the stimulated somatostatin levels decreased to basal levels prior to the termination of high potassium infusion. The more sustained increase in NPY secretion compared to somatostatin may be due to different regulatory pathways in neurones and endocrine cells such as those containing somatostatin.

Since no direct release studies have been performed on NPY-containing neurones innervating the stomach, the actions of cholinergic agonists and antagonists on NPY secretion in the isolated perfused rat stomach were investigated.

The effect of ACh on the basal secretion of NPY was initially studied. Following the infusion of ACh, there was rise in the perfusion pressure. The secretion of NPY increased to a steady stimulated level upon ACh infusion (Fig.18). Upon termination of ACh infusion there was a decrease in NPY secretion to basal levels.

In order to further characterize the cholinergic mechanisms involved in the secretion of NPY, the action of the muscarinic antagonist atropine was investigated (Fig.19). The results suggested that atropine had no effect on basal NPY secretion, however in order to confirm this a longer time interval of infusion of the drug should be performed prior to concomitant infusion
with ACh. Atropine did not block the action of ACh on NPY release which suggests that there is a non-muscarinic regulatory mechanism for NPY release.

The cholinergically-stimulated release of NPY is at least partially mediated by the nicotinic receptor stimulation at the ganglionic level. Evidence for this came from studies on the effects of the cholinergic ganglionic antagonist hexamethonium (Fig.20) and the nicotinic ganglionic agonist DMPP (Fig.21) on NPY secretion.

Hexamethonium had an inhibitory effect on basal release of NPY into the stomach vasculature which was maintained throughout the infusion. This suggests that nicotinic ganglionic stimulation under basal conditions does contribute to the NPY released. The effect of hexamethonium on ACh-stimulated NPY release was then investigated. Upon concomitant infusion of hexamethonium and ACh, there was a significant initial partial reduction in NPY secretion. This decrease in NPY release was found to be transient. The observed effect may be partly due to the inhibitory effect of the drug on basal NPY secretion (Fig.20).

The effect of the nicotinic ganglionic agonist DMPP on basal NPY release was also investigated. Secretion of NPY increased upon the infusion of DMPP (Fig.21). There was a latent period prior to the stimulation which could be an indication that most of the ganglia of the NPY-containing neurones are not easily accessible to the drug. The pattern of perfusion pressure changes differed from that observed with ACh. Pressure changes occurred at regular time intervals and were maintained throughout the infusion of DMPP. The release pattern of NPY may thus be partly due to the vascular effect of DMPP. The action of DMPP on smooth muscle contraction is more pronounced and prolonged than the action of ACh since it is less enzymatically degradable. The NPY secretion returned to basal levels upon termination of infusion.

Since NPY is contained in both intrinsic and extrinsic neurones, the measured NPY-IR could originate from either source. The study with hexamethonium suggests that under basal conditions secretion is at least partially from intrinsic neurones since nicotinic receptors on the extrinsic adrenergic neurones are not accessible to the drug, and since denervation of extrinsic
vagal fibers has been shown not to affect the density or the distribution of NPY containing fibers in the gut (Lee et al., 1985; Sundler et al., 1983). In addition, it is possible that the intrinsic NPY neurones are under the control of both cholinergic and non-cholinergic, possibly peptidergic, neurones. The response to ACh was partially and transiently blocked by hexamethonium. It is possible that when nicotinic ACh receptors are blocked, the ACh is now more available for stimulation of muscarinic receptors on the axons of the peptidergic neurones innervating NPY neurones and this results in the apparent escape phenomenon.

The effects of α- and β-adrenergic agonists and antagonists in NPY secretion in the stomach vasculature was examined to elucidate possible adrenergic release mechanisms. The results did not conclusively demonstrate as to whether there is an α-adrenergic stimulatory or inhibitory action on NPY-containing neurones in the stomach.

The α-adrenergic agonist phenylephrine had a stimulatory effect on NPY release (Fig.22). There was an increase in perfusion pressure possibly due to the stimulatory effect of the α-adrenergic agonist on smooth muscle. The stimulatory effect of phenylephrine may be secondary to these pronounced vascular changes which occurred. However, the possibility of there being a stimulatory α-adrenergic input on intrinsic NPY-containing neurones can not be excluded.

In contrast, the infusion of the α-adreceptor antagonist phentolamine had a latent stimulatory effect on NPY secretion, suggesting an α-adrenergic inhibitory input on NPY-IR neurones (Fig.22). The delay in the onset of its action could be due to the displacement of endogenous NA bound to the α-receptors. This study is consistent with the literature showing an inhibitory action of NA on NPY release mediated via α2-adrenoceptors (Wahlestedt et al., 1986). It has been shown that there is a several fold larger release of NPY-IR relative to NA in isolated mesenteric arteries in vitro, upon stimulation with irregular frequencies at a high rate compared to a low continuous frequency (Lundberg et al., 1986b), therefore the α2-mediated inhibition of NPY release is thought to be important during conditions of low sympathetic activity (Dahlof et al., 1986). The interaction between NA and NPY was thought to largely
depend on whether they are colocalized or distributed in separate neurones (Wiley et al., 1987). The inconclusive results may therefore be partly explained by the dual localization of NPY in intrinsic and extrinsic neurones. It is possible that the action of NA on NPY release may also depend on the relative density of α- and β-adrenoceptors in a particular region. The relatively greater density of α-adrenergic receptors compared to β-adrenoceptors in a tissue, the more likely that NA would have an inhibitory effect on NPY release.

The β-adrenergic mechanisms involved in NPY secretion were next investigated. The β-adrenergic blocker propranolol produced an initial increase in mean basal levels of NPY followed by a reduction (Fig.23). These changes in NPY release were not statistically significant and further studies are therefore required to confirm these changes. The increase in perfusion pressure which was observed may be due to the blockade of the vasodilatory β-adrenoceptors by propranolol, which then results in the unmasking of the α-adrenoceptor-mediated vasoconstrictive effect of endogenous NA.

The effect of the β-adrenergic agonist isoproterenol was also investigated to further elucidate any β-adrenergic mediated effects on NPY release. Isoproterenol had a stimulatory effect on NPY secretion throughout the infusion (Fig.24) which suggests a β-adrenergic stimulatory action. There was a transient reduction in perfusion pressure which may be due to the vasodilatory action of the β-adrenergic agonist on the stomach vasculature.

The adrenergic regulation of NPY release is probably mediated via actions on intrinsic neurones and extrinsic nerve terminals containing NPY originating in the coeliac ganglion, which has been shown to stain for NPY and supply the sympathetic innervation of the stomach (Lee et al., 1985).

NPY may have different functions in the GI tract depending on whether the peptide is localized in nonadrenergic intrinsic neurones or extrinsic adrenergic nerve terminals. In the nonadrenergic intrinsic neurones NPY may have an action on gut motility. The cholinergically-stimulated NPY release from these neurones could possibly have a role in fine regulating the degree of motility in the GI tract. NPY has been shown to inhibit smooth muscle contraction
resulting from electrical stimulation (Holzer et al., 1987; Allen et al., 1987a; Lundberg et al., 1982a). NPY has also been shown to inhibit the ascending enteric reflex contraction of the circular muscle in the guinea pig small intestine; both the cholinergic and non-cholinergic components of the ascending enteric reflex were suppressed (Holzer et al., 1987). Therefore, the cholinergically-stimulated release of NPY may have a negative feedback on the neurally-mediated excitatory cholinergic mechanism involved in motility in the GI tract.

The NPY present in the adrenergic extrinsic nerve terminals may interact with NA and have a role in blood flow regulation in the GI tract. The major actions of NA on the vasculature are generally considered to be mediated via \( \alpha \)-receptors. Therefore the stimulation of NPY release through \( \alpha \)-adrenoceptors inputs of NA may be desirable, since this would allow a prolonged amplification of the catecholamines vasoconstrictive action. NPY has been shown to have a long-lasting potentiative effect on the vasoconstrictive action of NA (Wahlestedt et al., 1986), as well as a direct vasoconstrictive effect which has been shown to be 50 times more potent than NA (Lundberg et al., 1986b).

In conclusion, the present results suggest that there are adrenergic, cholinergic and possibly non-cholinergic mechanisms of regulation of NPY secretion. The cholinergic stimulatory mechanisms are partially mediated via nicotinic receptor stimulation at the level of the intrinsic ganglia. The adrenergic effects on NPY release may depend on the relative number of \( \alpha \)- and \( \beta \)-adrenoceptors in a particular tissue. The \( \alpha \)-adrenoceptor has an inhibitory and/or stimulatory effect, while preliminary studies suggest that the \( \beta \)-adrenoceptor has a stimulatory effect on NPY release.

In order to elucidate the regulatory pathways involved in NPY secretion, further release studies are required. The relative contribution of muscarinic and nicotinic receptor stimulation on NPY release could be determined by the simultaneous infusion of hexamethonium and atropine on the ACh-stimulated and basal NPY secretion. The inconclusive results on the \( \alpha \)-adrenoceptor effect on NPY secretion may be clarified by determining the effect of NA on the basal release of the peptide. The action of \( \alpha_2 \)-adrenoceptor antagonists on the effects of NA and
phenylephrine on NPY release may also be another useful avenue of investigation. In order to confirm the β-adrenergic stimulatory action on NPY release, the effects of β₁ and β₂-adrenoceptor antagonists on basal and NA-stimulated levels of NPY should be studied. Extrinsic sympathetic denervation of the stomach, or the depletion of NA in the adrenergic nerve fibers using 6-OHDA and reserpine could also provide clues to the regulatory mechanisms involved in the release of this major gut peptide.
REFERENCES


APPENDIX

Table VI: Representative raw data of the effect of isoproterenol (15µM) on basal NPY release. The NPY-IR is expressed in fmole/min.

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