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Pancreatic Polypeptide Increases Gastric Activity Through A Vagal Dependent Mechanism

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Dana Marie McTigue, M.S.

*****

The Ohio State University

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Dissertation Committee:

Richard C. Rogers
Robert L. Stephens, Jr.
Jackie D. Wood
William B. Zipf
Ian L. Taylor

Approved by

Advisor

Department of Physiology
To my Parents, Joan and Ronald, whose love and unshakable belief in me have been constant in everything I have chosen to do. They invariably fostered my curiosity and always encouraged me to find answers to my seemingly endless questions.

And to my husband, Phil. I really cannot describe how thankful I am for his constant love and unbelievable support for me. I know I would not be where I am today without him in my life.
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VITA

August 20, 1966 ......................... Born -- Philipsburg, Pennsylvania

1989 .......................................... B.S., Pennsylvania State University, University Park, Pennsylvania

1993 .......................................... M.S., Ohio State University, Columbus, Ohio

PUBLICATIONS


ABSTRACTS


FIELDS OF STUDY

Major Field: Physiology

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CHAPTER I

INTRODUCTION

For most healthy individuals, the concern for what actually happens to their food once swallowed is absent from their daily routine. They simply enjoy the taste of the food and then go about their business once their meal is completed. Fortunately, neural circuitry within the gastrointestinal (GI) tract and the central nervous system (CNS) can take over the processes of digestion, absorption of nutrients, and removal of ingested toxins and waste products. Assuming everything runs properly, the control of digestive activity is not brought to the attention of the individual. Typically, the central nervous system and the GI tract work in conjunction to regulate GI activity. This report will focus mainly on regulation of digestive functions by the CNS. Specifically, modulation of gastric-related vagal reflexes by the hormone pancreatic polypeptide will be addressed.

Organization of Gastrointestinal Reflexes

Digestive processes within the gastrointestinal (GI) tract are controlled through several hierarchical mechanisms. The first level of organization begins with
the enteric nervous system (ENS) which consists of interconnected ganglia within the submucosa and between the circular and longitudinal muscle layers of the gut (178). The ENS is fully capable of initiating and modulating several reflexes within the GI tract (178). For instance, in the extrinsically denervated GI tract, enteric neurons generate functional migrating myoelectric complexes. The enteric nervous system receives substantial influence, however, from vago-vagal parasympathetic reflexes. Vagal reflexes represent the next level of hierarchy in the overall coordination of digestive processes. In turn, vagal reflex activity can be modified to a great degree by other central structures such as the cortex and hypothalamus (8,26,135,172). In the following sections, the anatomy, physiology, and modulation of vago-vagal reflexes will be addressed.

**Anatomy of Vago-Vagal Reflexes**

A typical gastrointestinal vago-vagal reflex consists of three basic components: an afferent limb, an interneuronal relay in the nucleus tractus solitarius (NTS), and an efferent limb (139). The afferent limb transmits a compilation of sensory signals from the GI tract to the brainstem NTS. The NTS, after integrating the sensory data, projects to the subjacent dorsal motor nucleus (DMN) which then provides the efferent fibers innervating ganglia in the ENS.

**Vagal Afferents.** The subdiphragmatic vagus nerve contains 50,000 fibers. Of these, 45,000 are afferents (56). Clearly there is a vast amount of sensory data
relayed to the CNS concerning the status of the gut. Vagal afferent innervation of the
GI tract extends from the esophagus, stomach, small intestine, pancreas, and initial
segments of the colon (6,13,105,120,140). Thus, the state of the entire digestive
apparatus is continually monitored by vagal afferent fibers and the information
relayed to the brainstem.

Afferent Receptors. The sensory information transmitted to the CNS mainly
consists of mechano- and chemoreceptor signals (56,105,120). Through these
receptors, the brain is continually informed of the magnitude and duration of muscle
contractions and also the chemical content in the lumen of the gut. Also found within
the gut are thermoreceptors which are important for coordination of whole body
temperature (105). The anatomical structure of the sensory receptors within the GI
tract has been difficult to determine (96,140). Only a few encapsulated endings
similar to Pacinian corpuscles have been described (105). However, it is commonly
thought that the free nerve endings of the afferent fibers serve as sensory receptors
(96,105,140,143).

Vagal sensory endings innervate both the mucosal and muscle layers of the
GI tract (96,105,120,143). Mucosal receptors are typically polymodal responding to
mechanical deformation of the mucosa and also to chemical stimulants within the
lumen (96,120,140). These receptors have been described as "rapidly adapting
mechanoreceptors that act also as slowly adapting chemoreceptors" (140). The
mucosal receptors thereby inform the brain of the local luminal environment.
Afferent endings within the musculature are referred to as tension or stretch receptors given that they may respond to distension and contraction of the smooth muscle (56,96,120,140). Within the stomach, antral stretch receptors are very abundant and respond to both distension and peristaltic contractions (56,140). Stretch receptors in the proximal stomach, however, are activated only by changes in volume, which is not surprising due to the reservoir function of this region (56,140). Thus, mechanoreceptors located throughout the GI tract may be responsive to different types of stimuli corresponding to each specific region.

**Nodose Ganglia.** Afferent fibers within each vagus originate from cell bodies located in the nodose ganglia (71,72,81). These cells are typical sensory neurons in that they are bipolar having a peripheral branch to the gut and central branch projecting to the brainstem. Several different neurochemicals have been identified in nodose neural cells and vagal afferent fibers including substance P, calcitonin gene-related peptide, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), somatostatin (SST), and galanin (20,36,96). Unfortunately, the specific function of these neurochemicals in the transmission of sensory information to the CNS is largely unknown. Given that the vagus not only innervates the subdiaphragmatic organs but also those within the thoracic cavity, it should not be assumed that the potential neurotransmitters found in the nodose are specifically related to gastrointestinal activity.
Central Distribution of Vagal Afferents. The central projections of vagal afferent fibers have been studied using a variety of neuronal tracing techniques. These techniques demonstrated that the afferent fibers enter the dorsolateral brainstem, travel through the medulla in the tractus solitarius (71,72) and eventually terminate in the area postrema (AP) and NTS (71,72,81,130). If one branch of the vagus nerve is labeled unilaterally with a neuronal tracer, afferent terminals can be visualized throughout the area postrema suggesting that this structure is the recipient of extensive bilateral vagal sensory information (71,72). Likewise, labeling of one branch of the vagus results in very dense labeling of the ipsilateral NTS and lighter, yet prominent, labeling of the contralateral NTS (71,72). Afferent terminations from different digestive organs exhibit a significant degree of overlap within the NTS (114). This overlapping pattern suggests the NTS is involved in complex organization and integration of the sensory status of the entire gut.

In addition to the NTS and AP, some regions of the DMN also appear to be innervated by vagal afferent fibers. For instance, following application of a neuronal tracer to the vagus, labeled afferent terminals can be visualized within the DMN (19). Even more notable, several investigators have demonstrated that dendrites from DMN neurons project out of the nuclear boundaries and into the NTS and AP -- regions of dense vagal afferent innervation (6,102,130,131). Thus, the motor neurons of the vagus are in a position to be directly affected by vagal afferent fibers. Indeed, Rinamin et al. (130) demonstrated a gastric monosynaptic circuit using electron
microscopy. Analysis of the subnucleus gelatinosus of the NTS revealed synaptic contacts between vagal afferent terminals and dendrites of gastric-projecting DMN neurons. The physiological significance of this potential reflex pathway, however, has not been determined.

**Nucleus Tractus Solitarius.** The dorsal vagal complex (DVC), consisting of the NTS and DMN, is the site for integration of visceral sensory information and organization of vagal reflexes which in turn innervate the abdominal organs. The NTS receives a vast amount of information not only from the GI tract and the thoracic organs but also from higher brain structures such as the cortex and amygdala (82,129,137). This nucleus, therefore, must organize all this incoming data and then activate the proper efferent pathways necessary for maintaining appropriate activity within the viscera. The primary source of efferent pathways utilized for coordinating GI activity is the DMN (12-14,19,38,75,81). Some efferent fibers may also derive from nucleus ambiguus (NA) neurons (81,82).

Besides projecting to the DMN and NA (9,19), the NTS also projects to several other CNS structures (9,19,82). For instance, anatomical studies have revealed direct projections to the parabrachial nucleus, ventral posteromedial thalamus, amygdala, bed nucleus of the stria terminalis, hypothalamus, and cortex (9,26,29,64,142,172). Also receiving afferents from NTS is the contralateral ventral horn in cervical, thoracic, and lumbar spinal cord and areas of the ventral medulla involved in cardiovascular regulation (19). Thus, the NTS coordinates all functions
which depend on visceral afferent input and therefore is truly an overall integrating center for visceral information.

**Suspected Modes of Neurotransmission within the DVC.** Although the importance of vagal innervation to the GI tract is not debated, the chemical nature of these reflex pathways is not completely understood. For instance, a wide variety of neurotransmitters are known to be present in the nodose ganglia (see above). Which transmitters relay specific GI information, however, is not known. McCann and Rogers examined this problem in a recent report (101). When the stomach is distended, neurons in the NTS referred to as "ON cells" are activated by vagal afferents; the excitatory transmitter released from afferents onto NTS neurons is thought to be glutamate. McCann and Rogers demonstrated that if a glutamate antagonist is injected into the NTS, the neuronal activation induced by gastric inflation is reduced or eliminated indicating that glutamate may be important in the signal transduction from gastric sensory fibers to the NTS (101).

Neuropharmacologic studies have revealed that a wide variety of transmitters are present in the NTS (19,80). These include norepinephrine (NE) and dopamine, glutamate, g-aminobutyric acid (GABA), substance P, and enkephalin (19,80). Thus, both classical excitatory and inhibitory transmitters are produced within the NTS. However, the transmitters important in gastric reflexes are presently unknown. For example, gastric distension activates a vagal reflex resulting in activation of NTS neurons (ON cells) which in turn inhibit cholinergic DMN neurons (OFF cells).
(This is part of the accommodation reflex which will be described in more detail later.) While the inhibitory transmitter from the NTS remains unknown, two lines of evidence suggested it may be adrenergic. First, NE is found within NTS neurons (80), and second, adrenergic axons have been shown to form symmetrical synapses on dendrites from DMN cells that project to the stomach (155). Thus, a plausible hypothesis might be that the inhibitory NTS-DMN synapse may involve release of NE. What has been shown, however, is that neither alpha nor beta antagonists blocked the inhibition of DMN cells induced by gastric inflation (101). Thus, the transmitter utilized by NTS in this reflex probably is not epinephrine or norepinephrine and presently remains unknown.

**Vagal Efferents.** Vagal efferent innervation of the GI tract includes fibers from the NA and the DMN. The main digestive-related target for NA innervation is the esophagus, although some fibers may also innervate the abdominal viscera (140). The DMN provides vagal efferent innervation to all other regions of the gastrointestinal tract (6,12-14,38,75,81,140). In a thorough study by Berthoud et al. (13), the fluorescent tracer Dil was used to label vagal efferent fibers. Their study revealed that myenteric ganglia in the stomach, small intestine, and entire colon through the descending segment receive vagal efferent fibers and terminals. The densest efferent vagal innervation was observed in the stomach and proximal duodenum compared to the remaining GI tract. Interestingly, very few to no efferent
vagal fibers were seen in the submucosa and mucosa suggesting that vagal influence over the GI tract is transduced mainly via synapsing within the myenteric ganglia.

The anatomical details of vagal innervation to most viscera are fairly straightforward. However, the exact nature of vagal input to the pancreas has been the topic of debate for several years. Research had been impeded by a lack of reliable neuronal tracers and also by the "leakiness" of the pancreas following injection of dye. With the technical improvements over the last ten years, it has been demonstrated that the pancreas does receive some direct vagal innervation (112,131,152). Afferent fibers from the vagus innervate approximately 10% of the pancreatic islets (112,152) whereas vagal efferent fibers innervate about 8% of ganglia within the pancreas (14). Further, no efferent fibers were identified in either the islets or exocrine tissue (14). Thus, it appears that direct vagal innervation to the pancreas is relatively sparse and consists of sensory innervation of the islets and efferent terminals within the ganglia. Interestingly, Kirchgessner and Gershon (76) demonstrated that serotonergic and cholinergic myenteric neurons within the stomach and duodenum project to the pancreatic ganglia, islets, acini, and ducts. In addition, application of veratridine to the duodenal lumen increased cytochrome oxidase, an indicator of cellular activity, within these pancreatic tissues suggesting that neural pathways from the stomach and duodenum may be capable of altering the intrinsic pancreatic neural and parenchymal tissue activity (76). These data indicate an extensive entero-pancreatic innervation and provide the basis for the theory that
vagal input to the stomach and duodenum may indirectly affect pancreatic functions through these local connections.

**Peripheral Target of Vagal Efferents.** In the classical description of parasympathetic efferent pathways, preganglionic fibers from the DMN are cholinergic and synapse on nicotinic receptors on postganglionic cholinergic enteric neurons. These enteric neurons then synapse at muscarinic receptors on the target tissue. For the most part, this description holds true. However, transmitters other than acetylcholine (ACh) are located within both the DMN and within the ENS. For instance, galanin, glutamate, VIP, substance P, and enkephalin have been detected in the DMN (89,140,150). Besides ACh containing neurons within the ENS, vagal efferents also innervate VIP- and serotonin- (5HT) containing enteric neurons (75).

Not surprisingly, not all enteric neurons are excitatory. Thus, inhibitory vagal reflexes also exist in which cholinergic vagal fibers synapse on inhibitory enteric neurons. This pathway was demonstrated in 1959 by Harper et al. (59) who showed that stimulation of the vagal trunk resulted in a small increase in gastric contractions superimposed on a reduction in gastric tone. If the vagus is stimulated during a background of atropine, only the inhibitory effects are observed (140). The neurotransmitter utilized by this inhibitory pathway is known to be non-cholinergic and non-adrenergic (NANC) (140). While a definitive identification has yet to be provided, transmitters such as VIP and, more recently, nitric oxide have been suggested (89,107).
Viscerotopy in NTS and DMN. Several studies have focused on a possible viscerotopic organization within the NTS and DMN (6,12,43,81). What has been determined is that the DMN is discretely organized into longitudinal columns with the gastric branches originating from the medial two-thirds of the nucleus and the celiac branches corresponding to the lateral third; the hepatic branch derives from a few neurons scattered throughout the left medial DMN (43,114). The results of these studies suggest that within the DMN, a viscerotopic organization is detectable with the proximal GI tract, e.g., the stomach and duodenum, represented medially and caudal viscera represented more laterally (6,7,43,114).

Similar to vagal efferents, sensory fibers from the five branches of the vagus innervate the NTS in a loose viscerotopic array (114). Compared to the DMN, the viscerotopic arrangement within the NTS is not as rigid (114). For instance, the terminal field for the gastric afferent branches is centered on the medial "gastric region" of the NTS (114). However, some afferent terminations from the celiac branches also are found in the medial NTS implicating that integration of sensory information from several digestive organs may occur at this level.

Examples of Gastric Vago-Vagal Reflexes

As stated above, vago-vagal reflexes are important for coordinated activity along the entire GI tract. Many of the best understood reflexes involve control of the
Receptive Relaxation. When deglutition occurs, the stomach is "forewarned" in order to prepare for entrance of the food. This is accomplished through the receptive relaxation reflex. Swallowing or esophageal distension initiates this reflex which produces relaxation of the proximal stomach (108, 140). Given that the stomach is under tonic excitatory input resulting in a baseline level of gastric tone, this reflex is important for allowing an increase in volume with minimal increase in intraluminal pressure. Vagotomy eliminates this gastric relaxation indicating that receptive relaxation occurs via a vago-vagal reflex (140). The reflex relaxation is an active process in that vagal afferents stimulated by esophageal distension activate inhibitory NANC vagal efferent pathways; this inhibitory reflex is accompanied also by disfacilitation of excitatory vagal efferents projecting to the proximal stomach (108, 140).

Accommodation. In 1973, Abrahamsson demonstrated in cats that distension of the antrum caused pronounced relaxation in the proximal stomach (1). He also showed that the reflex was not blocked by atropine, adrenergic antagonists, or spinal cord transection, but was eliminated by vagotomy. It has been shown since that slowly adapting antral tension receptors activated either by antral distension or strong antral contractions stimulate a vago-vagal reflex causing relaxation of the proximal stomach (1, 2, 140, 149). The degree of corporal inhibition is related to the volume of
distension of the antrum (149). Thus, if a large amount of food has entered the antrum, the proximal stomach will undergo significant relaxation to allow for retropulsion of the digesta. This accommodation reflex is due to both removal of excitatory vagal input and, even more so, stimulation of vagal efferents projecting to NANC postganglionic neurons in the proximal stomach (140). Spinal sympathetic reflexes also may play a role in inhibiting cholinergic transmission to the stomach (2). This reflex takes over the job of maintaining low intragastric pressure once swallowing has ceased.

**Antro-Antral Reflex.** Aside from accommodation, the antrum also participates in excitatory vagal reflexes in which distension of the antrum can stimulate antral contractions. This effect is diminished following vagotomy and is completely eliminated by atropine suggesting that both local enteric and long vago-vagal cholinergic pathways are involved (57). Incidentally, distension of the corporal region of the stomach also may lead to reflex contraction of the antrum (149). Stretch receptors in the corpus activate a vago-vagal reflex which can directly stimulate antral motility. In this reflex, vagal efferents play a permissive role by augmenting local reflex action (149). This coporo-antral reflex is thought to be important in gastric emptying (149).

**Distension-Induced Acid Secretion.** Not only can antral distension cause an increase in antral contractility, this stimulus also will stimulate acid release from the proximal stomach through a vagal-dependent mechanism (32,63,171). This action is
not mediated via gastrin release since distension with an acidic solution inhibits gastrin secretion but still produces the acid response (32,63). In addition to antral-induced acid release, fundic distension also stimulates acid secretion through a vagal-dependent mechanism (55). These gastro-gastric reflexes thereby enhance the efficiency of gastric digestion by ensuring acid release throughout the duration of food-induced gastric distension.

**Distension-Induced Pancreatic Secretion.** Proper regulation of pancreatic enzyme and bicarbonate release is absolutely essential for normal digestion to occur. Although to a large extent the control of exocrine secretion involves hormonal regulators, the vagus nerves also can have potent effects on exocrine secretion (59,111). It was determined in the 1960's that antral distension stimulates a vago-vagal cholinergic reflex which produces the observed elevation in exocrine secretion (7,16). Gastric distension and secretin may have synergistic effects on pancreatic protein release (171). A previous study demonstrated that minimal distension of the stomach did not cause enzyme release, but the same distension during a subthreshold dose of secretin potently stimulated exocrine secretion. Through these pathways, pancreatic enzyme release may be stimulated during the gastric phase of digestion in preparation for movement of the food into the duodenum.

**Enterogastric Reflex.** It has been known for almost a century that either acidification or distension of the duodenum results in reflex relaxation of the
stomach; this has been termed the enterogastric reflex (17,167). Mucosal receptors in the duodenum responding to chemical or mechanical stimuli, e.g., acid or mucosal deformation, probably produce the afferent signal for this reflex (140). The vagal mechanisms involved include reducing the firing rate of vagal excitatory fibers and stimulating NANC pathways innervating the antrum (31,108,140). This reflex may function as a way in which gastric emptying is regulated around the amount and type of digesta entering the duodenum (140). The end result of the reflex is a reduction in gastric emptying following entry of chyme into the duodenum.

Central Modulation of Vagal Reflex Activity:

Input from Other Brain Structures

Although vagal reflexes provide the basic circuitry required for coordinated digestive activity, other CNS structures may modify vagal activity leading to altered GI behavior. For instance, it was obvious to investigators such as Pavlov (124), Beaumont (110), and Cannon (21) in the nineteenth and early twentieth centuries that the emotional state of an individual can have a profound influence on gastrointestinal tract activity. Cannon noted that events provoking hostility are associated with a dramatic increase in gastric motility and acid secretion (21). In addition, it was during this era that the well-known experiments by Pavlov demonstrated that the central nervous system clearly alters digestive activities in anticipation of feeding (124). However, identification of the neural pathways responsible for these early
observations did not begin until the late twentieth century. Neuroanatomical tracing techniques revealed that the DVC receives significant afferent input from several structures which may be involved in coordinating gastrointestinal activity with ongoing activity in the rest of the body (137).

**Cerebral Cortex.** Data from the 1950's revealed that the cerebral cortex can exert an inhibitory influence over gastrointestinal activity (8,153). More recent anatomical studies revealed a direct projection from the medial and insular cortex to the DVC (67,166,172). These areas of cortex are appropriately known as the "visceral sensory and motor" cortex (166). Given that this cortical region also is considered part of the limbic system (166), this corticobulbar pathway may be responsible for the effect of perceived emotions and threats on digestive tract activity.

**Amygdala and Bed Nucleus of the Stria Terminalis.** The central nucleus of the amygdala (CNA) and bed nucleus of the stria terminalis (BNST) are intimately involved in limbic activity. These forebrain regions form a continuous band of "pre-vagal" neurons that have reciprocal projections with the DVC (29,129,145,146,172). These nuclei, therefore, are most likely involved in coordination of emotional events with autonomic functions. Electrophysiological experiments have revealed that stimulation of the CNA influences the activity of NTS and DMN neurons (28,133). Our laboratory has demonstrated that somatostatin (SST) may be the transmitter involved in CNA influence over DVC activity.
Stimulation of the CNA or microinjection of SST into the DVC produces an augmentation of gastric acid secretion and motility (137). Furthermore, if a SST antagonist is microinjected into the DVC, CNA stimulation no longer produces an elevation in gastric activities (137). These data suggest that stimulation of the CNA, perhaps by emotional or stressful situations, leads to augmented gastric activity due to the CNA-DVC SST-ergic pathway.

The BNST also is involved in control of gastric functions. Stimulation of the BNST either electrically or with glutamate results in an atropine-sensitive elevation of gastric motility, probably as a result of the BNST-DVC projection (61). Although several neuropeptides have been located in the BNST, including SST, enkephalin, and corticotrophin releasing factor, the neurotransmitter involved in the BNST projection to the DVC is not known (53,137).

**Hypothalamus.** Several lines of evidence indicate that this structure has substantial influence over DVC activity. Anatomical studies revealed a direct projection from the paraventricular nucleus (PVN) to the NTS and DMN (26,79,129,142,144). Our laboratory also has demonstrated electrophysiologically the existence of an afferent projection from the PVN to the NTS (135). Within this pathway, several putative neurotransmitters have been identified including vasopressin, angiotensin II, neurotensin, and oxytocin (OT) (36,144,157).

A function of this PVN-DVC projection may be to modulate vagal influence over gastric functions. Electrophysiological experiments have determined that PVN
stimulation can bias gastric-related NTS neurons such that they are more responsive to incoming sensory signals (135). Studies also have revealed that stimulation of the PVN will result in augmented gastric acid secretion and increased gastric motility superimposed on a reduction of circular muscle tone (137). Microinjection of OT, a candidate peptide transmitter for the PVN-DVC pathway, into the DVC produces an elevation of acid secretion and a reduction in gastric tone (134,136). Thus, OT may be used by the PVN fibers to produce several of the events observed following stimulation of this nucleus. In support of this, if an OT antagonist is injected into the DVC prior to PVN stimulation, the increased acid output and reduced gastric tone are eliminated (136). Furthermore, in vivo and in vitro studies demonstrated that DVC neurons are excited by microinjection of OT (22,99). Given that OT activates gastric-related DVC neurons, the explanation for the observed gastric effects produced by this peptide may entail stimulation of cholinergic pathways responsible for acid secretion and also stimulation of NANC pathways causing a reduction in gastric tone. That the central nervous system is capable of such precise control is quite remarkable. These OT pathways to the DVC may be important in cephalic phase events since both increased acid secretion and decreased tone are observed within the stomach during this early stage of digestion (140).

Given the position of the PVN, CNA, BNST, and cortex in overall coordination of physiological events in the body, it seems quite reasonable that they should be involved in keeping the digestive tract "in tune" with the rest of the body.
These forebrain regions receive significant sensory and emotional information about the state of the individual and therefore need to integrate the data and make executive decisions concerning the appropriate recourse. Thus, these structures provide another layer of hierarchy in the control of digestive activities.

**Raphe Nuclei.** The raphe nuclei, as their name implies, are midline structures in the brainstem. One of these nuclei, the nucleus raphe obscurus (nRO), is emerging as an important regulator of autonomic functions, including gastric activities (159,160). Neurotransmitters found within this nucleus include serotonin (5HT) and thyrotropin releasing hormone (TRH) (106,160,170). As such, both of these transmitters probably are involved in the modulatory role the raphe plays on gastric functions. Fibers containing TRH and 5HT have been identified projecting from the raphe into the DVC (18,106,121,169). Also, specific receptors for each of these transmitters are found on NTS and DMN neurons (91,92,168). Several studies have revealed that injection of TRH into the DVC causes potent long-lasting stimulation of gastric acid secretion and motility through a vagal cholinergic mechanism (49,65,69,134,136). In addition, electrophysiological studies from our laboratory revealed an interesting pattern of activation by TRH on the DVC cells (98). Application of TRH onto NTS neurons resulted in 50% being inhibited while 50% were unaffected. In contrast, 50% of DMN neurons were activated by TRH with the other half being unaffected. These results suggest that TRH may induce long-lasting elevations in gastric activity by a simultaneous stimulation of vagal
cholinergic efferent fibers and suppression of the afferent limb of the reflex (98). It is interesting to note that while both TRH and OT lead to elevated gastric acid secretion, the actions of these two peptides on DVC neurons differ.

Serotonin also may be important in raphe-induced alterations in gastric behavior. Studies from this laboratory revealed an interesting relationship between 5HT and TRH -- these transmitters appear to potentiate the action of one another and thereby lead to potent stimulation of gastric acid secretion and motility (97,104). Given that the nRO has been implicated in the production of stress ulcers and that TRH is the candidate peptide transducing this action, the ability to pharmacologically block this TRH effect would be important clinically. Unfortunately, TRH antagonists are not available. However, given the potent interaction between TRH and 5HT, it would be valuable to determine if a 5HT antagonist would be capable of inhibiting TRH action in the DVC. Hopefully, future studies will address this important and interesting interaction.

Central Modulation of Vagal Reflex Activity: Effect of Circulating Agents

As stated above, the dorsal vagal complex is influenced by a variety of structures within the central nervous system. In addition to these neuronal inputs, the DVC also can be affected by substances within the circulation, the extent of which was not appreciated until very recently. It is well known that the structure dorsal to the DVC, the area postrema, is a classical circumventricular organ. Within the area
postrema are fenestrated capillaries located within enlarged Virchow-Robins spaces. These capillaries have high diffusional permeability and therefore are said to be outside the blood-brain barrier. It was not realized until 1990 that the dorsomedial region of the NTS also contains fenestrated capillaries morphologically similar to those in the AP (54). Because of this special vasculature, the NTS may be exposed to circulating molecules.

Several anatomical studies have revealed a second mechanism whereby DVC neurons may be influenced by circulating agents. Dendrites from both NTS and DMN neurons enter the dorsomedial NTS, the AP, and even travel so far as the border of the fourth ventricle (130,138,151). Due to these dendritic projections, cells within the DVC may be capable of "sampling" the contents of both the circulation and the cerebrospinal fluid (CSF). What this means, of course, is that these nuclei may themselves be affected by agents such as circulating hormones or cytokines. Some examples of hormonal influences on the DVC are given below.

**Angiotensin II.** Studies examining the action of angiotensin II (Ang II) have indicated that some of the effects produced by this hormone may be due to a direct action on brainstem neurons (109). Receptors for Ang II are found in the AT, NTS, and DMN (36,158) and microinjections into this area produces altered heart rate and blood pressure (35,88). In addition, *in vitro* experiments have revealed a direct action of Ang II on DVC cells when applied during synaptic blockade (109). Although Ang
II is present in some neuronal pathways projecting to the DVC (36), circulating angiotensin also most likely reaches the DVC and thereby alters vagal tone to the cardiovascular system (94). Unfortunately, the results to date reveal controversy as to the exact role Ang II plays in the mediation of vagal reflexes.

**Somatostatin.** This gastrointestinal hormone/neurotransmitter is known to produce several effects such as inhibition of gastric and pancreatic secretion. Part of these effects may be transduced by a direct action within the brainstem. A recent study by Li and Owyang (83) demonstrated that, as previously shown, SST inhibited pancreatic enzyme release. However, while SST iv blocked exocrine secretion induced by 2-deoxy-glucose (2-DG), SST was not acting at vagal afferent or efferent terminals, at the level of muscarinic receptors within the pancreas, or via the sympathetic nervous system. Since 2-DG is a potent vagal stimulator, it was concluded that SST was acting within the brainstem DVC to inhibit enzyme secretion.

**Pancreatic Polypeptide.** Several lines of evidence indicate that pancreatic polypeptide (PP) may be capable of altering vagal tone to the GI tract and thereby affect visceral activity. Thus, PP may function as an "endoneurocrine" agent given that it may directly alter neuronal activity. The details of the hormone are provided in the next section.
Pancreatic Polypeptide

Although this hormone was initially identified in the 1960's, the function and site of action were not clearly understood until very recently. Several studies have demonstrated that pancreatic polypeptide (PP) produced a variety of actions on the GI tract. These effects, however, appeared to be indirect and possibly mediated through a neural mechanism (see Chap 3 and Chap 5). More recent data suggest that PP may alter GI activity by a direct action on DVC neurons in the brainstem. In this way, PP may be capable of altering vagal influence over digestive organs thereby affecting their activity. Thus, the following section will describe the necessary background information for this peptide and provide the logic behind the hypothesis that is the basis for this dissertation.

Background. In the late 1960's, Kimmel and colleagues noted that an unknown contaminant was persistently found in their insulin extractions from chicken pancreas (8). After seven years of disregarding the stable molecule, they decided to isolate this peptide and, since they had no idea what its function may be, gave it the lackluster name "Pancreatic Polypeptide" (73). They determined that the amount of PP within the pancreas was equal to or greater than the amount of extractable insulin and which led them to postulate that PP may function as a newly-found hormone (73). It was subsequently demonstrated in several mammalian species that specific PP-producing cells are located within the pancreatic islets and also spread throughout the pancreatic exocrine tissue (78).
Analysis of the amino acid sequence revealed that the carboxy-terminal tyrosine is amidated -- an indication that the peptide may be physiologically active (73). Tatemoto et al. used this information to investigate the possibility that other structurally related peptides may exist and were rewarded with the discovery of peptide YY (PYY) in 1980 (162) and neuropeptide Y (NPY) in 1982 (161), both of which contain an amidated C-terminal. These three peptides are said to comprise the "PP-fold" family (162). To date, no other members have been added to this peptide family.

**PP Release**

*Basal Secretion.* In the fasted state, PP release occurs at low levels (~20nM) which tend to fluctuate in synchrony with interdigestive pancreatic secretion and duodenal contractions (25). Application of atropine will reduce the basal level of PP in the circulation indicating that this release of PP is mainly driven by cholinergic tone over the pancreas (148). This PP release occurs in phase with the migrating myoelectric complex (MMC) which led some investigators to hypothesize that PP may be involved in regulation of the MMC. Studies have indicated since, however, that PP probably does not control interdigestive motility but simply is released as a consequence of neural activity driving the MMC (30).

*Postprandial Release.* Release of PP is potently stimulated by ingestion of a meal (46,163,164). Postprandial PP secretion follows a predictable pattern including
an early peak occurring at approximately 30 min and then a later plateau during which PP levels may be elevated as long as four hours (46,47,164).

As is true with many gastrointestinal functions, pancreatic secretion of PP begins during the cephalic phase of digestion (47,53,163). Cephalic phase events are stimulated by the thought, sight, smell and taste of appetizing of food. Different groups have demonstrated that sham feeding produces immediate (within 10 min) release of PP which can continue for an hour (39,53,163). This cephalic release is dependent entirely upon intact vagal cholinergic pathways (39,53,163).

Not surprisingly, PP release continues throughout the gastric phase of digestion (46,163,164). Both gastric distension and the presence of food in the stomach stimulate PP secretion (46,163,164). This gastric phase release can be blocked by cholinergic antagonists suggesting again that vagal cholinergic pathways are important in PP release (46).

PP secretion also occurs during the intestinal phase of digestion (47). Several studies have demonstrated that perfusion of the duodenum with a meal, an amino acid solution, or oleate significantly stimulates PP release (3,10,48). A thorough study by Fried et al. (47) clearly demonstrated that the late plateau of PP release seen following a meal correlates with the delivery of fats and protein to the duodenum. Studies have indicated that, similar to the earlier stages of PP release, cholinergic pathways are important for intestinal phase PP secretion (3) Thus, release of this
pancreatic hormone occurs promptly at the initiation of a meal and continues throughout the entire digestive process.

**CCK and PP Release.** As indicated above, vagal cholinergic pathways are essential for postprandial PP release. Other agents, however, may play a role in PP secretion. One potentially important regulator of PP release is the hormone cholecystokinin (CCK). Unfortunately, results from the CCK studies are not clear. While some investigators have demonstrated that intravenous administration of CCK or a CCK analog stimulates PP release (3,45), others have shown no apparent PP response to CCK (10). A study in which circulating levels of several hormones were correlated with emptying of various portions of a meal from the stomach demonstrated the well characterized pattern of PP release, i.e., an early peak at 30 min with a later plateau at 150-180 min (47). However, the pattern of CCK release in no way indicated that CCK was responsible for PP secretion. CCK levels did not peak until 60 min following ingestion and then began to decline so that at the time of the PP plateau, CCK levels had returned almost to baseline.

However, as with most physiological relationships, the one between CCK and PP is more complicated than first thought. Recent studies have indicated that CCK may function in conjunction with the cholinergic system. An interesting study by Hosotani et al. (66) used 2-DG, a well known central stimulator of vagal cholinergic pathways, to stimulate PP release. When 2-DG was applied concomitant with a CCK antagonist, PP release was reduced by ~60% even though 2-DG does not stimulate
CCK release. Other studies have demonstrated intestinal phase PP release was completely eliminated by administration of either atropine or loxiglumide, a CCK antagonist (3). Thus, an emerging hypothesis concerning PP release is that CCK may be acting in alliance with ACh either as a neurotransmitter or neuromodulator. Indeed, it has been demonstrated that nerve terminals within the pancreas contain CCK-4, the terminal tetrapeptide of CCK (128). Interestingly, Mawe et al. have demonstrated in the gall bladder that CCK acts presynaptically to release ACh from nerve terminals (95). Perhaps vagal pathways innervating the pancreas are also a target for CCK allowing both ACh and CCK to play a role in PP release.

Pancreatic or Antral Denervation and PP Release. The role of antral and pancreatic innervation in the regulation of PP release has been the subject of several studies (33,41,77,125,165). For instance, investigators have shown that denervation of the antrum or transection of the pylorus eliminates postprandial PP release (33,125,165). These results lead to the conclusion that vagal fibers innervating PP cells pass through the antral and pyloric region of the stomach before terminating within the pancreas. Indeed, this was demonstrated anatomically by Kirchgessner and Gershon (76). However, some studies in which the pancreas was denervated exhibited typical postprandial PP release (33,77). Although several hypotheses have been put forth to explain these discrepancies, including secretion of a PP releasing factor by the antrum, the exact mechanism for the vagal-dependent postprandial PP release has yet to be unambiguously identified.
Functions of PP

Pancreatic Exocrine Secretion. Although PP has been known to exist since the 1960's, the role this hormone plays during digestion is still speculative. The most thoroughly examined potential function of this peptide involves regulation of pancreatic exocrine secretion. Several studies have demonstrated that PP given intravenously potently inhibits both basal and stimulated pancreatic exocrine secretion (11,85-87,93,117,126). Agents used in these studies to stimulate pancreatic secretion included CCK, secretin, 2-DG, and intraduodenal instillation of amino acids. Following administration of one of these stimulators, PP was capable of significantly inhibiting release of both enzymes and bicarbonate from the pancreas (11,85-87,117,126). Although PP itself is released from the pancreas, the action of this hormone on its organ of origin appears to be through an indirect, most likely neural, mechanism. For example, vagotomy has been shown to abolish PP-induced inhibition of exocrine secretion (93). Interestingly, in rats pretreated with capsaicin to destroy vagal afferents, the inhibitory action of PP was unaffected suggesting that PP acts through the vagal efferent pathway (93). Further evidence was provided by Louie et al. who revealed that PP inhibits CCK-stimulated pancreatic enzyme release in intact rats (87). However, using in vitro preparations of pancreatic acini, PP no longer reduced protein release provoked by CCK nor did radiolabeled PP bind to the acini. This study indicates that receptors for PP are not located within the pancreas and, therefore, PP must alter pancreatic exocrine secretion via an indirect route.
Although a seemingly overwhelming body of evidence exists suggesting an indirect inhibitory action of PP on the pancreas, there are reports to the contrary. One such study using pancreatic slices provided data indicating that PP may presynaptically inhibit ACh release from efferent terminals within the pancreas thereby reducing enzyme release (70). Another study using bovine PP provided evidence suggesting that in isolated rat acini, PP acted as a muscarinic cholinergic antagonist (122). A third study even provided data indicating that human PP stimulated enzyme release from pancreatic acini in a dose-dependent, atropine-insensitive manner (37). Thus, even though an impressive amount of data has been generated concerning the effect of PP on pancreatic exocrine secretion, a definitive answer remains elusive.

**PP Effects on Gastrointestinal Tract.** Several investigators have examined the role PP may play in the regulation of gastrointestinal behavior. Studies from the late 1970's focusing on the effect of PP on gastric acid secretion produced conflicting results with one study showing inhibition of acid output while another study demonstrated no effect of PP (85,123). Using conscious pigs, Adrian et al. pursued the action of PP on the gall bladder (5). This group demonstrated that infusion of PP produced a dose-related inhibition of gall bladder pressure, again indicating an inhibitory action of PP on digestive organs. More recently, Davison and colleagues have examined the role of PP in control of the duodenum and colon (173). They revealed that an iv bolus of porcine PP into rats produced an increase in both
duodenal and colonic motility. This same group also demonstrated that an iv bolus of PP was capable of increasing pentagastrin-stimulated gastric acid secretion (174). However, the mechanism behind this PP-evoked acid secretion was not examined.

**PP Receptors**

Almost as soon as it was characterized, the hunt was on to determine a target for PP. The first demonstration of binding sites for PP, however, was not until the late 1980's. Gingerich and colleagues established that bovine PP bound to the basolateral surface of intestinal mucosa in dogs (50). They characterized the binding by demonstrating the necessity for an intact carboxy- and amino-terminal on the PP molecule (51). More recently, it was shown that both liver membranes and the adrenal gland exhibit binding sites for PP (113,177). These sites are specific in that the closely related peptides PYY and NPY did not inhibit PP binding (113,177). Interestingly, saturable binding sites for PP also have been identified in the dorsal medulla (176). Radiolabeled PP injected intravenously specifically bound to the area postrema and the dorsal vagal complex in rats. This binding was not prevented by NPY or PYY suggesting that specific receptors for PP exist on these neurons.

**Hypothesis**

To date, pancreatic polypeptide has been shown to have a variety of effects on the gastrointestinal tract. Although some of the data is contradictory, one fairly
clear point is that the effects of PP are indirect. Thus, PP appears to alter the neuronal input to the digestive tract resulting in modified activity. Given that specific receptors for PP are located in the DVC and that the vagus nerve is quite influential over gastrointestinal activity, binding to DVC neurons may be the mechanism whereby PP exerts its effects on digestive activity. Although PP is strictly an endocrine factor, i.e., PP is not detectable within the CNS (34), the fenestrated capillaries within the dorsal NTS may provide access to the DVC parenchyma (54). Further, the anatomical data demonstrating dendritic projections of NTS and DMN neurons into the regions of high vascular permeability argues that circulating PP may have access to these neurons.

Based on the data outlined above, a general hypothesis was developed concerning the action of PP on gastric functions. Namely, the hypothesis tested is that PP may indirectly affect gastric activities by altering vagal tone to the stomach. This may be accomplished by a direct action of PP on the DVC neurons. Several experimental models were designed to test this hypothesis including intravenous infusion of PP (Chap 2), direct brainstem injections of PP (Chap 3), and intracisternal injection of PP into conscious rats (Chap 4). These paradigms were used to determine if PP produces a change in gastric acid secretion, motility, or emptying. In addition, in vivo electrophysiological experiments were conducted to elucidate the action of PP on the neuronal activity of identified DVC neurons (Chap 5).
CHAPTER II

Pancreatic Polypeptide Stimulates Gastric Acid Secretion and Motility Through a Vagal Mechanism

Introduction

Pancreatic polypeptide (PP) is a 36 amino acid hormone produced by "F" cells within the pancreatic islets (73,162). Secretion of PP occurs during all stages of digestion and is dependent upon intact vagal cholinergic fibers (162,164). To date, observed effects of PP include inhibition of pancreatic exocrine secretion, inhibition of gall bladder motility, and altered gastric acid secretion (5,85,93,162). Results from these studies suggest that the action of PP on these organs is indirect and most likely mediated through a vagal pathway (87,93,126).

The exact mechanism of action for PP was rather difficult to interpret until recently. Although it was speculated that PP acts via a vagal pathway, its specific site of action was unknown. In 1990, a significant study by Whitcomb et al. demonstrated that specific receptors for PP exist within the dorsal vagal complex and the area postrema (176). Given that the dorsomedial region of the DVC contains fenestrated capillaries with high permeability (54), agents within the circulation, such
as PP, probably have direct access to these nuclei. Thus, PP may be capable of altering gastric functions by directly affecting the activity of the vagal nuclei which, in turn, may alter vagal tone to the stomach. To test this hypothesis, the effect of an intravenous infusion of rat PP (rPP) on vagal control of gastric acid secretion and motility was examined. Different routes of injections and pharmacological manipulation of vagal-dependent mechanisms were used to determine which segments of vago-vagal reflex circuitry PP may act upon. In light of contradictory results presented in previous reports concerning the effect of PP on gastric acid secretion (85,123), we examined the possibility that the conflicting data is due to species differences. For instance, several studies have used the bovine form of PP (bPP) in heterologous experimental models (85,123). Thus, included in the present study is a comparison of the effects of rPP and bPP on gastric acid secretion.

**Methods and Materials**

Following an overnight fast, male Long-Evans rats (210-350g) were anesthetized with urethane (1.5mg/kg, ip; Sigma, St. Louis, MO). The trachea was cannulated (PE-240) to ensure a patent airway and a cannula (PE-50) was inserted into the left jugular vein for peptide infusion.
**Gastric Acid Secretion**

**Intravenous Infusion.** To prevent reflux of gastric contents, the esophagus was ligated. The abdominal cavity was opened and the stomach was exposed. The pylorus was ligated with suture to inhibit stomach contents from draining into the duodenum. A double-lumen cannula was inserted into the stomach through a small incision in the forestomach and then sutured in place. Tubing from the gastric cannula exited through the abdominal incision which then was closed with suture. The animal was placed onto a heating pad and its temperature maintained at 36-37°C throughout the experiment.

The amount of gastric acid secreted was measured by flushing a 5 ml bolus of saline (0.9%, pH7) through the gastric cannula followed by a 5 ml bolus of air. The collected perfusate was autotitrated with 0.01 N NaOH to pH7 (Radiometer, Copenhagen). Baseline acid secretion was monitored in 15 min intervals until 3 consistent readings were recorded after which time infusion into the jugular vein was begun. Infusions consisted of varying doses of either rPP (Bachem California), bPP (Bachem California), or sterile saline (0.9%). Acid secretion was monitored in 15 min intervals for the next 120 min. The doses of rPP used for the infusion were 10 pmol, 50 pmol, or 100 pmol dissolved in a total volume of 0.9 ml sterile saline (n=5 per dose). Animals receiving bPP were given 100 pmol in 0.9 ml sterile saline (n=5) and control animals were infused with 0.9 ml sterile saline alone (n=5). The infusion
The system was calibrated to deliver 0.1 ml solution over 5 min thereby delivering the entire infusion volume over 45 min. Each animal received only one infusion.

**Vagotomy or Atropine.** To determine the reliance of any rPP-evoked changes in acid secretion on intact vagal cholinergic reflexes, a separate group of rats received either bilateral cervical vagotomy during the surgical preparation (n=3) or pretreatment with atropine methyl nitrate (0.2 mg/kg, ip; Sigma, St. Louis, MO; n=3) 15 min prior to the infusion. These animals were administered 100 pmol rPP and treated as above.

**Close Intra-arterial Infusion.** Although complete acute vagotomy reveals whether PP-induced actions depend on intact vago-vagal reflexes, the possibility of peripheral stimulation of vagal afferents by rPP is not eliminated. Thus, a separate group of rats received a close intra-arterial (ia) infusion of rPP into the gastric circulation to determine whether PP directly affects vagal afferents. This model has been used previously as a reliable method for exposure of gastric afferent fibers to infused agents. For example, investigators have clearly shown that cholecystokinin, a hormone known to act by binding to vagal afferent fibers (127,156), can activate vagal afferents when administered in a close ia infusion (15,147). Thus, the possibility of PP altering gastric acid output by stimulation off vagal afferent terminals located in the stomach may be studied using this model.

Each animal (n=7) was anesthetized as above following which the trachea was intubated and the esophagus ligated at the cervical level. The animal was
laparotomized and the spleen deflected and placed onto moist gauze. The splenic artery, following isolation from the vein, was cannulated using a 30-gauge needle attached to PE-10 tubing filled with heparin. The arterial cannula was secured in place and flushed with a small volume of heparin (<0.1ml). The position of the cannula in the artery was such that flow of the infusion was against arterial flow thus allowing the infusate to move from the splenic artery into the gastric circulation.

Next, the stomach was exposed, the pylorus was ligated and a cannula was placed into the stomach as above. The abdominal cavity was closed with suture; gastric and arterial cannulas exited through the incision. Once baseline acid secretion had stabilized, a 45 min close ia infusion of either sterile saline (0.9 ml) or rPP (100 pmol in 0.9 ml sterile saline) was begun and acid secretion was monitored in 15 min intervals for a total of 90 min.

**Intraperitoneal (ip) Infusion.** Although close ia infusion exposes gastric vagal afferents to rPP, the possibility exists that this peptide may act upon afferents located elsewhere along the gastrointestinal tract. By delivering an ip infusion, afferent fibers located throughout the abdominal cavity may be exposed to the peptide. This route of injection has been used previously by investigators to demonstrate that CCK increases *c-fos* expression in the brain (24,118,132). Since it is well known that CCK acts by altering vagal afferent activity (84,156), the previous studies suggest that CCK given ip is capable of reaching and binding to vagal afferent fibers. To examine whether rPP interacts with abdominal afferent fibers,
fasted rats were surgically prepared as described above with the exception that the infusion cannula was placed into the peritoneal cavity rather than the jugular vein. Once a stable acid baseline was established, a 45 min ip infusion of rPP was begun. Acid secretion was monitored for 90 min. The infused doses of rPP were 100 pmol and 1000 pmol (n=4 per dose). The higher dose was chosen to overcome dilution within the peritoneal cavity and also to increase the possibility of exposure of PP to afferent fibers.

_Gastric Motility_

Following laparotomy, a miniature strain gauge was sewn onto the antral serosa in an orientation such that circular muscle contractions would be detected. Motility signals from the strain gauge were amplified by a conventional Wheatstone bridge-based amplifier and the data displayed on a chart recorder (Gould 2600).

_Intravenous Infusion._ Following surgery, antral motility was monitored until a stable baseline was observed. Baseline motility was recorded for a minimum of 10 min followed by an infusion of either sterile saline (0.9%, pH 7; n=5) or rat PP (Bachem, California) into the jugular vein. Motility was monitored for 60 min. Doses of PP used were 2, 20, and 200 pmol (n=5 per group) dissolved in 0.9 ml sterile saline. The injection system was calibrated to deliver 0.1 ml solution per 5 min resulting in delivery of the entire dose of PP over 45 min. Each rat received a single infusion.
Vagotomy or Atropine. To determine if PP-induced changes in motility require intact vagal reflexes, a separate group of rats received either bilateral cervical vagotomy (n=4) or atropine methyl nitrate (0.2mg/kg, ip.; n=4) 10 min prior to peptide infusion. Experiments were carried out as above with each animal receiving an iv infusion of 200 pmol PP (the highest dose).

Close Intra-Arterial Infusion. As stated above, this paradigm may be used to determine the effect of rPP on gastric afferent fibers. Rats were anesthetized (n=6) and the splenic artery cannulated as described previously. A strain gauge was sewn onto the antrum and motility data collected until a stable baseline was observed. At the start of the infusion, the cannula was flushed with a small volume of heparin (<0.1ml) and then attached to a syringe containing either 0.9 ml sterile saline or 200 pmol rPP in 0.9 ml saline. The solution was infused as above and motility was monitored throughout the infusion.

Data Analysis

Acid Secretion. Acid secretory rates in each group were compared using a one-way analysis of variance (ANOVA). A post-hoc Dunnett t-test was used for the time course data and a Bonferroni test for the remaining data. Statistical significance was set at p<0.05.

Motility. Motility results were converted to numerical motility indexes (MI) using the method of Ormsbee and Bass (119). Briefly, the motility index assigns a numerical value to an epoch of strain gauge data according to the formula:
\[ MI = (n \times A_{1,2}) + 2(n \times A_{2,4}) + 4(n \times A_{4,8}) + 8(n \times A_{8,}) \]

where \( MI \) = motility per unit time (in this case, 5 min);

\((n \times A_{1,2})\) = number of contractions in the amplitude range from "just-detectable" to twice the just-detectable contraction;

\((n \times A_{2,4})\) = number of contractions in the amplitude range from 2 to 4 times the just-detectable contraction;

\((n \times A_{4,8})\) = number of contractions in the amplitude range from 4 to 8 times the just-detectable contraction;

\((n \times A_{8,})\) = all those contractions that are at least 8 times greater than the just-detectable contraction.

To reduce variability due to inter-rat differences in baseline motility, MI data were converted to difference scores by subtracting the baseline value from subsequent values in each record.

Data were compared statistically using an ANOVA. Post-hoc tests included a Dunnett \( t \)-test for comparing time course data following PP infusion with control values; Bonferroni multiple comparisons test to compare data from intact, vagotomized, and atropinized animals; and a Students \( t \)-test for close ia data (given that there were only two groups). Significance was set at \( p < 0.05 \).
Results

Gastric Acid Secretion

*Intravenous infusion.* Intravenous infusion of 100 pmol rat PP resulted in potent stimulation of gastric acid secretion [Fig 1]. Acid output rose significantly during the first 15 min of the PP infusion and peaked at 30 min when acid levels attained 950% of baseline (25 ± 7 mEq) (p<0.01). Acid secretory levels began to decline during the last 15 min of the infusion although remained significantly elevated over baseline for 60 min (p<0.01) [Fig 1]. The intermediate dose of rPP, 50 pmol, appears to be the threshold dose. Infusion of 50 pmol PP resulted in a slight but non-significant increase in acid secretion throughout the infusion [Fig 1]. Acid secretory levels were not affected by the lowest dose of PP, 10 pmol [Fig 1]. Infusion of rPP at doses greater than 100 pmol (e.g., 1 nmol or 5 nmol) produced no further incrementation in acid secretion (data not shown). Thus, 100 pmol rPP appears to produce a maximal response. Infusion of the saline vehicle did not alter gastric acid secretion [Fig 1].

*Vagotomy or Atropine.* Stimulated acid secretion due to infusion of 100 pmol rPP was abolished by pretreatment with atropine methyl nitrate or bilateral cervical vagotomy [Fig 2]. In this group of animals, the amount of acid secreted over 90 min was significantly lower than acid output in intact rats receiving an equivalent dose of rPP (p<0.001) [Fig 2].
FIGURE 1: Effect of 45 min infusion of rat PP into the jugular vein on gastric acid secretion. PP was administered at the indicated doses in a volume of 0.9 ml saline; vehicle infusion consisted of 0.9 ml saline alone (n=5 per group). Baseline for each rat prior to the infusion considered 100%. Subsequent values represent % baseline. Data are means ± SE; *p<0.05, **p<0.01 compared to vehicle.
FIGURE 2: Comparison of acid secretion in either intact rats or rats prepared with bilateral cervical vagotomy (vx; n=3) or pretreated with atropine methyl nitrate (atr; 0.1 mg/kg, ip; n=3). Following baseline acid collection (-30 to 0 min), animals received a 45 min infusion of 100 pmol rat PP (or vehicle) into the jugular vein beginning at time=0; acid secretion was monitored for a total of 90 min. Data are means ± SE for acid secreted over previous 30 min. ***p<0.001 compared to other groups.
Close Intra-Arterial or Intraperitoneal infusion. Close ia infusion of 100 pmol rPP into the gastric circulation had no effect on gastric acid secretion [Fig 3]. Acid output in this preparation was not different from that in animals receiving close ia infusion of saline and was significantly lower than that observed following iv PP (p < 0.001) [Fig 3]. Similarly, ip infusion of either 100 pmol or 1000 pmol rPP produced no change in acid secretion. The acid responses following ip infusions also were significantly lower than that produced by systemic infusion of rPP at 100 pmol (p < 0.001) [Fig 3].

Rat versus Bovine PP. Intravenous infusion of 100 pmol bovine PP resulted in a small but non-significant elevation of acid secretion [Fig 4]. When compared to an equimolar dose of rPP, the bovine PP response is delayed and significantly lower [Fig 4].

Gastric Motility

Intravenous Infusion. Intravenous infusion of rat PP produced a dose-dependent increase in antral motility [Fig 5]. The highest dose of rPP, 200 pmol, produced a significant increase in antral contractions within the first 10 min (p < 0.01) which remained significantly elevated throughout the infusion. The intermediate dose of rPP, 20 pmol, also significantly stimulated antral motility [Fig 5]. At this dose, contraction amplitude began to rise during the first 10 min, but did not reach significance until 20 min into the infusion. Rat PP given at the lowest dose, 2 pmol, produced a delayed non-significant increase in antral motility [Fig 5]. This
FIGURE 3: Comparison of acid secretion in animals receiving either an intravenous (iv) infusion of 100 pmol rat PP (rPP) or an intraperitoneal (ip) infusion of 100 or 1000 pmol (rPP), or a close intra-arterial (ia) infusion into the gastric circulation of either vehicle (veh) or 100 pmol rPP. Infusions began at time=0 and lasted 45 min. Data are means ± SE and represent acid output over previous 30 min. ***p<0.001 compared to other groups.
FIGURE 4: Comparison of acid secretion in response to 45 min jugular vein infusion of 100 pmol or either rat PP (rPP) or bovine PP (bPP) or saline vehicle (n=5 per group). Average baseline value per rat is considered 100% and all subsequent values are % over baseline. Data are means ± SE; *p<0.05, **p<0.01 compared to vehicle.
FIGURE 5: Dose dependent effect of intravenous infusion of rat PP on the amplitude of antral contractions. Rats were infused with indicated doses of PP or saline vehicle (n=5 per dose) beginning at time=0 and ending at time=45 (indicated by arrowheads). Motility records were divided into 5 min epochs and then converted to motility indexes. Data represent means ± SE; *p<0.05, **p<0.01 compared to control.
dose, therefore, appears to be a threshold dose. Control infusions of 0.9 ml sterile saline had no effect on motility [Fig 5].

**Vagotomy or Atropine.** Bilateral cervical vagotomy eliminated the motility response to 200 pmol rPP iv [Fig 6]. Similarly, rPP-induced motility was abolished by pretreatment with atropine methyl nitrate [Fig 6].

**Close ia Infusion.** Close ia infusion into the gastric circulation of 200 pmol rPP had no effect on antral motility. Likewise, sterile saline given close ia did not alter antral contractility [Fig 7].

**Discussion**

Results from the present study demonstrate that intravenous infusion of rat pancreatic polypeptide significantly stimulates gastric acid secretion and motility. The range of effective doses of rPP for acid stimulation was relatively sharp with no response at 10 pmol and maximal stimulation at a 10-fold higher dose. As for motility, effective doses also spanned only one order of magnitude, between 20 and 200 pmol. It is interesting to note that the threshold for acid secretion (50 pmol) was greater than that for motility (2 pmol), as has been described previously (40). Acid and motility responses to PP infusion were eliminated by pretreatment with peripheral atropine or bilateral cervical vagotomy suggesting that circulating PP requires intact vagal cholinergic pathways to stimulate gastric activity.

The different routes of peptide delivery utilized in this study allow us to make certain conclusions with regard to potential target sites for PP. Given that rPP
FIGURE 6: Effect of bilateral cervical vagotomy (n=4) or atropine methyl nitrate (n=4) on antral motility evoked by iv infusion of 200 pmol rat PP. PP infusion began at time=0 and ended at time=45. Motility data represent antral contractions over previous 15 min periods and are expressed as means ± SE. Both atropine and vagotomy abolished the PP-induced antral motility. *p<0.05, **p<0.01, ***p<0.001 compared to atropine and vagotomy data.
FIGURE 7: Effect on antral motility of a 45 min close intra-arterial (ia) infusion of sterile saline (n=3) or 200 pmol rat PP in sterile saline (n=3) beginning at time=0. Scale of Y-axis same as Fig 5 to aid visual comparison. Data represent means ± SE. Administration of PP through this route had no effect on antral motility as compared to saline controls. Thus, no values reached statistical significance.
infusion into the gastric circulation was without effect, it is unlikely that PP stimulates acid secretion or motility by activating vagal afferent fibers located within the stomach. Furthermore, ip infusion of PP was ineffectual. Thus, stimulation of vagal cholinergic reflexes is probably not mediated by binding of PP to afferents located within abdominal viscera. If PP were to function in this manner, one would predict that either ip or close ia infusion of PP into the gastric circulation would be effective in stimulating acid output or motility. In support of this, preliminary data from another laboratory also demonstrated a lack of effect of PP on acid secretion when given intraperitoneally (115). Acute cervical vagotomy eliminated the effect of PP deeming it unlikely that PP stimulates presynaptic release of acetylcholine from vagal efferent terminals. Lastly, since close ia infusion of PP was without effect, it is unlikely that PP acts directly within the stomach to elevate acid secretion and motility. Thus, convergent evidence suggests that circulating PP relies on intact vagal cholinergic pathways to increase gastric activity, but appears not to act directly on vagal afferent or efferent fibers.

Given that PP does not act peripherally, the most likely target of this peptide is the dorsal vagal complex in the medulla. Radiolabeled PP given intravenously has been demonstrated to specifically bind to the DVC and area postrema (176). Although the DVC is not a circumventricular organ per se, fenestrated capillaries with enlarged perivascular spaces indicative of an open blood-brain barrier have been demonstrated within the dorsomedial region of the NTS (54). These capillaries are
morphologically similar to those within the area postrema which thereby may provide access to the neuronal parenchyma for circulating agents such as PP. Furthermore, dendrites from both NTS and DMN cells may project into the dorsomedial NTS and area postrema (138,151). Thus, it is indeed plausible that these neurons may be directly affected by substances diffusing out of the circulation through the fenestrated capillaries. Given that specific binding sites for PP are found within these brainstem nuclei and that no other region of the brain demonstrated specific binding when PP was given intravenously (176), it seems likely that these nuclei are a target for circulating PP. In addition, PP immunoreactivity is not detectable within the CNS indicating that the ligand for the DVC receptors probably originates from the pancreas (34). Based on our findings and those of other laboratories (34,176), we conclude that PP released during a meal may travel through the circulation to the brainstem, enter the DVC, and stimulate vagal cholinergic pathways resulting in enhanced gastric activity.

Our results are in agreement with those of Wager-Pagé and colleagues (174) who measured the effect of PP on pentagastrin-stimulated acid secretion and demonstrated that iv PP significantly augmented acid output. The results of our studies contrast, however, with earlier work performed in conscious dogs examining the effect of PP on gastric acid secretion. One group infused relatively high doses of bovine PP and demonstrated that basal gastric acid secretion was stimulated whereas gastrin-stimulated acid output was reduced by the bPP (85). In a separate study, PP
was predicted to inhibit acid release and was infused during a background infusion of a maximal dose of gastrin (123). PP did not inhibit acid secretion, however, and was thought therefore not to play a role in gastric acid regulation. The use of a heterologous form of the peptide may account for the absence and/or conflicting results observed in these studies. Our present data demonstrate that bovine PP given iv was much less potent than rPP in stimulating gastric acid secretion in the rat. In light of this, future studies with this hormone ought to be conducted using the homologous form of the peptide whenever possible.
CHAPTER III

Pancreatic Polypeptide in the Dorsal Vagal Complex Stimulates Gastric Acid Secretion and Motility

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Introduction

Pancreatic polypeptide (PP), a 36 amino acid hormone, is synthesized and released from endocrine cells within the pancreatic islets (162). PP is the founding member of a family of structurally similar peptides which also includes neuropeptide Y and peptide YY (161,162). Release of PP from the pancreas occurs at a low rate in the fasted state and is greatly incremented throughout all phases of digestion (53,162).

Receptors for PP are limited regionally. Although the adrenal gland and liver contain saturable PP binding sites, the function for these is not known (113,177). Receptors for PP also are located in the dorsal medullary region of the brainstem. High affinity, specific receptors for PP are found in the area postrema (AP), nucleus tractus solitarius (NTS), and dorsal motor nucleus (DMN) (176). These nuclei, in
particular the NTS and DMN, are directly involved in vago-vagal regulation of gastrointestinal behavior (see Chap 1).

Although most of the dorsal vagal complex (DVC), i.e., the NTS and DMN, is endowed with an intact blood-brain barrier, the dorsomedial region of the NTS was recently shown to contain fenestrated capillaries that are morphologically similar to those in the area postrema (54). Similar to those within the AP, these fenestrated DVC capillaries have relatively high vascular permeability. This attribute provides the dorsomedial region of the DVC with the functional characteristics of a circumventricular organ and, therefore, may allow circulating peptides access to the NTS and neighboring DMN. Whitcomb et al. provided support for this hypothesis with the demonstration that radiolabeled PP given intravenously at postprandial concentrations binds to NTS and DMN regions in a saturable and specific manner (176). Thus, circulating PP may modulate autonomic regulation of gastrointestinal functions by gaining access directly to the dorsal vagal neurons and altering their activity. Indeed, our previous studies (see Chap 2) demonstrated that PP given intravenously potently stimulated gastric acid secretion and motility through a vagal-dependent mechanism. The data from the study indicate that the site of action for PP is not located in the periphery. Thus, the aim of the present study was to examine the influence of pancreatic polypeptide on vagal control of gastric motility and acid secretion. These studies examined the effect of microinjecting PP directly into the dorsal vagal complex to evaluate the probable site of action.
and effective dose range of this peptide with regard to affecting acid output and motility.

**Methods and Materials**

Male Long-Evans rats, 250-400g, were fasted overnight and then anesthetized with urethane (1.5 g/kg, ip) on the day of the experiment. Dexamethasone (0.8 mg) was administered subcutaneously to prevent cerebral edema. Animals then were equipped for measurement of either gastric acid secretion or antral motility.

**Acid Secretion**

*DVC Injection During Basal Acid Secretion.* The esophagus was ligated at the cervical level and the trachea was cannulated (PE-240). Following laparotomy, the pylorus was ligated, and a double-lumen cannula was inserted into the stomach through a small incision into the forestomach. Animals were placed into a stereotaxic frame and the brainstem was exposed by resection of the dorsal cervical musculature and removal of the occipital plate. Following brainstem surgery, basal acid secretion was recorded in 10 minute intervals by flushing the stomach with 5 ml of 0.9% saline (pH 7.0) followed with 5 ml of air. The acid level in the perfusate was determined by autotitration with 0.01N NaOH (Radiometer Copenhagen). Basal collection continued until 3 consistent readings were recorded.
Once the level of basal acid secretion was established, a double barrel micropipette was stereotaxically placed into the DVC using coordinates of 0.3 mm lateral to the midline, 0.2 mm anterior to calamus scriptorum, and 0.46 mm ventral to the brainstem surface. One pipette barrel contained either the vehicle solution (isotonic phosphate buffered saline [PBS], pH 7.4) or different concentrations of rat PP dissolved in PBS vehicle \([10^{-4}\text{ M}, 10^{-6}\text{ M}, \text{ or } 10^{-10}\text{ M PP}]\). The adjacent barrel contained a 1% pontamine blue dye solution which was used to iontophoretically mark the injection site at the end of the experiment. Bilateral injections, consisting of 20 nl PP or PBS, were administered into the left and right DVC. Thus, total PP doses equaled 4.0 pmol for group 1 \([10^{-4}\text{ M } \times 40\text{ nl}]\), 0.04 pmol for group 2 \([10^{-6}\text{ M } \times 40\text{ nl}]\) and 0.004 fmol \([10^{-10}\text{ M } \times 40\text{ nl}]\) for group 3. Since each 20 nl volume fills a cube with sides of approximately 300 microns, each injection should just fill both the DMN and adjacent NTS regions. Six animals were assigned to the vehicle and each of the PP-injection groups.

Following DVC injections, acid output was recorded for 120 min after which the pontamine dye was iontophoresed into the DVC. At the termination of dye ejection, each animal was transcardially perfused with 0.9% saline followed by 10% formalin. The brainstem was removed and post-fixed in a 10% formalin/20% sucrose solution for at least 24 hours. The tissue was cut (60 mm sections) on a freezing microtome, stained with Neutral Red, and examined for pipette placement.
Injection sites outside the DVC produced no observable results and were excluded from the study.

**Vagotomy or Atropine Pretreatment.** To determine the role of vagal cholinergic pathways in the PP response, another group of animals, which were prepared as above, received either an injection of atropine methyl nitrate (0.2 mg/kg, ip; n=5) or bilateral cervical vagotomy (n=3) 10 minutes prior to injection of 4.0 pmol of PP (the largest dose) into the DVC. Acid output then was monitored for 120 min.

**Pentagastrin-Stimulated Acid Secretion.** To determine whether PP may alter previously stimulated acid secretion, a separate group of rats (n=3) were prepared as above. In addition, they were fitted with a jugular cannula to infuse pentagastrin (PG; 8mg/kg/hr). Basal acid secretion was monitored until 3 stable readings were collected after which time PG infusion was begun. After 60 min, stimulated acid secretion due to PG stabilized and PP (4.0 pmol) was injected into the DVC as above. Acid output was monitored for an additional 60 min.

**Motility Studies**

Following cannulation of the trachea, a laparotomy was performed to expose the stomach. A strain gauge was carefully sewn onto the serosa of the antrum in an orientation for detection of circular muscle contractility. The incision was closed with suture; strain gauge leads exited through the incision. The animal was placed
into a stereotaxic frame and the dorsal surface of the brainstem was exposed as above.

Motility signals from the strain gauge were amplified by a conventional Wheatstone bridge-based amplifier. The amplified signals were digitized and stored on computer [RC Electronics, Santa Barbara, CA]. Motility records were analyzed in 400 second epochs and subjected to motility indexing analysis (see Chap 2 for details). Note that each strain gauge was calibrated with specific weights and a calibration curve was generated prior to experimental use. From this curve, voltage data from the amplifier could be converted to approximate grams of force. Although this method does not give absolute values of gram-force of stomach contractions, it does allow measurement of relative changes in motility and has been used previously (49).

Following brainstem exposure, gastric motility was recorded for at least 30 minutes to determine baseline activity. Once a stable baseline was established, a double-barrel pipette was placed into the left DVC as above. The pipette contained different doses of rat PP in one barrel and a 1.0% pontamine dye solution in the second barrel. The doses of PP used were 2.0 pmol, 0.02 pmol, and 0.002 fmol ejected in a 20 nl volume of PBS [10^{-4} M x 20 nl; 10^{-6} M x 20 nl; 10^{-10} M x 20 nl; n=6 per dose]. Unilateral injections into the left DVC were performed in the motility experiments to reduce variability. Control experiments consisted of 20 nl PBS injected into the left DVC (n=6). Following injection of PP, gastric motility was
continuously monitored until baseline values returned. For control animals, motility was followed for a minimum of 1.5 hours.

**Vagotomy or Atropine Pretreatment.** An additional group of animals were prepared as above and then pretreated with either atropine methyl nitrate (0.2mg/kg, ip; n=5) or bilateral cervical vagotomy (n=3) 10 minutes prior to injection of 2.0 pmol PP into the DVC.

At the completion of the experiments, pontamine dye was iontophoresed into the DVC to mark the injection site. Animals were perfused and the brainstem tissue processed as above. Again, injections occurring outside the DVC resulted in no change in motility and were excluded from the study.

**Data analysis.**

**Acid Secretion.** The percent change in acid secretion over baseline was calculated and then used to determine the mean value for each dose of PP. These values were compared with corresponding control values using a one way ANOVA followed by a post-hoc Bonferroni multicomparisons test. The effect of PP on pentagastrin-stimulated acid output was evaluated by comparing mean acid output values before and after DVC injection of PP with the Sign Test.

**Antral Motility.** Motility data were converted to 400 second motility indices for statistical and graphical purposes according to Ormsbee and Bass (119) (as detailed in Chap 2). Motility indexes at each time point were compared with the
corresponding control value using a one way ANOVA followed by a post-hoc Bonferroni test. Significance for all statistical procedures was set at p < 0.05.

Results

Acid Secretion

Effect on Basal Acid Secretion. Microinjection of PP into the dorsal vagal complex produced a long-lasting dose-dependent increase in gastric acid secretion. As seen in Figure 8, the two higher doses of PP, 4.0 and 0.04 pmol, stimulated acid secretion to levels significantly greater than those following control injections at almost all timepoints post-injection [p < 0.05]. The lowest dose of PP, 0.004 pmol, significantly stimulated acid output at only 2 timepoints following brainstem injection [Fig 8]. Acid secretion was unaffected by PBS injections into the DVC [Fig 8].

Effect on Pentagastrin-Stimulated Acid Secretion. Infusion of pentagastrin produced a 5-fold increase in acid secretion. Bilateral injection of 4.0 pmol PP into the DVC during pentagastrin infusion significantly elevated acid output over that due to PG alone (p < 0.05) [Fig 9].

Vagotomy or Atropine Pretreatment. Following pretreatment with atropine methyl nitrate, injection of PP (4.0 pmol) into the DVC produced no change in acid secretion [Fig 10]. Likewise, bilateral cervical vagotomy abolished the acid response to DVC injection of 4.0 pmol PP [Fig 10].
FIGURE 8: Dose dependent effect on gastric acid secretion following bilateral microinjection of indicated doses of PP or vehicle (PBS) into the DVC (n=6 per group). All brainstem injections occurred at time=0. Baseline acid output is considered 100% and all subsequent values are % over baseline. Data represent means ± SE. *p<0.05 compared to vehicle.
FIGURE 9: Effect of PP on pentagastrin-stimulated acid secretion. Microinjection of 4 pmol PP into the DVC (arrowhead) resulted in a significant increase in % acid secretion over that due to pentagastrin alone (n=3). Data represent means ± SE; *p<0.05 compared to pentagastrin-induced acid secretion.
FIGURE 10: Effect of bilateral cervical vagotomy (n=3) or atropine methyl nitrate (n=5) on acid output induced by microinjection of PP into the DVC. Injection of 4 pmol PP occurred at time=0. Data are means ± SE.
Motility

Effect of PP on Antral Motility. Microinjection of pancreatic polypeptide into the dorsal vagal complex produced a potent increase in contraction amplitude of the gastric antrum. This response occurred rapidly (within 2 minutes) upon brainstem injection, was dose-dependent, and long-lasting (e.g., following injection of the highest dose of PP, elevated motility was observed for over 3 hrs). The two higher doses of PP, 2.0 pmol and 0.02 pmol, produced significant increases in motility indices at several timepoints following DVC injection while motility following the lowest dose of 0.002 fmol was not significantly different from control data [Fig 11]. Microinjection of PBS into the DVC had no effect on antral contractions [Fig 11].

Vagotomy or Atropine Pretreatment. Pretreatment with atropine methyl nitrate or bilateral cervical vagotomy completely eliminated the increased motility evoked by PP injection into the DVC [Fig 12]. Thus, vagal cholinergic innervation is essential for the PP-evoked stimulation of antral contractions.

Discussion

The results of this study demonstrate that pancreatic polypeptide microinjected into the dorsal vagal complex produced a dose-dependent stimulation of gastric acid secretion and antral motility. DVC injections of PP significantly increased basal acid secretion at several post-injection timepoints. Microinjection of PP into the DVC
FIGURE 11: Dose dependent effect on amplitude of antral contractions following microinjection of indicated doses of PP or vehicle into the left DVC (n=6 per group). All DVC injections occurred at time=0. Data represent means ± SE; p<0.05 compared to vehicle.
FIGURE 12: Effect of bilateral cervical vagotomy (n=3) or atropine methyl nitrate (n=5) on antral motility evoked by microinjection of 2 pmol PP into the left DVC. Injection of PP occurred at time=0. Data are means ± SE.
also significantly elevated acid secretion above that produced by pentagastrin. Thus, central injection of PP enhanced both basal and stimulated acid secretion.

Motility studies revealed microinjection of PP into the DVC caused a long-lasting dose-dependent increase in the amplitude of antral contractions. The PP-induced stimulation of acid secretion and motility was eliminated by bilateral cervical vagotomy or peripheral treatment with atropine thereby suggesting vagal cholinergic pathways mediate these responses. These results support the view that pancreatic polypeptide alters digestive functions by acting directly on vagal nuclei within the brainstem.

The most likely target for PP within the brainstem is the DVC or AP given that these are the only regions within the brain, with the exception of the interpeduncular nucleus, that contain specific receptors for PP (176). Thus, concerns that the observed responses are a result of PP interacting with brainstem nuclei other than the DVC are minimal. This is further supported by the fact that microinjection of PP outside of the DVC produced no change in acid secretion or antral contractions. A second consideration involves the fact that PP is structurally similar to PYY and NPY and receptors for all three peptides are located within the DVC (161,162). Previous studies have determined, however, that concentrations of NPY or PYY up to 1.0 mM were unable to inhibit binding of radiolabeled PP to the AP or DVC during in vitro receptor autoradiography suggesting that the receptors for PP are specific (176). Additionally, studies from our laboratory demonstrate that injection of
PYY into the DVC has an inhibitory effect on gastric motility (23) suggesting that PP does not act at the PYY-NPY receptors.

Although previously it was thought that PP synthesis occurred within the central nervous system, radioimmunoassay studies revealed that all PP-like reactivity within the CNS is attributable to the structurally-related peptide NPY (34). These results indicate that the source of PP for the DVC receptors lies within the periphery. Since all detectable levels of circulating PP disappear following pancreatectomy (4), PP-secreting cells within the pancreatic islets must provide the ligand for the medullary receptors. Once PP is released into the circulation, the peptide most likely enters the DVC through fenestrated capillaries located within the dorsomedial NTS (54). Although the DMN is located subjacent to the NTS, dendrites from DMN neurons project into both the AP and the dorsomedial NTS (151) -- areas containing an open blood-brain barrier. This would allow DMN neurons to be directly affected by PP exiting the circulation as well as PP that diffuses the distance from the NTS into the DMN. Binding of PP to DVC receptors probably alters the neuronal activity which then may stimulate vagal cholinergic reflexes with the end result being enhancement of gastric functions.

Earlier work performed in conscious dogs examined a role for PP in the regulation of gastrin-stimulated acid secretion (123). The investigators predicted that PP administered intravenously would inhibit acid secretion and therefore performed the experiments against a background infusion of gastrin at a dose producing
maximal acid output. When PP was infused concomitant with the gastrin, no change in acid secretion was observed. Thus, PP was not thought to play a role in the regulation of gastric acid secretion. A separate study by Lin et al. (85) using conscious dogs demonstrated that intravenous infusions of relatively high doses of bovine PP alone increased basal gastric acid secretion. However, when the same doses of PP were administered during a submaximal infusion of gastrin, PP caused a reduction in the elevated acid secretion. In contrast to this prior work, our present study using an anesthetized rat model demonstrated that a modest stimulation of acid output induced by pentagastrin is further augmented by rat PP injected into the dorsal vagal complex. In addition, our previous data (Chap 2) clearly indicates that iv infusion of rPP into rats potently stimulates gastric acid secretion and motility through a vagal pathway. As also evidenced by our previous study (Chap 2), the discrepancies of the earlier studies may result from previous use of a heterologous form of PP.

The present data demonstrate that pancreatic polypeptide, a hormone produced in peripheral endocrine tissue, can directly modulate the activity of central autonomic nuclei resulting in enhanced gastric function. This suggests an exciting new model whereby circulating peripheral hormones may directly regulate parasympathetic control of gastrointestinal functions.
CHAPTER IV

*Pancreatic Polypeptide Stimulates Gastric Emptying in Rats*

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**Introduction**

Pancreatic polypeptide (PP) is a 36 amino acid hormone belonging to a family of structurally related peptides which also includes peptide YY and neuropeptide Y (161,162). Specific "F" cells located within the pancreatic islets and scattered throughout the exocrine tissue synthesize and secrete PP into the circulation (78,162). PP secretion occurs at low basal levels in the fasted state and is potently stimulated during all phases of digestion (141,164). This postprandial secretion is mainly dependent upon intact vagal cholinergic pathways (141,164).

Given that release of PP occurs during digestion, research aimed at determining the function of this hormone has focused on a role in regulating gastrointestinal events. Several studies have examined the effectiveness of PP as a feedback modulator of pancreatic exocrine function (11,87,93). The majority of results suggest that PP potently reduces pancreatic enzyme release through an indirect mechanism, i.e., PP neither binds to nor acts directly on pancreatic tissue
It has been suggested, therefore, that PP may alter the neural input controlling pancreatic function (87,93,175).

Although binding sites for PP are located on the liver and adrenal gland (113,177), specific binding of PP to the pancreas or gastrointestinal tract has not been observed, with the exception of the basolateral surface in canine small intestinal mucosa (50). Thus, explanations as to how PP altered gastrointestinal functions were not immediately obvious. In 1990, a possible site of action was discovered by Whitcomb et al. who demonstrated that radiolabeled-PP given intravenously at postprandial concentrations specifically bound to the area postrema and subjacent dorsal vagal complex (DVC) (176). Therefore, it is plausible that circulating PP may directly affect the activity of the DVC neurons leading to altered vagal outflow and ultimately modification of digestive activities. Indeed, recent studies from our laboratory revealed that microinjection of rat PP directly into the DVC of rats significantly increased gastric acid secretion and motility through a vagal cholinergic pathway (103) (see Chap 3).

Presently, we attempted to further explore the role PP plays in regulating gastric activities by assessing the effect of central injection of PP on the rate of gastric emptying. The reason for examination of emptying is twofold. First, a lack of confluence exists in the results from previous investigations. Namely, PP has been demonstrated both to augment gastric activities and potently reduce pancreatic enzyme release. This would seemingly lead to elevated gastric activities with a
simultaneous suppression of pancreatic secretion, a paradoxical combination. We hypothesized that although antral motility and acid secretion are augmented by PP, perhaps the rate of gastric emptying is not affected and the gastric phase of digestion thereby could be enhanced. Indeed, gastric emptying and gastric motility are not necessarily synonymous. For example, pentagastrin stimulates antral contractions while concomitantly decreasing the rate of gastric emptying (27). Additionally, previous studies have revealed that the rate of gastric emptying is not necessarily correlated with the amplitude or absolute number of antral contractions (44,58).

Thus, examining the effect of PP on gastric emptying would help to delineate if the function of PP is to prolong gastric phase digestion (i.e., increased motility without increased emptying) OR to amplify gastric activities while concomitantly increasing transit to the intestinal tract.

A second reason for performing these studies was that the action of PP on gastric emptying could be monitored in a conscious animal. In addition to examining the action of rat PP, central injection of bovine PP (bPP) was included in this study in order to compare the effectiveness of bPP in altering gastric activities with that of rat PP (rPP). The inclusion of these experiments was important since much of the previous work has been performed using bPP in heterologous models and, although similar, the structures of the peptides are not identical (74).
Methods and Materials

_Gastric Emptying Measurement_. Methods were modified from those described by Maeda-Hagiwara and Taché (90). The non-nutrient "meal" to be administered was prepared prior to the experimental day by making a 2% methylcellulose solution in distilled water. The mixture was maintained at 80°C under continuous stirring until the methylcellulose completely dissolved. The solution was allowed to cool to 37°C at which time the non-absorbable marker phenol red (50 mg/100 ml) was added. The meal was refrigerated until needed.

Male Long-Evans rats weighing between 200-350 g were deprived of food 20-22 hours prior to the experimental day but allowed free access to water. Animals were lightly anesthetized with ether and then mounted in a stereotaxic frame. An intracisternal (ic) injection was performed consisting of either 10 ml artificial cerebrospinal fluid (aCSF) alone (n=9) or aCSF containing varying doses of rPP (Bachem California), bPP (Bachem California), or the thyrotropin releasing hormone analogue (TRH), RX77368 (Reckitt and Colman, Kingston upon Hull, England). Intracisternal administration of 260 pmol RX77368 (0.1 mg) has been shown previously to evoke approximately 80% emptying of this test meal (90). Thus, inclusion of this group of animals provided a positive-control with which the PP response could be compared. The doses of rPP included 2.6 (n=5), 26 (n=4), or 260 pmol (26 mM; n=7); bPP doses included 260 (n=7) or 780 pmol (n=3); RX77368 was administered at a dose of 260 pmol (n=3).
Once the animal regained the righting reflex following ic injection, 1.5 ml of the phenol red meal (37°C) was placed into the stomach by gavage. After 20 min, the animal was sacrificed by CO₂ inhalation, the abdominal cavity was opened, and the pyloric and esophageal regions of the stomach clamped. The stomach was removed and placed into 100 ml of 0.1 N NaOH. The sample was homogenized for 30 sec (Biohomogenizer, Biospec Products) and then allowed to settle for 60 min at room temperature. Next, 5 ml of supernatant from the suspension was added to 0.5 ml of trifluoroacetic acid and the mixture centrifuged at 2000 g for 20 min. After centrifugation, the supernatant was added to 4 ml of 0.5 N NaOH and the absorbance of samples read on a spectrophotometer at a wavelength of 560 nm (Bausch and Lomb, Spectronic 20). This absorption reading is an index of the amount of phenol red that has not been cleared from the stomach.

On each experimental day, one rat was included which received no central injection but was fed 1.5 ml of the meal and then sacrificed immediately. The stomach was removed and treated as above. This animal served as a standard in which the amount of phenol red contained within the stomach was considered 100%.

Gastric emptying for each rat was determined by the following formula:

\[
\% \text{ gastric emptying} = 1 - \frac{\text{absorbance from test stomach}}{\text{absorbance from standard}} \times 100. \quad (2)
\]

**Atropine Pretreatment.** To determine if alterations in emptying following ic injection of rPP (260 pmol) were dependent on muscarinic cholinergic pathways, a separate group of animals was pretreated with atropine methyl nitrate (0.2 mg/kg, ip;
n=5) prior to central injection. Controls for this section consisted of two groups: rats receiving sterile saline ip and 260 pmol rPP ic (n=5) OR rats receiving sterile saline ip and aCSF ic (n=4). In this portion of the study, ip injection of saline or atropine was timed to precede ic injection by 8 min and the meal by 10 min. The protocol was then followed as above.

**Gastric Motility Studies.** In order to further characterize the potencies of rat versus bovine PP following central injection, an additional group of animals (n=11) was prepared for examination of antral motility as previously described (see Chap 3). Briefly, male Long-Evans rats (200-300g) were anesthetized with urethane (1.5mg/kg, ip) following an overnight fast. A strain gauge was sewn carefully onto the serosa of the antral region. The animal was placed in a stereotaxic frame and the dorsal surface of the brainstem exposed. Baseline motility was recorded for at least 20 min following which a microinjection into the left DVC was performed using the coordinates of 300 mm lateral and 300 mm rostral to calamus scriptorum and 450 mm ventral to the brainstem surface. The location of the injection site was verified histologically by injection of dye after completion of the experiment. Microinjections into the DVC included 20 nl of either phosphate-buffered saline (PBS, pH 7) alone, 20 nl of $10^{-6}$ M bPP, or 20 nl of $10^{-6}$ M rPP. The resulting dose of rat and bovine PP was 20 fmol each. This dose of rPP has been shown previously to produce a significant but submaximal stimulation of antral motility upon injection into the DVC (Chap 3). Antral motility was monitored for 60 min subsequent to brainstem
injection and then quantified using the method of Ormsbee and Bass (119) (see Chap 2 for details). For statistical and graphical purposes, a motility difference score was used for each rat in which the baseline motility value was subtracted from post-injection values.

In these experiments, the action of the two peptides when delivered directly into the region containing PP receptors (as opposed to the cisternal space) could be compared. Thus, inclusion of the bPP-DVC injections would allow determination of whether disparate emptying results with rPP and bPP were due to differential access to the DVC following ic injection or an actual difference in the activity of these two peptides when interacting with brainstem receptors.

**Data Analysis.** The percentage of gastric emptying following ic injection of rPP, bPP, or RX77368 was compared with controls using one-way ANOVA followed by a Dunnett t-test. For atropine and motility data, one-way ANOVA followed by a Bonferroni multicomparison test was used in which all values in each group were compared. Data are represented as mean ± SEM; significance was set at p < 0.05.

**Results**

**Rat PP ic.** Intracisternal injection of 260 pmol rat PP produced a significant increase in the rate of gastric emptying [Fig 13]. This dose of rPP caused 72 ± 6% of the meal to empty in 20 min. This was significantly different from rats given aCSF in
FIGURE 13: Influence of intracisternal injection of aCSF vehicle (Veh), rat PP (rPP), the TRH analogue RX77368, or bovine PP (bPP) on gastric emptying. Dose of injected drugs listed along bottom of figure are in pmol; injection volume consisted of 10 μl. Data represent means ± SE; *p<0.05 compared to vehicle.
which only 32 ± 8% of the meal emptied in 20 min (p<0.05) [Fig 13]. Although the intermediate dose of rPP, 26 pmol, caused a small elevation in the amount of meal emptied (41 ± 7%), this response was not significantly different from controls. The lowest dose of rPP, 2.6 pmol, produced no change in gastric emptying [Fig 13].

**Bovine PP ic.** In contrast to an equimolar dose of rPP, 260 pmol bPP did not alter the proportion of meal emptied over 20 min (22 ± 10%) [Fig 13]. When the dose of bPP was increased 3 fold (780 pmol), gastric emptying was still no different from emptying in control animals (20 ± 5%) and actually appeared to be somewhat reduced [Fig 13].

**TRH analogue ic.** In the positive-control experiments, intracisternal injection of RX77368 (260 pmol) provoked 75 ± 4% of the meal to empty in 20 min [Fig 13]; this response was significant compared to control animals receiving central aCSF (p<0.05). Note that percent emptying following RX77368 was not different from that seen following equimolar rPP. Thus, rPP was equally as potent as the TRH analogue at stimulating gastric emptying.

**Atropine Methyl Nitrate.** The stimulated gastric emptying provoked by central injection of 260 pmol rPP was eliminated by pretreatment with systemic atropine methyl nitrate (17 ± 10% emptied in 20 min) [Fig 14]. In these animals, the amount of meal emptied was not different from control animals receiving saline systemically and aCSF centrally (12 ± 8%; p>0.05) [Fig 14]. To ensure that elimination of the PP response was due to interruption of cholinergic pathways by
FIGURE 14: Effect of atropine methyl nitrate on the stimulated gastric emptying response produced by intracisternal injection of 260 pmol rat PP. Animals were placed into one of three groups: 1) V+V: ip sterile saline followed by ic vehicle; 2) V+PP: ip sterile saline followed by ic PP; 3) Atr+PP: ip atropine followed by ic PP. Data represent means ± SE; **p<0.01 compared to other two groups.
atropine and not simply stress due to an ip injection, an additional group of animals received saline ip followed by ic injection of rPP. In this control group, rPP (260 pmol) produced a 67 ± 7% increase in gastric emptying which was significantly greater than emptying in both the atropine animals (p<0.01) and the control animals (p<0.01) [Fig 14]. Thus, pretreatment with the muscarinic antagonist atropine was responsible for disrupting PP-evoked stimulation of emptying.

**Rat vs. Bovine PP Effect on Gastric Motility.** To determine if the absence of effect following ic bPP was due to impaired accessibility to the vagal nuclei or simply due to lack of peptide activity, the separate group of urethane-anesthetized rats was included. In these animals, microinjection of 20 fmol bPP directly into the region containing PP receptors, i.e., the DVC, significantly increased the amplitude of antral contractions compared to vehicle injections which were without effect [Fig 15]. Microinjection of 20 fmol rPP into the DVC also stimulated antral motility, the extent of which was similar to that seen previously (103). In contrast to bPP, stimulation of antral contractions following rPP occurred earlier (within the first 5 min) and achieved a significantly higher peak (p<0.05) [Fig 15]. The rPP-induced elevation in motility was also longer-lasting than that following bPP.

**Discussion**

The principal findings of this study are 1) intracisternal injection of rPP into rats potently stimulated gastric emptying and 2) bPP, given intracisternally at doses
FIGURE 15: Influence on gastric motility of vehicle (PBS), 20 fmol rat PP (rPP), or 20 fmol bovine PP (bPP) into the left DVC (n=11). Brainstem injections occurred at time=0 and consisted of a volume of 20 nl. Data represent means ± SE; *p<0.05, **p<0.01, ***p<0.001 compared to vehicle; +p<0.05 for comparison between rPP and bPP.
equal to or greater than those used for rPP, was ineffective. Increased emptying following rPP was comparable to that observed after ic injection of an equal dose of a TRH analogue -- a known potent stimulator of gastric emptying (90). The stimulated emptying induced by rPP was eliminated by pretreatment with atropine suggesting that PP acts through a muscarinic receptor mediated mechanism to augment gastric activity. It is highly probable that the cholinergic pathways stimulated by PP are vagal. A very recent report by Okumura et al. revealed that PP applied ic into vagotomized rats had no effect on emptying whereas PP stimulated emptying in intact rats (116).

Although the ic injection model used in the present study has several advantages, not the least of which is the use of conscious animals, there are some caveats. The first involves a lack of specificity due to spread of injectate throughout the brainstem. Fortunately, receptors for PP within the brainstem are restricted to the DVC and area postrema (176). Therefore, results observed in this study are due most likely to interaction of rPP with DVC neurons leading to enhanced vagal cholinergic outflow to the stomach. This is supported by previous data demonstrating that DVC injection of rPP stimulated vagal cholinergic pathways innervating the stomach whereas injections outside the DVC were without effect (Chap 3).

A second concern with intracisternal injections involves the ease with which the peptide reaches its target neurons. Both the flow of the cerebrospinal fluid (CSF) from the cisterna magna and peptide metabolism by cellular elements lining the
cisternal space may influence the length of time an injected peptide has to access the appropriate neurons. In the present study, the target neurons for PP are within the DVC, namely the nucleus tractus solitarius (NTS) and the dorsal motor nucleus (DMN). Two lines of anatomical evidence suggest that, barring the caveats of metabolism and CSF flow mentioned above, peptides given ic ought to have access to DVC neurons. First, Gross et al. demonstrated in 1990 that the dorsomedial region of the NTS contains fenestrated capillaries with enlarged Virchow-Robins spaces (54). These enlarged perivascular spaces may form conduits through which molecules in the CSF may travel from the cisternal space to the NTS. Secondly, anatomical studies have demonstrated that dendrites from NTS and DMN neurons extend into the area postrema (138,151) which thereby would expose these cells to substances in both the circulation and CSF. This anatomical evidence suggests that injection into the cisternal space most likely exposes NTS and DMN target neurons to PP. Given that a physiological response was produced by ic injection of rPP (but not vehicle), it is probable that neither flow of CSF out of the cisterna magna nor metabolism of PP within the CSF produced an effect substantial enough to eliminate the effectiveness of PP. In spite of the fact that CSF levels of PP do not rise following a meal, at least not within the third ventricle (68), injection into the subarachnoid CSF of the cisterna magna appears to be a reliable technique for elevating brainstem levels of this peptide.
Although bovine PP is structurally similar to rPP, when administered intracisternally in the present study it surprisingly produced no effect on the rate of gastric emptying. This was true for doses equal to or even three times greater than the effective dose for rPP. Several possible explanations exist for this observation. One hypothesis, which was explored in the present study, is that bPP given ic may have a reduced ability to access DVC neurons compared to rPP. To determine if bPP produces a physiological response when in direct contact with DVC neurons, separate studies were included in which bPP was microinjected into the DVC following which gastric motility was monitored. The results demonstrate that bPP is capable of significantly stimulating gastric motility when injected directly into the DVC. However, the delayed onset of action, lower peak effect and shortened duration of response produced by bPP compared to rPP [Fig 15] suggest that the intrinsic activity of bPP may not be as great as rPP, at least within rats.

Several possible explanations exist for the markedly different bPP responses following intracisternal and intra-DVC injections. As mentioned above, entry into the DVC from the cisterna magna may be more difficult for bPP compared to rPP, perhaps due to differences in the amino acid sequence of the two peptides. A previous study has determined that the rat form of PP differs from bovine at 8 out of the 36 amino acids (74). Structural differences may cause less efficient transport of bovine PP into the DVC. Once bPP contacts the receptors, however, the receptor binding region of the peptide is probably similar enough to the natural rat ligand to
cause receptor activation. An alternate explanation for the disparate results may be that rat and bovine PP have different degradative rates within the CSF. It is possible that the rat form of the peptide is relatively resistant to degradation while the bovine peptide is more susceptible. Finally, the markedly different responses produced by bPP in the two models may be due to different arousal states, i.e., anesthetic effects. Given that it is technically impossible to perform precise stereotaxic microinjections into the brainstem of awake animals, it was necessary to use anesthetized rats for placement of bPP directly into the vicinity of the PP receptors. On the other hand, emptying studies in anesthetized rats are not well characterized, and thus the two phenomena are best studied with the models presently utilized. However, a potent stimulatory effect was observed in both models with rPP indicating that the state of arousal or anesthesia did not impinge upon the activity of the peptides. It seems more likely that the different results for bPP in emptying and motility studies are due to transport and/or metabolic issues.
CHAPTER V

Effect of Pancreatic Polypeptide on Rat

Dorsal Vagal Complex Neurons

Introduction

Pancreatic polypeptide (PP) is a 36 amino acid hormone released from the pancreas during all stages of digestion (46,163,164). When given peripherally, PP has diverse effects on gastrointestinal functions including, but not limited to, inhibition of pancreatic enzyme release (87,93,126) and stimulation of gastric acid secretion and motility (173,174; also see Chap 2). These actions of PP, however, are indirect. For instance, stimulated gastric functions observed following intravenous PP are abolished by vagotomy (Chap 2). Furthermore, studies using close intra-arterial infusion of PP into the gastric circulation or capsaicin pretreated-animals indicate that vagal afferent terminals are not target for PP (93; also Chap 2). In addition, receptors for PP have not been demonstrated within the gastrointestinal tract with the exception of the liver (113) and the basolateral intestine (50). Thus, PP-induced alterations in digestive functions rely on intact vagal reflexes;
the site of action of PP, however, does not appear to be either the peripheral segments of vagal pathways or within the viscera.

In 1990, evidence for the site of action of PP was provided by Whitcomb and colleagues who demonstrated that specific, high affinity receptors for PP are located in the area postrema and dorsal vagal complex (DVC) (176). Thus, this central site is a prime location for PP to modify neuronal activity and thereby indirectly alter gastrointestinal functions. Indeed, previous work demonstrated microinjection of PP directly into the DVC produced a long-lasting significant elevation in gastric acid secretion and motility (103). This PP-evoked enhancement of gastric activity was eliminated by vagotomy and atropine suggesting that PP augments vagal cholinergic tone to the stomach. Preliminary evidence also suggests that DVC injections of PP may alter vagal tone to the pancreas leading to reduced exocrine secretion (117).

Based on evidence presented above, the hypothesis may be drawn that PP influences the activity of DVC neurons thereby altering vagal reflex activity. The following series of experiments evaluates what effect PP has on the cellular activity of DVC neurons using methods based on those previously published by our laboratory (99). In the present study, brainstem neurons were identified using two techniques. First, DVC neurons were identified as either NTS or DMN cells by stimulation of the cervical vagus and recording orthodromic and antidromic activation, respectively. Second, DVC cells responding to gastric mechanoreceptive
information were identified by inflating the antrum. Once identified, a neuron was tested for responsiveness to both pancreatic polypeptide and a vehicle control.

Methods and Materials

Male Long-Evans rats (n=30; 250-400g) were fasted overnight. On the experimental day, rats were anesthetized with urethane (1.5g/kg, ip) and given dexamethasone (0.8 mg, sc) to reduce cerebral swelling. Also, the trachea was cannulated to ensure a patent airway.

Surgery

Vagal Nerve Stimulating Electrode. Following tracheal intubation, the left cervical vagus nerve was isolated from the carotid artery and surrounding nerve sheath. A bipolar stimulating electrode (30 ga silver wire) was placed next to the exposed nerve. A small piece of parafilm was inserted under the nerve-electrode combination and sutures were placed around the parafilm to ensure contact between nerve and electrode. The cavity around the nerve and electrode was filled with petroleum jelly to prevent current spread into the surrounding musculature. The electrode wires were secured to the cervical musculature with suture and the neck incision was closed. Electrode leads were attached to a constant-current stimulus isolation unit (WPI) that was driven by a Grass S88 stimulator.
**Gastric Surgery.** Rats were laparotomized allowing exposure of the stomach and proximal small intestine. A suture was placed around the small intestine 1 cm distal to the pylorus; a small incision was made between the suture and the pylorus. A balloon, consisting of a latex glove fingertip attached to Silastic tubing, was inserted through the pylorus into the antral region of the stomach and secured in place. The abdominal musculature and skin were sutured, with tubing from the balloon exiting via the incision. The tubing was connected to a Stratum P23 pressure transducer to monitor gastric inflation pressure induced by injection of air into the balloon. Enough air was injected into the balloon to just allow inflation of the antrum. Inflation signals were monitored on a chart recorder and saved on magnetic tape for subsequent analysis. A subset of rats was instrumented for both gastric inflation and vagal nerve stimulation.

**Brain Stem Exposure.** Following the above surgical preparation, rats were placed in a stereotaxic frame. An open-ended tube supplying a gentle flow of oxygen was attached to the tracheal cannula. The dorsal surface of the brainstem was exposed by removal of the occipital plate (described in Chap 3).

**Procedure**

**Extracellular Recording.** A triple-barrel recording-injection pipette array was constructed by attaching a single recording barrel (OD 1mm) to a double-barrel
pipette (OD 10-15mm). The recording barrel was filled with 2 M NaCl; injection barrels were filled with phosphate buffered saline (PBS) or a solution of PP dissolved in PBS (10^4 to 10^8 M PP). Injection barrels were connected by pneumatic tubing to a Medical Systems BH-2 micropressure injection system. A silver wire was inserted into the recording barrel; recorded signals were amplified using Grass P-15 amplifiers (10,000x), displayed on a storage oscilloscope (Tektronix, R5031), and stored on magnetic tape for subsequent analysis. Signals also were routed through a combination window discriminator / rate meter circuit (Winston Electronics, RAD 1). A rate meter record of spike frequency was displayed on a variable persistence storage oscilloscope (Tektronix 5441) and routed to a Grass Model 79 chart recorder.

**Identification of NTS versus DMN Neurons.** Cells within the DVC were identified as NTS or DMN using orthodromic and antidromic activation, respectively. Given that NTS neurons are activated through a synapse, these cells can be identified using the following criteria: 1) variable latency in response to vagal stimulation; 2) inability to follow high frequency twin-pulse stimulation (> 50 Hz). In contrast, vagal stimulation causes antidromic activation of DMN axons. Because of this, DMN cells can be identified using the following criteria: 1) constant latency following vagal stimulation; 2) ability to follow high frequency twin-pulse stimulation (>50 Hz). Stimulation parameters for the vagus were typically 0.5 mA, 0.5 Hz, and 0.5 ms duration.
To search for NTS and DMN neurons, the electrode array was lowered into the brainstem in 10-15 mm steps using coordinates of approximately 0.2-0.6 mm lateral to the midline and 0.2-0.6 mm anterior to calamus scriptorius. Vagal stimulation continued until a neuron was encountered. Once a cell was identified as belonging to either NTS or DMN, the stimulator was turned off and baseline activity was recorded for at least 1 minute. Next, PBS was microinjected from the electrode and spontaneous activity of the neuron monitored for 1-2 minutes. If cellular activity was altered by PBS, the cell was rejected from the study. If PBS had no effect on firing rate of the cell, then PP was microinjected using the same pressure and duration settings used for PBS injection. Cellular activity following PP was observed for 1-2 minutes to determine if the cell was responsive. A cell was considered responsive if the activity was altered by 50% or more.

Identification of Gastric-Related Neurons. The electrode array was placed into the medial NTS using the same coordinates as above. The electrode was lowered into the brainstem in 10-15 mm steps. Following electrode movement, the stomach was inflated with 2-3 ml air and inflation maintained 2-5 s. This cycle was continued approximately every minute until a cell responsive to inflation was encountered. Cells were considered responsive if their firing rate was altered 50% or more by inflating the stomach. Gastric-related cells, i.e., cells that increased or decreased
firing rate during gastric inflation, were tested for responsiveness to PBS and PP as described above.

After monitoring the response of a cell to PP, the volume of the injected solution was determined microscopically by measuring the distance the meniscus in the pipette moved while performing injections at the same pressure and duration. The volume per injection was calculated by determining the number of injections needed to move the meniscus a given distance, which was previously calibrated to contain a certain volume.

Results

Vagal Nerve Stimulation

NTS and DMN Identification. Using orthodromic identification, 18 NTS neurons were identified. Of these identified NTS cells, 10 were stimulated by PP (56%), 5 were unaffected by PP (28%), and 3 were inhibited by PP (16%) (Table 1). An example of an orthodromically identified NTS neuron and its response to PP is shown in Fig 16.

Twelve DMN neurons were identified using antidromic stimulation. This identification included constant response latency following vagal nerve stimulation and ability to follow high frequency twin-pulse stimulation [Fig 17] One DMN cell was rejected from analysis because it was activated by PBS. Of the remaining cells, 6 were activated by PP (55%), 2 were unaffected (18%), and 3 were inhibited by PP
TABLE 1

Number and type of neuron tested with PP and the cellular response following application of PP, i.e., activation, inhibition, or no effect.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>stimulated by PP</th>
<th>no effect</th>
<th>inhibited by PP</th>
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<tbody>
<tr>
<td>ON cells</td>
<td>25</td>
<td>14</td>
<td>11</td>
<td>--</td>
</tr>
<tr>
<td>OFF cells</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td>NTS cells</td>
<td>18</td>
<td>10</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>DMN cells</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>3</td>
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</table>
FIGURE 16: Effect of PP on an NTS neuron. (A) 3 continuous sweeps showing orthodromic identification. * above stimulus artifact. Note variable latency of response following stimulation. (B) Integrated activity of cell shown in A. Injection of 0.2 nl PBS (Veh) had no effect on activity. Oscilloscope trace of record denoted by star is shown on following page (D). (C) Microinjection of PP (0.002fmol in 0.2 nl PBS) caused an abrupt increase in firing rate. Oscilloscope trace of record indicated by star shown in E. (D) and (E): Parameters are 500 mV/div and 1 sec/div.
FIGURE 17: Effect of PP on firing rate of DMN neuron. (A) Antidromic identification of cell. Stimulus artifacts indicated by *. Note constant response latency and ability to follow twin-pulse stimulation. (B) and (D) Effect of PBS (V) or PP injection on cellular activity. Corresponding oscilloscope traces to areas denoted by * are shown to right (C,E). PBS (0.2nl) had no effect on cellular firing rate while 0.002fmol PP in 0.2nl inhibited cellular activity. (C) and (E) Vertical axis: 500 mV/div; horizontal axis: 2 sec/div.
(27%) (Table 1). An example of an antidromically identified neuron that was stimulated by PP is shown in Fig 17.

**Gastric Inflation**

**ON and OFF Cells.** A total of 25 neurons stimulated by gastric inflation were encountered. These neurons are classified as ON cells. Of these cells, 14 were stimulated (56%) and 11 were unaffected by PP (44%). None of the ON cells were inhibited by PP.

Seventeen cells that were inhibited by inflation, i.e., OFF cells, were encountered. Of these, 11 cells (65%) were activated by PP, 6 exhibited no response to PP (35%), and none were inhibited. These data are summarized in Table 1. An example of an ON cell stimulated by PP is shown in Fig. 18; an OFF cell inhibited by PP is demonstrated in Fig. 19.

**Characteristics of Response to PP.** Neuronal activation following PP was quite abrupt, occurring usually within 1 min. The response to PP typically lasted several minutes; the firing rate of a few cells, however, was altered for over an hour. When higher doses of PP were used, e.g., $10^{-4}$ M to $10^{-6}$ M, cells typically were massively stimulated followed by a period of what appeared to be conduction blockade and sometimes cell death. Lower doses of PP produced effects equally as potent, however cellular activity did not display conduction blockade as frequently
FIGURE 18: Effect of PP on firing rate of an ON cell. (A) Oscilloscope trace showing ON response. Bottom line corresponds to gastric inflation. Vertical axis: 500 mV/div; Horizontal axis: 1 sec/div. (B) Record of cell shown in A. Injection of 0.12nl Veh had no effect on firing rate. (C) Injection of PP (1.2 pmol in .12nl) stimulated cellular activity. Firing rate was elevated for 13 min before returning to baseline.
FIGURE 19: Effect of PP on the activity of an OFF cell. Lower trace is a continuation of upper trace. Letters in two traces correspond to oscilloscope tracings shown on following page. Gastric inflation with 3 ml air (shown along bottom of A) completely inhibited neuronal activity (A). Baseline activity is shown in (B). Injection of 0.2 nl PBS produced no change in cellular activity (C). Injection of 0.002 fmol PP in 0.2 nl PBS produced marked stimulation in firing rate (D) that lasted approximately 1 min. In A-D, horizontal axis is 1 sec/div. Vertical axis is 500 mV/div.
nor did cell death occur. When a second injection of PP was attempted subsequent to the initial PP response, rarely was a second effect observed. In instances when a second response did occur, it was usually following a particularly long-lasting initial response. The effective doses of PP included pmol to subfemtomol quantities injected in volumes of <1 nl.

Discussion

When gastric inflation is used for identifying gastric-related DVC neurons, the located cells most likely are involved in the accommodation reflex [see Fig 20]. Inflating the stomach activates vagal afferents which excite NTS neurons (ON cells). According to this model, these NTS cells send inhibitory projections to tonically active cholinergic DMN neurons which project to the stomach. Thus, antral inflation results in inhibition of these DMN neurons (OFF cells) which will produce relaxation of the stomach (as described in Chap 1). Indeed, previous studies from our laboratory revealed that 92% of ON cells were NTS neurons and 64% of the OFF cells were located in the DMN (100). Of the neurons identified using gastric inflation, the vast majority were activated by PP. Interestingly, none were inhibited. Stimulation of OFF cells by PP is actually not surprising since these are most likely excitatory cholinergic neurons innervating the stomach. These cells may be responsible for the PP-induced gastric motility observed previously (Chap 3). In addition, the effect of
Antral distension produces disfacilitation of DMN resulting in gastric relaxation.

NTS cell corresponds to ON cells
DMN cell corresponds to OFF cells

Distension of the antrum stimulates gastric acid secretion
(May also stimulate antral motility and pancreatic secretion)

Distension of the esophagus actively inhibits gastric tone.
This reflex is identical to intestinal distension induced gastric relaxation.

Figure 20: Diagram of accommodation reflex, receptive relaxation, and distension-induced gastric acid secretion.
PP on motility typically was long-lasting which may be a reflection of the long-lasting effects PP had on the neuronal activity in the present study.

Activation of ON cells by PP is not as easily interpreted. In the typical description of reflexes, including vago-vagal reflexes, the sensory afferent limb synapses on an interneuron which in turns synapses on a motor neuron that projects to the area innervated by the sensory neuron. The interneuron, in this case the NTS neuron, is thought to be inhibitory. In this way, firing of the motor neuron may be regulated by sensory feedback relaying information about the activity of the motoneuron. For example, vagal cholinergic efferents stimulate gastric motility. This activation of the gastric musculature is detected by vagal afferents which project to the NTS. The NTS then may reduce the activity of the DMN neurons so that unbridled gastric activity does not occur. However, in the present study, activation of both NTS and DMN neurons, as well as both ON and OFF cells, by PP does not fit with this model since it is well established that PP causes long-lasting activation of gastric functions. If PP does stimulate inhibitory NTS cells and excitatory DMN neurons, one possible consequence may be an oscillatory firing rate of the vagus nerve. This in turn may lead to a cyclical effect on gastric functions. Indeed, this was observed in a number of animals in our previous study (103). Higher doses of PP microinjected into the DVC often produced cyclical motility patterns.

It is also possible that a number of the activated NTS and ON cells do not inhibit the cholinergic DMN cells, but instead are involved in separate reflexes. For
instance, these neurons could be involved in the distension-induced acid secretion reflex [Fig 20]. Activation of NTS neurons in this reflex by PP would produce a stimulation of cholinergic pathways projecting to the stomach resulting in enhanced acid output.

In reflexes such as receptive relaxation, excitatory projections from NTS neurons to DMN cells in the NANC pathway are predicted [Fig 20]. However, it is possible that the NTS projection to DMN-NANC pathway also is involved in reflex control of the pancreas. Conceivably, this may be one mechanism whereby PP inhibits pancreatic enzyme release. Given that some DMN neurons identified by vagal stimulation (but no gastric-related DMN neurons) were inhibited by PP, it may be that these are excitatory cells which cause pancreatic exocrine secretion. Inhibition of these cells by PP may be an alternate way in which PP reduces pancreatic output.

The observation that NTS and DMN neurons may be either stimulated or inhibited suggests that either unique receptor subtypes exist on DVC neurons or the same receptor may be linked to different second messenger systems in different cells. Although which second messengers associated with the DVC-PP receptors are presently unknown, research has shown that the binding sites for PP within the liver may be coupled to G-proteins (113). If this is also true for the neuronal receptors, it may partially explain the observed long-lasting effects of PP.
It is interesting to compare the action of PP on DVC activity with that of thyrotropin releasing hormone (TRH). Both PP and TRH potently stimulate gastric motility and gastric acid secretion (49,65,103,134). However, while PP mainly activates DMN and NTS cells, TRH inhibits NTS cells and activates DMN cells (98). It is easy to visualize the consequences of this TRH effect -- activation of cholinergic pathways to the stomach and reduction of the inhibitory sensory feedback due to inhibition of the ON cells. If one now examines the action of oxytocin (OT), the story becomes more complicated. Like PP, OT activates both ON and OFF cells (99). In addition, OT stimulates gastric acid secretion when placed into the DVC (134). In contrast, injection of OT into the DVC produces a reduction in gastric motility (136). Thus, it is obvious that the precise circuitry within the DVC responsible for regulating gastric functions is quite complicated and in vivo electrophysiology is only the first step towards a complete understanding of peptidergic regulation of gastric activity.
CHAPTER VI

Perspectives

Summary. The underlying theme of this dissertation is the exploration of the role PP plays in vagal control of gastric activities and the mechanisms whereby PP produces its effects. Several previous studies generated data indicating that PP may act within the brainstem to alter gastric activity. To examine this issue, several experimental designs were utilized and described in the previous chapters. From the results of these studies, we have learned that circulating PP acts at a central locus to stimulate gastric acid secretion and motility through a vagal cholinergic mechanism. Direct placement of PP into the DVC also potently enhances vagal cholinergic activity influencing the stomach resulting in long-lasting elevation of gastric acid output and motility. In addition, PP administered centrally produces marked gastric emptying. Given that brainstem injections of PP outside the DVC produced no change in gastric activity, it was concluded that PP stimulates vagal reflex circuitry within the DVC to produce augmented vagal cholinergic activity. In light of these data, it became obvious that PP must be capable of altering neuronal activity in the DVC nuclei. To describe the action of PP on cellular elements within the DMN and
NTS, *in vivo* extracellular recordings were performed. When DVC neurons were identified using vagal stimulation, the majority of NTS and DMN neurons were activated by PP. There were a small number of cells, however, that were inhibited by application of PP. These results indicated that PP is capable of altering cellular activity within the DVC and that both excitation and inhibition are produced by PP. When the technique of gastric inflation was used to locate gastric-related DVC neurons, the vast majority of cells were activated by PP while none were inhibited. This suggests that the effect of PP on DVC neurons involved in gastric function is purely excitatory. This may explain the previously described results which demonstrated that microinjection of PP into the DVC produces massive gastric acid secretion and motility through a vagal cholinergic reflex.

The electrophysiological data from the vagal stimulation experiments may be extrapolated to the action of PP on the pancreas. Since the activity of some encountered NTS and DMN neurons was reduced by PP, but no gastric inflation-related cells were inhibited, it is likely that the neurons inhibited by PP were not involved with gastric activity. Thus, a logical prediction is that the neurons inhibited by PP are involved in excitatory pathways projecting to the pancreas. In this way, PP may reduce vagal-induced exocrine secretion. It may seem paradoxical that this hormone which is released throughout digestion may inhibit pancreatic exocrine secretion since this function is essential for proper digestion. However, previous research has demonstrated that the exocrine secretion occurring during
digestion actually is submaximal (60). Perhaps the end result of the array of hormones and neural pathways activated by digestion is a balanced stimulation of pancreatic secretion which is regulated around a submaximal setpoint. Future studies hopefully will provide more details concerning the function of PP in the regulation of the pancreas. For now, the above results indicate that PP is a unique hormone in that it provides the afferent limb of an "endo-neurocrine" vagal reflex.

**Future Directions**

The presently described studies have provided insight into the role PP assumes in the vagal regulation of gastric activity. Not surprisingly, however, there remain several unanswered questions.

*Extracellular Electrophysiology.* The present electrophysiology studies have provided basic information involving the effect of PP on cellular activity of neurons within the DVC. Using vagal stimulation alone provides one with the identification of the recorded cell, i.e., NTS vs. DMN. However, the function of the neuron in terms of which GI organ is innervated is unknown. To achieve information on the action of PP on DMN neurons projecting to specific organs, dye injections into viscera could be performed prior to the extracellular recording. For instance, a neuronal tracer such as Fluro-Ruby could be injected into the pancreas and allowed to transport to the DMN. On the experimental day, the preparation would continue as
before with the addition of biocytin in the recording barrel of the electrode. Once a PP-sensitive DMN neuron is located, biocytin could be iontophoretically ejected from the pipette to label the recorded cell. At completion of the experiment, the brainstem would be examined for double-labeled neurons. In this way, the effect of PP on pancreatic-projecting neurons (or neurons projecting to other specified viscera) may be observed.

*In vivo* electrophysiology may also be used to determine if circulating PP affects neuronal activity within the DVC. For instance, vagal stimulation or gastric inflation would be used to locate DVC neurons. Next, a bolus of PP would be injected iv and the activity of the recorded neuron observed. If a significant alteration in the cellular firing rate is observed, this would provide evidence that indeed circulating PP is capable of affecting the activity of brainstem neurons. One drawback to this type of experiment would be that only one cell could be recorded with confidence per animal. If multiple cells were recorded, one could not be sure if any observed effects were due to pretreatment with the circulating PP. However, this type of experiment could be combined with other experimental paradigms such as the one described above. One the experimental day, the iv injection of PP would be the last manipulation performed.

Use of gastric distention allows identification of NTS and DMN neurons that are most likely involved in the accommodation reflex. It would be interesting to determine the action of PP on neurons involved in other reflexes. For instance,
esophageal distention should activate DMN neurons involved in receptive relaxation. These activated DMN cells would most likely participate in NANC pathways by which gastric motility and tone are reduced. Thus, since these are inhibitory neurons, one would predict that their activity would be reduced by PP.

**In Vitro Electrophysiology.** Although neurophysiological experiments performed using the whole animal setup provides important information, not all the relevant questions may be answered with this technique. By using *in vitro* recording of brainstem slices, one may delve further into the role PP plays in regulating neuronal activity. This preparation has the advantage of using synaptic blockade media which allows determination of *direct* effects on the DVC neurons. In addition, prelabeling of stomach or pancreas (described above) could be used in this setup, thus allowing determination of direct actions of PP on pancreatic- or gastric-projecting neurons.

In addition to extracellular recordings, the slice setup also may be used to perform both sharp-tip intracellular recordings and patch clamp recordings. Much valuable information may be garnered from these experimental designs. For instance, the ionic basis for PP-induced alterations in cellular firing rate may be examined. Additionally, using the perforated patch setup, second messenger systems involved in the response to PP may be investigated. In this way, one may determine if the different observed actions of PP on DVC neurons are due to activation of different intracellular cascades.
**Immunohistochemical Examination of Immediate Early Genes Following iv PP.** A new technique that has evolved over the past decade is examining the effect of a given agent on expression of immediate early genes (IEG). Using these methods, one could examine if IEG expression is altered following intravenous injection of PP. Preliminary data from our laboratory suggest that iv PP does increase expression of at least one IEG, c-fos, within the NTS and DMN. Although the exact function of c-fos in these neurons is not known, this experimental paradigm would provide evidence that circulating PP is capable of altering the activity of the brainstem neurons.

**Understanding the Role PP Plays in Digestion.** While it is interesting and important to decipher the mechanisms utilized by PP to produce the observed experimental results, it is also quite important to understand what role PP plays in regular digestive activities. Most of the previously-described experiments are performed in anesthetized fasted animals. While this is necessary, it is also artificial. Experiments designed to determine what effect PP has on digestive activity in an awake animal consuming a meal would be quite relevant. One goal for these experiments could be to eliminate the action of circulating PP and observe whether digestive activities are altered. A way in which this could be done, and indeed has already been attempted (42,154), is the use of antibodies against PP. The idea would be to remove active PP from the circulation and thereby prevent any of effects
typically induced by the hormone. However, these experiments are sometimes difficult to interpret and results to date have not been straightforward. One possible explanation for the discrepancies is that circulating PP molecules may only exist in more than one form. A study by Gingerich (52) demonstrated that the circulating form of PP in the fasted state consists of the entire PP molecule (i.e., 36 amino acid peptide). However, only ~33% of postprandial circulating PP may consist of the whole PP molecule. Other detected PP-related protein may be fragments of the original peptide. Whether these fragments possess physiological activity is unknown. If they do, they may be responsible for some of the disparate results observed in the antibody studies.

An exciting technique that has emerged recently is the use of antisense RNA. If the amino acid sequence for PP receptors was determined, the mRNA sequence could also be determined. From this information, antisense RNA could be synthesized and then infused into the cisterna magna to essentially eliminate PP receptors within the brainstem. These animals could be used to determine if their postprandial digestive processes are different from controls. In this way, it could be determined if PP binding to brainstem nuclei has observable effects on digestive processes.

**Evaluation of Effect of Other Circulating Peptides.** The data within this dissertation suggest that a circulating peptide alters brainstem neuronal activity
leading to long-lasting changes in visceral functions. This finding obviously has far-reaching implications. For instance, there is no reason to believe that circulating PP is alone in its ability to alter central vagal activity. Other hormones also may function in a similar manner. Indeed, the closely-related hormone peptide YY may act within the brainstem. A recent study by our laboratory revealed that PYY injected into the DVC produces potent inhibition of gastric motility through a vagal-dependent manner (23). Other hormones, such as somatostatin and angiotensin II, have also be suspected of functioning by acting within the DVC. In addition to hormones, circulating agents such as cytokines may function in a similar manner. Hermann and Rogers have revealed that the cytokine tumor necrosis factor has potent inhibitory actions on vagal control of gastric motility (62). The site of action for this cytokine was in the DVC. Thus, PP-induced action on DVC control of visceral functions may provide a model for which the effect of other circulating agents may be compared.

The experiments outlined above would provide evidence, that when examined in addition to the present results, may significantly improve our understanding of this enigmatic hormone. Also, these experiments would almost certainly lead to presently unforeseen questions concerning peptidergic influences on the brainstem DVC and digestion in general.
BIBLIOGRAPHY


