Pharmacology of the Inhibitory Innervation in the Gastroduodenum of Sprague Dawley Rat : an *in vivo* study

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ABSTRACT (abbreviated)

Intrinsic inhibitory innervation of the mammalian gut is non-adrenergic and non-cholinergic (NANC) in nature. Candidate NANC inhibitory transmitters include ATP, VIP and nitric oxide (NO) and there is considerable evidence for multiple NANC inhibitory nerve types. The goal of the research presented here was to determine the nature of the neurotransmitter(s) mediating relaxant motor activity in the rat gastroduodenum *in vivo*. In the gastric antrum, contractions and relaxations were observed with relaxations being predominant. Duodenal motor activity was patterned into 'Grouped' (periods of intense motor activity) interposed with 'Intergroup' periods of low motor activity. All antral and 'Intergroup' relaxations were sensitive to the NO synthase inhibitor L-NAME (10 $mg \cdot kg^{-1}$,i.v.). In contrast 'Grouped' relaxations were enhanced by L-NAME. ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$,i.v.) stimulated relaxations in the stomach and the duodenum. Although ATP was able to induce relaxations in both gut regions, with respect to spontaneous motor activity, the P_2-purinoceptor antagonist suramin (60 $mg \cdot kg^{-1}$,i.v.) specifically inhibited only 'Grouped' relaxations. Furthermore, desensitization with the ATP_{2x} receptor agonist α,β-Methylene ATP (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$,i.v.) also specifically blocked 'Grouped' relaxations. By contrast, P_{2y}-purinoceptor agonist, 2-Methylthio ATP (360 $\mu g \cdot kg^{-1}$,i.v.) induced an L-NAME sensitive relaxation, and prolonged exposure resulted in the reduction of 'Intergroup' relaxations only. GABA interneurons via $GABA_A$ receptors target NO neurons in the mammalian gut. All antral and 'Intergroup' relaxant activity was reduced by the $GABA_A$ receptor antagonist bicuculline (350 $\mu g \cdot kg^{-1}$,s.c.). Furthermore, the $GABA_A$ receptor agonist 3-APS (100 $mg \cdot kg^{-1}$, s.c.), evoked relaxations in the antrum, and in the duodenum only during the 'Intergroup' period. In the antrum, VIP (6 $\mu g \cdot kg^{-1}$,i.v.) was able to evoke L-NAME sensitive relaxations of both the longitudinal and circular muscle layers. However, only VIP evoked relaxations of the antral longitudinal muscle layer could be blocked by bicuculline. **Conclusions:** Spontaneous relaxant activity in the rat gastroduodenum can thus be differentiated: antral and duodenal 'Intergroup' relaxations are NO dependent and $GABA_A$ receptors mediate the pathways controlling these relaxations. The pathway(s) controlling 'Intergroup' NO relaxations involve functional ATP_{2y} receptors. 'Grouped' relaxations are mediated by ATP via ATP_{2x} receptors and are modulated by NO. Lastly, VIP can evoke relaxations of **i)** the antral longitudinal smooth muscle by targeting the A-GABAergic-nitrergic pathway and **ii)** the antral circular muscle either directly or via a neural pathway, both of which involve NO.

ABSTRACT

The intrinsic inhibitory innervation of the mammalian gastrointestinal smooth muscle is non-adrenergic and non-cholinergic (NANC) in nature and may involve more than one transmitter. To date, adenosine triphosphate (ATP), vasoactive intestinal peptide (VIP) and nitric oxide (NO) have all been proposed as candidate NANC inhibitory neurotransmitters of the gut in a variety of mammals. Indeed, it appears that NANC inhibitory innervation involves more than one population of inhibitory motor neurons and these may be region specific. NANC inhibitory neurotransmitters may also be co-released. In addition to the direct effect of NANC inhibitory neurotransmitters on gut muscularis, these neurotransmitters may also have neurogenic actions. The goal of the research presented here was to determine the nature of the neurotransmitter(s) mediating relaxant motor activity in the rat gastroduodenum *in vivo*. This would provide a greater understanding of the nature of inhibitory innervation in this region and facilitate identifying which NANC neurons are affected in disease such as duodenal ulceration.

In order to address the issue of the nature and control of relaxant activity in the gut *in vivo*, it was necessary to employ techniques that would be both sensitive and easy to use. This was achieved by refining an *in vivo* recording technique using foil strain gauges, and a computerized recording and analysis system for the assessment of both contractions and relaxations, developed previously in this laboratory for use in conscious rats. In **Chapter 1**, I describe experiments where: **i)** the recording technique was refined to create a direct online IBM based data acquisition system, and **ii)** the recording technique was adapted for use in

anaesthetized rats.

The results show that the recording technique provided a reliable and sensitive method for recording motor activity simultaneously from multiple sites in the gastroduodenum of the anaesthetized rat. Spontaneous gastroduodenal motility comprised distinct patterns of motor activities. In the gastric antrum, contractions and relaxations were observed with relaxations being predominant. Duodenal motor activity was patterned into 'Grouped' (periods of intense motor activity) interposed with 'Intergroup' periods of low motor activity. Occasionally, random large amplitude events in the gastric antrum temporally coupled with large amplitude responses in the proximal duodenum, were also detected. These simultaneous responses were termed 'Coupled' events.

The motor activities in the gastroduodenum were found to be differentially sensitive to treatment with the ulcerogen cysteamine-HCl and this may be related to the involvement of different neuronal populations controlling these motor activities. Studies presented in **Chapter 2-5** sought to characterize these motor activities. When treated with the NO synthase inhibitor L-NAME (10 $mg \cdot kg^{-1}$, i.v.) antral and 'Intergroup' relaxations were reduced or abolished, whilst 'Grouped' relaxations were enhanced. These effects were reversed with the NO synthase substrate, L-arginine (300 $mg \cdot kg^{-1}$, i.v.). ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$, i.v.) stimulated relaxations in the stomach and the duodenum. Although ATP was able to induce relaxations in both gut region, with respect to spontaneous motor activity treatment with the P_2-purinoceptor antagonist suramin (60 $mg \cdot kg^{-1}$, i.v.), specifically inhibited only 'Grouped' relaxations. Thus spontaneous relaxant activity in the rat gastroduodenum of the anaesthetized rat can be characterized based on the involvement of different NANC transmitters. Antral

relaxations and duodenal 'Intergroup' relaxations are NO-dependent. 'Grouped' relaxations of the duodenum showed sensitivity to suramin indicative of the involvement of ATP via P_2-purinoceptors. Furthermore, these 'Grouped' relaxations appear to be under some tonic inhibitory modulation involving NO.

Examination of which P_2-purinoceptor subtype mediates 'Grouped' relaxations was the focus of the research presented in **Chapter 3**. Treatment with the P_{2x}-purinoceptor agonist α,β-Methylene ATP (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$, i.v.) resulted in an initial increase in 'Grouped' relaxation amplitude followed in time by a reduction or abolishment of 'Grouped' relaxant activity only, reminiscent of the development of tachyphylaxis. The results provide evidence that 'Grouped' relaxations are mediated by ATP via P_{2x}-purinoceptors. By contrast, systemic injection of the P_{2y}-purinoceptor agonist, 2-Methylthio ATP (360 $\mu g \cdot kg^{-1}$, i.v.) induced an L-NAME sensitive relaxation, and prolonged exposure to 2-Methylthio ATP resulted in the reduction of NO mediated 'Intergroup' relaxations only. Thus, it is concluded that ATP via P_{2y}-purinoceptors targets nitrergic inhibitory motor neurons and these excitatory ATP sites represent a functional component of the pathway mediating NO related inhibitory motor innervation of the duodenum.

GABA interneurons via $GABA_A$ receptors are known to target NO neurons elsewhere in the mammalian gut. Therefore, it was of interest to determine whether NO mediated responses in the rat gastroduodenum are targeted by GABAergic neurons. All antral and 'Intergroup' relaxant activity was reduced by the $GABA_A$ receptor antagonist bicuculline (350 $\mu g \cdot kg^{-1}$, s.c.). Furthermore, application of the specific $GABA_A$ receptor agonist 3-APS (100 $mg \cdot kg^{-1}$, s.c.), evoked relaxations in the antrum, and in the duodenum only during the

'Intergroup' period which in Chapter 2 were shown to be L-NAME sensitive. The functional evidence presented herein together with histoanatomical evidence in the literature that $GABA_A$ receptors exist on nitrergic neurons, suggest that $GABA_A$ receptors and hence enteric GABAergic neurons occur within the pathway(s) controlling spontaneous NO-mediated antral relaxations and duodenal 'Intergroup' relaxations.

The results summarised in **Chapter 2-4** show that all spontaneous relaxations of the rat gastroduodenum can be accounted for with respect to being either NO or ATP mediated. However, the actions of the putative NANC transmitter VIP needed to be assessed. In the antrum VIP (6 $\mu g \cdot kg^{-1}$, i.v.) was able to evoke L-NAME sensitive and hence NO mediated relaxations of both the longitudinal and circular muscle layers. However, only VIP evoked relaxations of the antral longitudinal muscle layer could be blocked by bicuculline. Therefore, it is possible that VIP can evoke relaxations of **i)** the antral longitudinal smooth muscle by targeting the A-GABAergic-nitrergic pathway and **ii)** the antral circular muscle either directly or via a neural pathway, both of which involve NO. In the proximal duodenum, VIP always evoked a relaxation however, this was insensitive to both L-NAME and bicuculline. Whether VIP targets the purinergic (ATP) inhibitory innervation of the duodenum remains to be determined.

VIP (i.v.) was also able to evoke a 'coupled' response in the rat gastroduodenum. Utilising *ex vivo* experiments, it was found that direct serosal application of VIP to the proximal duodenum was able to mimic the coupled responses observed with systemic application of VIP. In contrast, direct application of VIP to the gastric antrum only resulted in antral relaxation. These results suggest that a pathway projecting from the proximal

duodenum to the gastric antrum involves VIP.

Preliminary studies with cysteamine-HCl (within the validation experiments of Chapter 1) showed that this ulcerogen disrupts only motor activity of the proximal duodenum. Cysteamine-HCl, administered orally or subcutaneously induces duodenal ulcers (DU) in Sprague Dawley rats within 24 hours, and is used in this and other laboratories as a model for the human disease. Duodenal ulceration is a multifactorial disease involving increased acid secretion, impaired mucosal defense and altered gastroduodenal motility. Ulceration occurs when the increase in acid load overcomes the normal neutralizing capability of the mucosa in the duodenal bulb. Thus, gastroduodenal motility has important implications in the pathogenesis of DU. Motor activity of the gastroduodenum is disrupted early in the development of experimental DU. *In vivo* and *in vitro* evidence from experimental duodenal ulcer studies suggest cysteamine-HCl exerts effects on motor activity through inhibitory sites on both excitatory cholinergic and intestinal non-adrenergic, non-cholinergic (NANC) inhibitory motor neurons, and stimulatory sites on NANC inhibitory motor neurons. Therefore it is important that the nature of *in vivo* motility changes be fully characterized, especially with respect to the inhibitory innervation. The results of the research presented in this thesis shows how NO, ATP and VIP are involved in the control of inhibitory motor activity of the rat gastroduodenum. This affords an important basis for future identification of neurones and pathways disrupted during DU development, and for understanding gastroduodenal function per se.

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INTRODUCTION

Functional Anatomy

The gastrointestinal (GI) tract is a tube-like structure that originates at the proximal end of the esophagus and extends to the distal end of the rectum. Although, the components of the GI tract may vary in anatomy and functionality, the overall tissue organization of the gut wall remains consistent. Four basic layers make up the gut wall: the serosa, muscularis externa, submucosa, and the mucosa as shown in figure 1 (for review see [61,68]).

With the exception of the stomach, the muscularis externa is composed of two well defined layers of smooth muscle. These differ with respect to the orientation of the muscle cells that make up these layers. The outer muscle layer, referred to as the longitudinal muscle is so defined since its muscle cells are oriented in the longitudinal axis (in the oral to anal direction). The inner or circular muscle layer is composed of cells oriented perpendicular (circumferentially) to the longitudinal muscle layer. The stomach is unique in that it has an additional smooth muscle layer, an inner oblique layer which lies on the anterior and posterior surfaces and fuses with the circular layer in the distal stomach. The longitudinal layer in the stomach is incomplete on the anterior and posterior surface. In the colon of the human and guinea pig the longitudinal muscle layer also deviates from the general description, as the muscle layer is not evenly distributed around the circumference of the colon. Instead the longitudinal muscle layer is concentrated into three bands of muscle termed taenia coli. The bands are equidistant around the circumference and are separated by a thin sheet of longitudinal muscle.

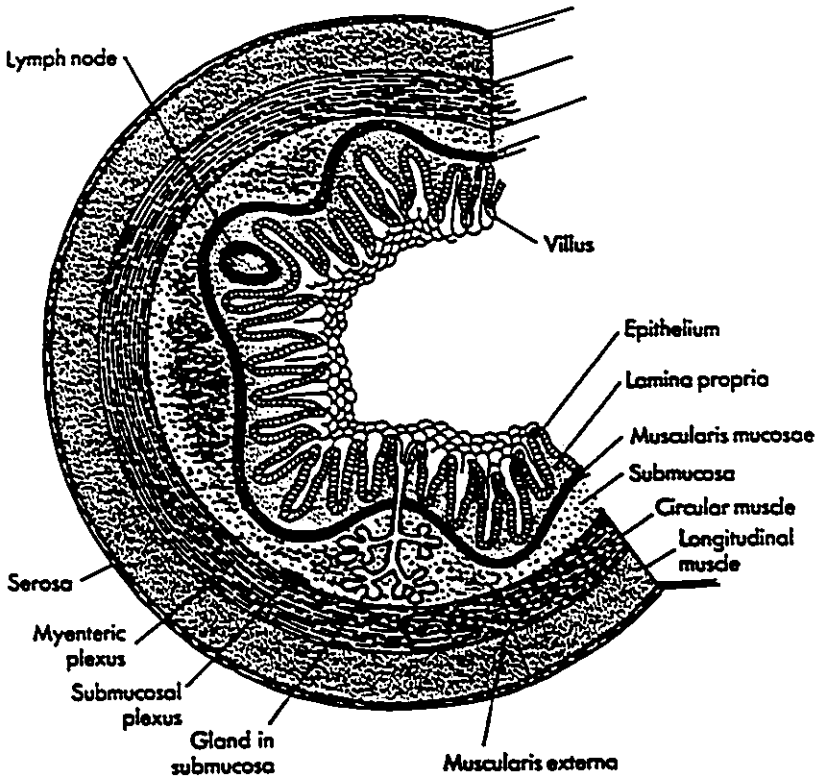


Figure 1 A cross section illustrating the general organization of the layers of the gastrointestinal tract (adapted from Physiology, ed. by Berne and Levy, C.V. Mosby Co, 1983).

Lying in between the muscularis externa and the mucosa is a region of loose connective tissue termed the submucosa. A distinctive characteristic of the submucosa is that it is comprised of distinct nerve layers as well as a vascular network which serves as the interface of the enteric blood supply with systemic vascular system. Lymphatics are also distributed within the submucosa. In some regions, the serosa also contains some glands.

The inner most layer, the mucosa, varies in organization depending upon the region of the gastrointestinal tract. This variation reflects the role of these regions in digestion and absorption. Irrespective of the region, the mucosa is basically made up of an epithelial surface, a muscularis mucosae and a connective lamina propria.

Also residing in the gut wall are several distinct nerve plexi of which the myenteric and secondarily the submucosal plexi are the most prominent (Fig. 2). These plexi are planar networks of interconnected nerve strands and ganglia. The myenteric plexus (also referred to as Auerbach's plexus) lies in a plane between the longitudinal and circular muscle layers. This plexus is continuous around the whole circumference and length of the gastrointestinal tract.

Within the connective tissue of the submucosa there exist up to three ganglionated nerve networks. The ganglia and nerve strands in these plexi are smaller compared to those of the myenteric plexus. In the case of the guinea pig, there are two interconnected plexi which are referred to as Henle's located close to the circular muscle layer and Meisseners plexi located near the muscularis mucosae.

Enteric Nerves and Motility:

The function of the gastrointestinal tract is to assimilate (digestion and absorption)

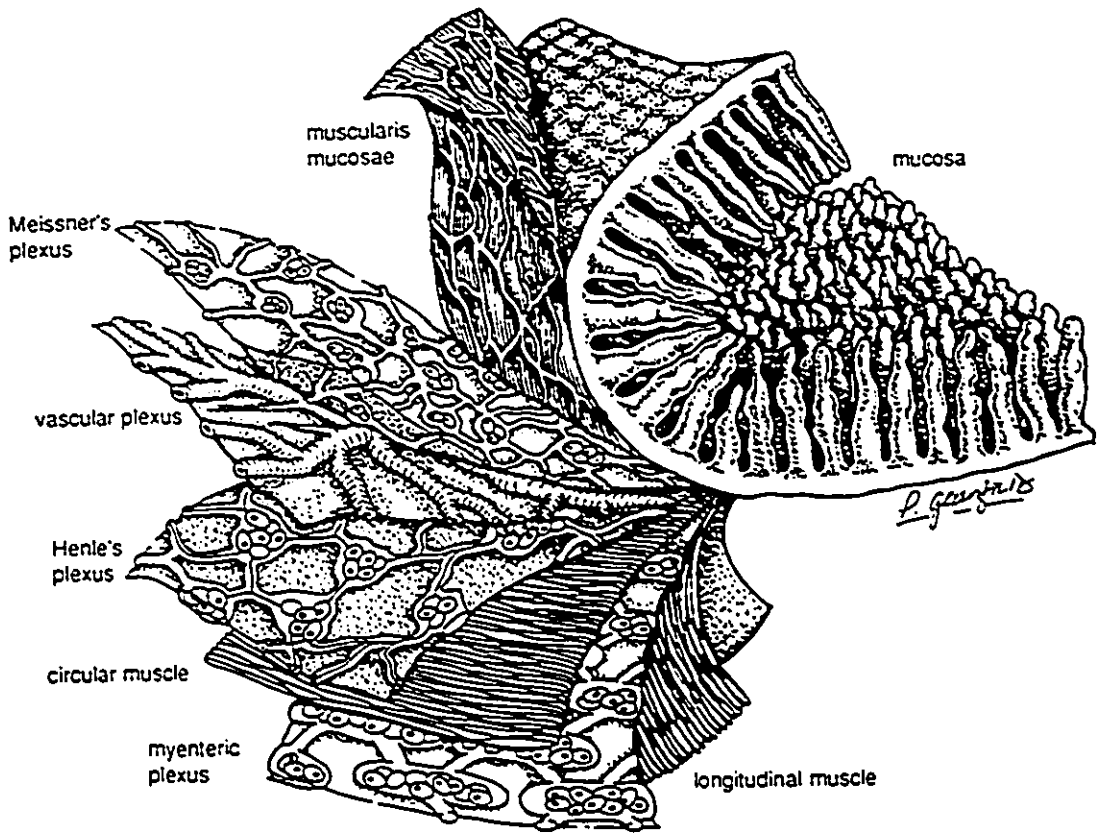


Figure 2 A schematic illustration of the wall of the gastrointestinal tract with the layers reflected to show the general arrangement of the smooth muscle layers and nerve plexi. N.B. Henle's plexus is not present in the rat. In the human submucosa, a third nerve network, the Intermediate plexus, occurs.

food. The process of food assimilation, and homeostasis during interdigestive periods require that secretory, absorptive and motor functions be co-ordinated. Several levels of control are responsible for this co-ordinated activity [19,33,68]. The primary control of gut function is achieved via the intrinsic nerve layers described above which together comprise the intrinsic nervous system of the gut, the so-called, enteric nervous system (ENS). Superimposed upon and connected to the ENS is the intrinsic endocrine/paracrine system. The gut represents one of the largest endocrine/paracrine organs found in mammals. In the absence of CNS connections, the gut is capable of sustaining much of its own co-ordinated activity in the gut. However, the CNS can influence enteric function. This is most evident under conditions of autonomic sympathetic activation. In addition, somatic nerve innervation of the gut wall allows region to region reflex responses to be coordinated. These neural pathways run extrinsic to the gut wall and neurally connect different regions via sympathetic ganglia. Both of these levels can be modulated by the autonomic nervous system via parasympathetic and sympathetic innervation.

The ENS achieves control of muscle tone and reflex motor activity via direct myenteric innervation of the smooth muscle cells of the muscularis externa. Although the submucosal plexus efferents may also affect the smooth muscle layers, submucosal nerves primarily terminate directly on submucosal neurones, blood vessels, the muscularis mucosa and cells in the mucosa [68]. This arrangement parallels functional evidence which indicates there is a division of labour; the myenteric plexus primarily exerts control over motor events, and the submucosal plexus controls mucosal epithelial activity (secretion, absorption, blood flow and motor activity of the muscularis mucosa) and to a lesser extent circular muscle

motility [19]. However, the myenteric plexus serves as the major integrative site for control of all gut function.

The coordination of these different gut functions occurs via the many nerve fibres that form connections between ganglia and the ganglionated networks. These connections allow the plexi to share information and act as an integrative unit to coordinate the gut processes[68]. Afferent nerve fibres from the myenteric/submucosa and mucosa also transmit information (chemical and mechanical stimulus) to the central nervous system (CNS).

Gut Motility

Efficient digestion and absorption of food requires that the gastrointestinal tract has an orderly motor system to ensure food is mixed and propelled (peristalsis) at the proper rate through the gut. Four basic patterns of GI motor activity have been identified (Fig. 3) with three of these patterns observed after the ingestion of food: i) peristalsis, for the propulsion of food, ii) rhythmic segmentation, for mixing purposes and iii) tonic contraction (for restriction) or tonic relaxation (for accommodation, not shown in figure). The fourth pattern is observable between meals or in the unfed or fasted state and is termed the migrating motility complex (MMC's). Regardless of the types of movements involved, these patterns all require coordinated contraction and/or relaxation of the gut smooth muscle layers. Most of what is known about the neuronal circuitry controlling GI motor activity has been derived through the study of peristalsis.

Bayliss and Starling (for review see [19,33,62]) first demonstrated that a pinch stimulus caused two types of intestinal reflexes to occur, a smooth muscle contraction on the oral side

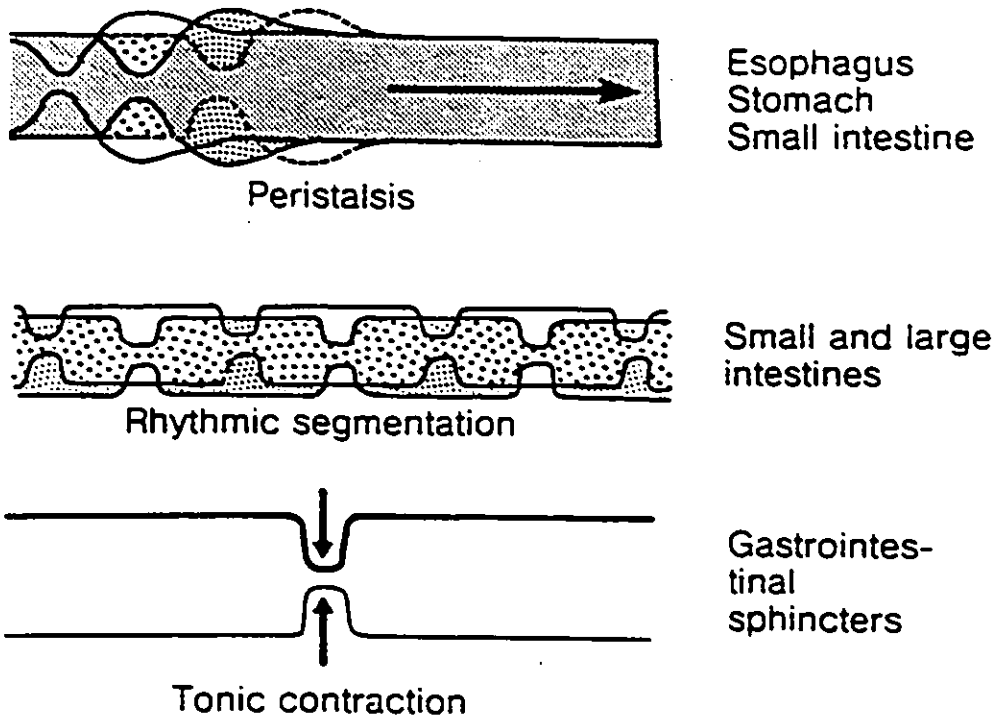
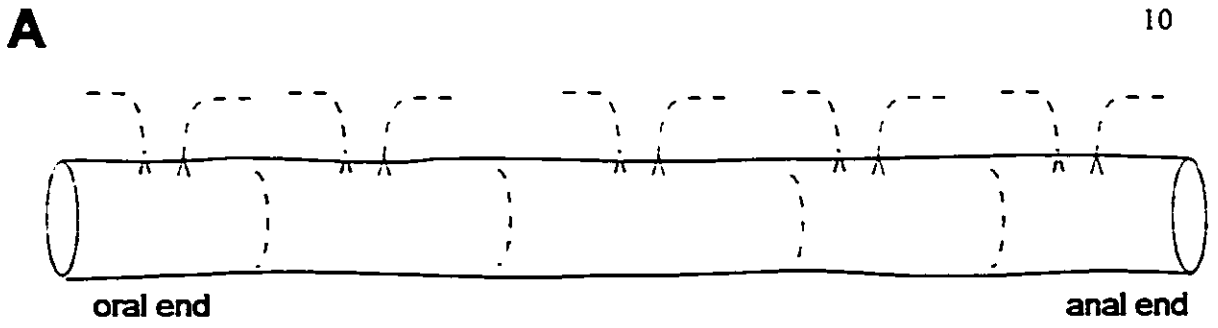


Figure 3 Typical tracings of the three basic motility patterns in the gastrointestinal tract and the regions of the GI tract where they are generally observed (adapted from Clinical Gastrointestinal Physiology, Granger, Barrowman & Kvietys, W.B. Saunders Co., 1985)

of the stimulus while a muscle relaxation occurred on the anal side. This was indirect evidence for the existence of inhibitory and excitatory motor neurons within the gut wall. These reflex responses were termed 'ascending excitatory reflex' for the oral side contraction and a 'descending inhibitory reflex' for the relaxation on the anal side. It should be understood that these polarized reflexes are standing reflexes. In other words, a stimulus at one point triggers two localized responses polar to each other. Investigators have proposed that enteric motor reflexes form the basis of gut motility.

Peristalsis occurs through a co-ordinated motor reflex behaviour resulting in propagated waves of contractions and relaxations, that travels anally [33,169,170]. This stereotypic behaviour requires the continuous activation of numerous excitatory and inhibitory neural reflex pathways involving sensory neurons, interneurons and motor neurons resident in the gut wall. Pathways necessarily overlap each other and are repeated at each point along the intestine. The final activity occurring at each point is a result of the balance between the reflex excitation and inhibition at that site. Peristalsis is evoked either by local distension stimulus such as a bolus of food, or generalized distension that occurs with fluid in the lumen. Figure 4 illustrates peristalsis evoked in a segment of gut as it would occur in organ bath experiments. The number of pathways used in the diagram has been reduced to simplify the description. The sequence of events are as follows: the intestine slowly distends resulting in the simultaneous activation of neuronal pathways underlying the ascending excitatory and descending inhibitory reflexes along the length of the intestine. The inhibitory reflex predominates. At a certain intraluminal volume (distension threshold) a contraction occurs at the oral side of the distension. The peristaltic contraction is initiated at the oral end because

Figure 4 A diagrammatic representation of the local enteric reflexes underlying peristalsis. The stylized segment of gut as shown is made up of a number of local functional units (delineated here by black dotted lines), the number of which has been reduced to simplify the diagram. Each local unit is innervated by an ascending excitatory pathway (blue) and a descending inhibitory pathway (red). The ascending excitatory pathway is activated by distension at a local unit immediately oral to the point of the pathway's innervation. The descending inhibitory pathway is activated by distension at a local unit immediately anal to the point of the pathway's innervation. **A)** When the gut is not distended, no pathways are activated (shown by dotted pathway axons). **B)** Upon distension ascending and descending reflex pathways are activated (solid lines). Note that at the oral end of the gut the descending inhibitory pathway is not activated as the pathway has been interrupted. Likewise, at the anal end the same is true with the ascending pathway. **C)** At the oral end, a contraction occurs throughout the local unit as the balance of innervation is in favour of excitation as shown in part B. As contraction occurs, the descending pathway originating at this unit and innervating the next unit becomes inactivated. This creates an imbalance in the next anally adjacent unit and a contraction occurs in this next unit **(D)**. The unit where the wave of contraction originated returns to rest since innervating pathways have been inactivated. The wave of contraction continues as there is a sequential de-activation of pathways.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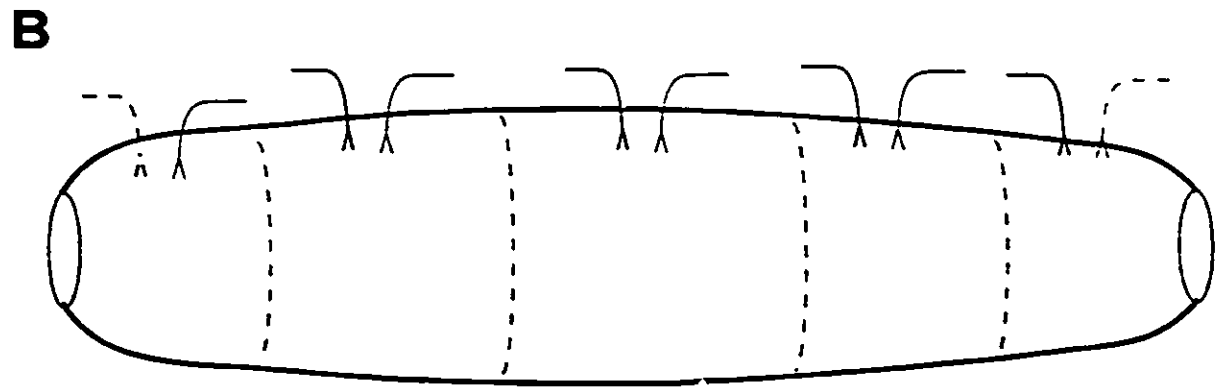
anal end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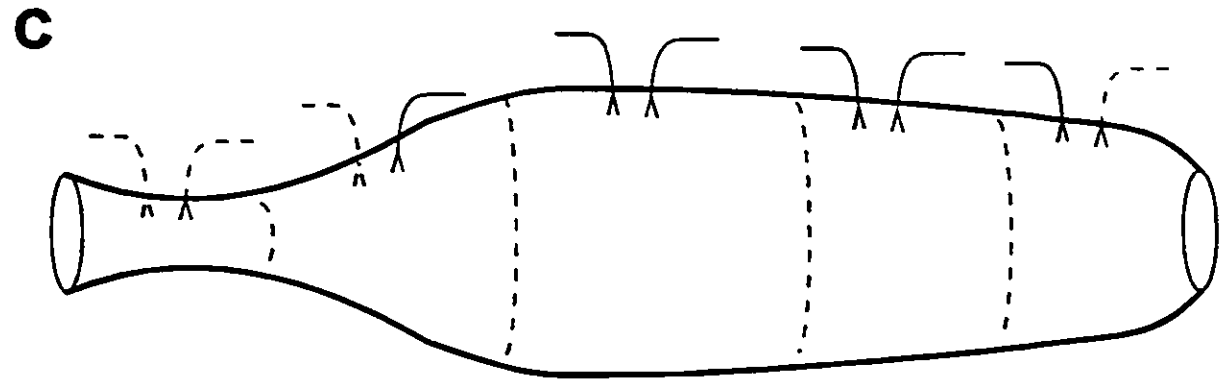
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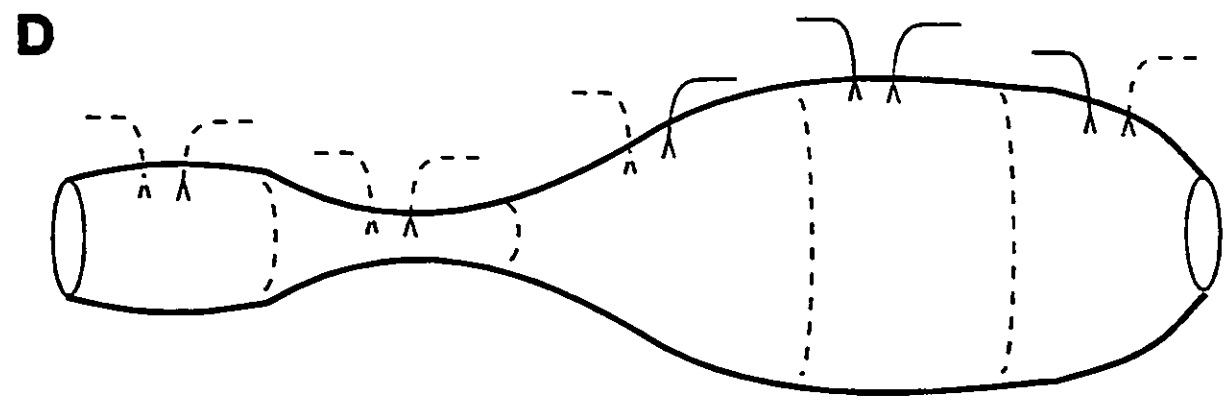

D

there is an imbalance between excitation and inhibition at this site in favour of excitation. At this region there is no descending inhibitory input (the pathway has been interrupted) but there is an activated ascending excitatory input from points immediately anal to the oral end. In the animal where the gut is continuous, the peristaltic contraction would still originate at the oral end of the distended area of the gut. There is no descending inhibitory input from the area just oral to the distended area because this area is not distended, hence there is no activation of descending inhibitory inputs. As a result of the contraction occurring at the oral end, this region is no longer distended and triggers no descending inhibitory reflexes. The region immediately anal to the contraction has now lost its descending inhibitory input but still has an activated ascending excitatory input from the distended section anal to this region. Therefore, a contraction occurs due to the net excitation and the apparent propagation of the contraction. As the new region contracts it deactivates ascending excitatory reflex to the original area of contraction. The original area of contraction returns to resting tonus since excitatory or inhibitory inputs are not activated (ie. the contents that was distending this region have been propelled anally). This process continues and the contraction propagates anally and terminates just short of the end of the segment of the gut. It cannot continue to the very end as the anal end of the gut has had its ascending excitatory reflex pathways interrupted. *In vivo*, the contraction would continue to propagate as long as the propelled contents in the lumen can distend the gut enough to trigger both reflex pathways.

Both reflex pathways are essential for peristalsis. Studies show that blocking the transmission of final excitatory motor neurons in the reflex prevents peristalsis [170]. Likewise, the descending inhibitory pathway is essential for the co-ordination of the propagated wave.

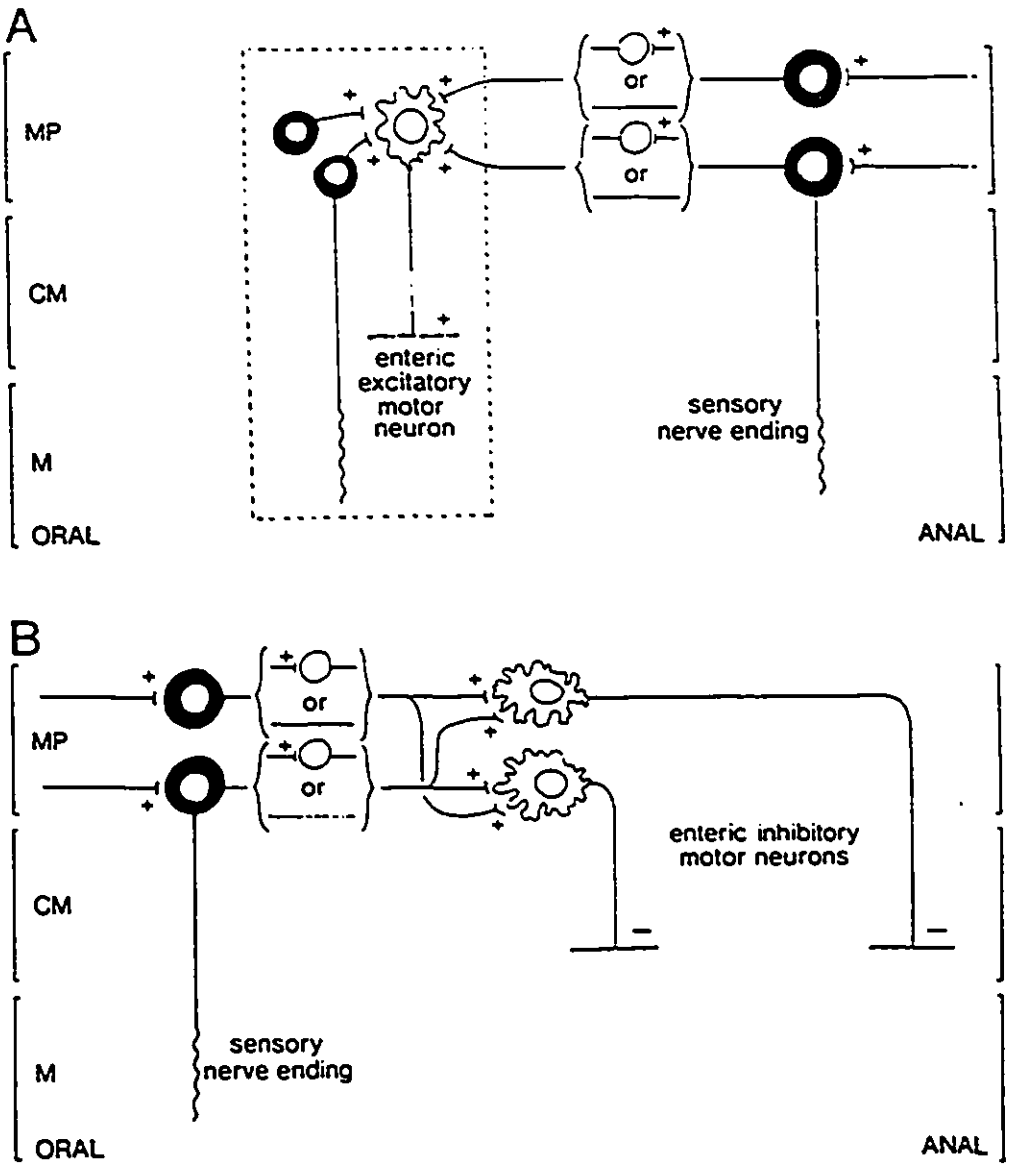
As the contraction propagates it sequentially removes the descending inhibition at the immediate region anal to the contraction. Loss of reflex inhibition would prevent this sequential removal and a contraction would occur almost synchronously along the whole length of the gut segment [170].

Peristalsis, *in vivo*, is much more complicated than the simple description presented above. For example, in a longer segment of distended intestine, the luminal contents may disperse as it is propelled anally. This would require a modification of the peristaltic wave (a change in distension threshold) for the propagation to continue. There may also be central nervous system, humoral or other gut region inputs that influence peristalsis in a region thereby modifying the rate of peristalsis based upon local digestive demands or emotional state.

Figure 5 is a simple model illustrating the basic intrinsic neuronal circuitry involving interneurons, sensory and final motor neurons (derived from functional studies) involved in local enteric reflexes [19,62]. Irrespective of the type of motility, there are two types of final neurons that innervate the muscularis: an excitatory motor neuron (induces contractions) and an inhibitory motor neuron (induces relaxations).

The mucosa is the gut tissue layer that experiences stimuli associated with luminal contents. Under physiological conditions various stimuli can evoke an enteric reflex are: i) irritation of the mucosa or mechanical stimuli, ii) exposure of the mucosa to chemical stimuli (ie salt, amino acids, fats and carbohydrates) present in the lumen of the gut, iii) distension of lumen (ie food entering the stomach). Therefore, information must be transmitted to the myenteric plexus to evoke the motility pattern. Mechanosensitive neurons located in the

Figure 5 Diagrams of basic circuit elements involved in local enteric excitatory (A) and inhibitory reflexes (B). Excitatory motor neurons are activated by the ascending excitatory reflex, while inhibitory motor neurons are activated by the descending reflex. The sensory neurons are shown in black. MP: myenteric plexus; CM: circular muscle; M: mucosa. (modified from The Enteric Nervous System, Furness & Costa, Churchill Livingston, 1987)


A
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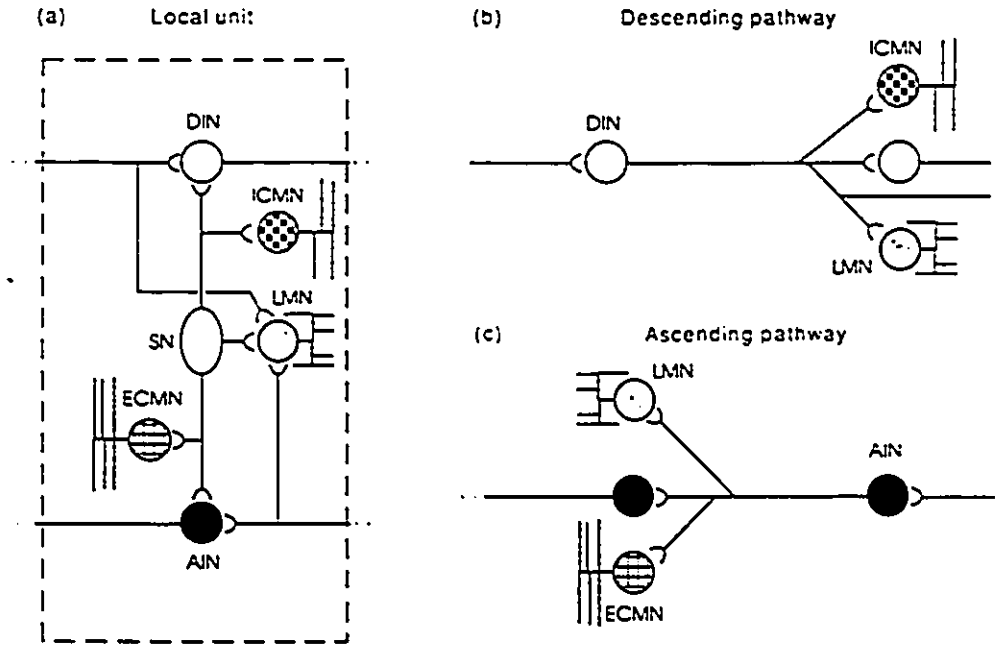
ANAL

Figure 6 1) The reflex circuit proposed by Bornstein *et al* [19]. The circuit is divided into three units: a local unit, the descending pathway and the ascending pathway. Within the local unit (a), sensory neurons (SN) project to contact descending interneurons (DIN), longitudinal muscle motor neurons (LMN), inhibitory circular muscle neurons (ICMN) and ascending interneurons (AIN). All outputs of the sensory neuron are excitatory. In the descending pathway (b), all interneurons are excitatory and contact other descending interneurons, longitudinal muscle neurons and inhibitory circular muscle motor neurons. In the ascending pathway (c), all interneurons are excitatory and contact ascending interneurons, LMN and ICMN.

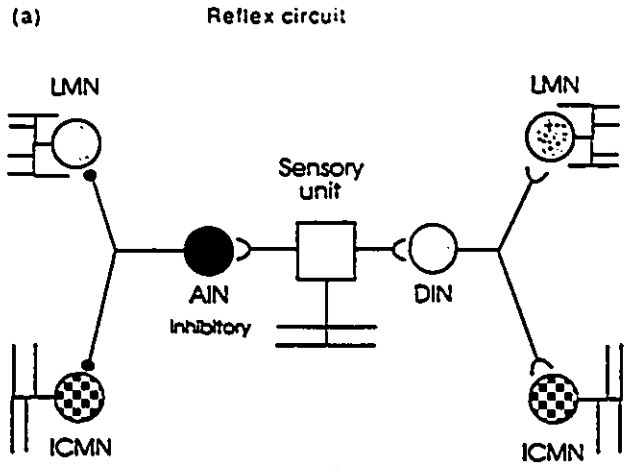
2) The reflex circuit proposed by Wood[19]. The sensory unit makes excitatory synapses with AIN and DIN interneurons. The AIN are inhibitory and contact LMN and ICMN. The ICMN are tonically active, and excitation of AIN would lead to reduction of an ongoing inhibition of the circular muscle leading to a net excitation. LMN are also tonically active, so activity in the AIN would lead to net inhibition of longitudinal muscle. On the anal side, the DIN are excitatory so that they produce increased inhibition of the circular muscle and excitation of longitudinal muscle.

Both figures were taken from Bornstein *et al* [19].

1


(a) Local unit
DIN
ICMN
LMN
SN
ECMN
AIN
(b) Descending pathway
ICMN
DIN
LMN
(c) Ascending pathway
LMN
AIN
ECMN

2


(a) Reflex circuit
LMN
LMN
Sensory unit
AIN
Inhibitory
DIN
ICMN
ICMN

submucosa and myenteric plexus have sensory nerve endings in the mucosa to respond to the stimuli. The number of sensory neurons involved is still not known. However, Bornstein *et al* proposed that sensory neurons are multi-axonal with circumferential projections that make contact with both the ascending and descending pathway interneurons, and other neuronal classes (Fig. 6) [19]. Although interneurons are involved in the descending and ascending pathways, there is disagreement on their nature. Based on combined electrophysiological, morphological and immunohistochemical data, Bornstein *et al*[19] propose a neuronal circuit where both interneuronal pathways are excitatory. In an alternative circuit model, Wood[19] proposes that the ascending pathway is inhibitory and the descending pathway is excitatory (Fig. 6). Irrespective of how these interneurons function, there are many similar local reflex units in adjacent portions of the gut. Therefore, there must be some kind of interaction between these units.

It is now accepted that enteric excitatory motor neurons are predmoninantly cholinergic, acting via muscarinic sites localized on the muscularis. Non-cholinergic excitatory motor innervation also occurs (that utilise substance P), but appears to be of little significance [33,62]. Although the intrinsic inhibitory motor innervation is primarily non-adrenergic non-cholinergic (NANC), the identity of the neurotransmitter(s) released by these neurons is controversial. Putative NANC inhibitory transmitters include: i) Purinergic, advocating adenosine triphosphate (ATP), ii) Peptidergic, forwarding vasoactive intestinal peptide (VIP) and iii) Nitrergic, involving nitric oxide (NO).

Putative NANC Inhibitory Neurotransmitters

Adenosine Triphosphate (ATP)

With the discovery that inhibitory motor control is under the influence of intrinsic NANC neurons, Burnstock and his co-workers were one of the first groups to propose a candidate inhibitory transmitter [28]. These investigators showed that NANC stimulation (via vagal stimulation) of isolated guinea-pig stomach resulted in the release of adenosine and inosine into the vascular perfusate. ATP added to vascular perfusate was found to be broken down to adenosine and inosine. In addition, application of ATP resulted in relaxations in a variety of gut preparations representing various species and sections of the GI tract. In guinea-pig ileum and rabbit stomach, however, ATP applied in high concentrations ($>10^{-4}$ $g \cdot ml^{-1}$) resulted in contractions instead of a relaxations. In rat gastric fundus not only did ATP cause a relaxation but it was followed by a rebound contraction. Relaxations in these gut segments could not be prevented by the tetrodotoxin (TTX: suppresses neuronal function through the prevention of an increase in sodium conductance in response to depolarization).

In a subsequent pharmacological study, Burnstock *et al* compared exogenous application of ATP with NANC nerve stimulation in guinea-pig (stomach, ileum, taenia coli and colon), human (colon), rat (duodenum and ileum) and mouse (duodenum and ileum) [29]. With the exception of the guinea-pig ileum, nerve stimulation caused NANC relaxations. Exogenously applied ATP also caused relaxations, and these relaxations responses matched the transmural stimulation induced responses, except in the mouse colon where the relaxation response to transmural stimulation was similar in shape but of greater amplitude. In the

guinea-pig ileum, relaxations almost always converted to a large contraction. This 'after contraction' is a well known phenomena and is deemed to be a rebound response. This ability of both neural stimulation and ATP to produce similar relaxations response was also observed in subsequent investigations in rat duodenum, guinea-pig fundus, small intestine and taenia coli, canine ileum and colon and rabbit duodenum muscle strips [5,7,15,86,87,118,120,145,146,154].

Investigators have shown that guinea-pig taenia coli can readily uptake ^{3}H-adenosine, that is converted largely to ^{3}H-ATP, and is released upon stimulation of NANC nerves [146]. Furthermore, in the guinea-pig taenia coli ATP is released during relaxation following nerve stimulation [146]. In contrast, direct muscle stimulation resulted in relaxations with no significant release of ATP.

Functional evidence for the involvement of ATP in NANC nerve mediated relaxations has been bolstered by morphological evidence in support of a neuronal localization of ATP in the gut. ATP has been localized in enteric neurons of some mammalian species using the quinacrine-staining technique[138]. Quinacrine has been shown to bind to ATP and in addition did not show fluorescence in tissue known to have only adrenergic and cholinergic nerves (ie. vas deferens) [39]. In the rat stomach, intestine and colon, rabbit stomach and intestine, and in the mouse GI tract quinacrine-positive nerve cells have been identified in the myenteric plexus [9,39,138]. Additionally in the rat ileum and colon, nerve fibres also showed quinacrine fluorescence [9]

In 1978, Burnstock suggested that ATP receptors could be classified into two groups, the P_1 and P_2-purinoceptors [119]. P_1-purinoceptors are defined by the rank order of agonist potency: adenosine> AMP > ADP > ATP [27,119]. Methylxanthines (ie. theophylline) have been

shown to be selective antagonists against these P_1-purinoceptors. The potency order for P_2-purinoceptors is opposite to that of the P_1-purinoceptors : ATP > ADP > AMP >adenosine. Currently there is no proven potent selective antagonist to P_2-purinoceptors. To date the best characterized P_2-purinoceptor antagonists are Evans blue, reactive blue and suramin [51,86,115,147,167,178]. Both groups of purinoceptors classes are not homogenous and can be further broken down into subclasses: i) for P_1-purinoceptors : A_1, A_{2A}, A_{2B} and A_3 and ii) for P_2-purinoceptors: P_{2x}, P_{2y}, P_{2u}, P_{2T}, P_{2z} of which only the subtypes 2x, 2y and 2u are present in the periphery [178]. The pharmacological subclassification of P_2 purinoceptors has been based mainly on the use of agonist potencies (see Table 1).

These classes and subclasses of ATP receptors have presented some problems in the investigation of purinergic involvement in the control of gut motility. Okwuasaba *et al* showed that theophylline caused effective antagonism of both ATP-induced and nerve-mediated relaxant responses in guinea-pig stomach fundus [5]. However, other investigators showed the opposite, in that theophylline was not effective against ATP or nerve-stimulated effects in guinea-pig fundic strips [5,87]. Small and Weston demonstrated, that although theophylline inhibited adenosine responses in rabbit duodenum, it had no effect on ATP- and electrically-evoked relaxations [154]. Using the P_1-purinoceptor antagonist 8-phenyl theophylline and 8-(p-bromo phenyl) theophylline, Frew and Lundy demonstrated that one could antagonize ATP-induced relaxations, but not electrical field-stimulated relaxations, in the guinea-pig stomach [60]. On this basis it was suggested that ATP was not a NANC neurotransmitter. It has to be noted that early studies have neglected to use antagonists to P_2-purinoceptors.

TABLE 1
Pharmacology of P_2-purinoceptors in the GUT

P_2-purinoceptor subclasses	Agonist	Antagonist	
P_{2x}	α,β-methylene-ATP = β,γ-methylene-ATP >ATP= 2-methylthio-ATP	- desensitization by α,β-methylene ATP	suramin, evans blue and reactive blue
P_{2y}	2-methylthio-ATP >> ATP> α,β-methylene ATP = β,γ-methylene-ATP	N.A.	

References: [178,48,27]

In rat gastric fundus, Matharu and Hollingsworth found purinoceptors to be responsible for mediating relaxations, and that 8-sulpho-phenyltheophylline (8-SPT: a P_1-purinoceptor antagonist) inhibited adenosine and 5'-N-ethylcarboxaminoadenosine(NECA: a selective agonist at P_1-purinoceptors) induced relaxations, but was unable to prevent ATP induced relaxations [119]. By contrast, suramin (a selective P_2-purinoceptor antagonist) antagonized ATP, but not adenosine induced relaxations. Suramin has also been found to antagonize P_2-purinoceptor agonist (α,β-methylene ATP) and electrically evoked relaxations in guinea-pig taenia-coli [86]. Additionally, in guinea-pig stomach, suramin inhibited inhibitory junctional potentials [137].

In the feline *in vivo*, ATP (0 5 -1.0 mg, i.a.) induces strong gastric contractions. However, after adding indomethacin (to prevent prostaglandin synthesis), ATP produces only gastric relaxations with time courses identical to relaxations induced by vagal stimulation [47,48]. Following administration (i.v.) of large doses of the ATP analog α,β-methylene ATP (to produce desensitization to ATP actions), vagal stimulation failed to produce relaxations whereas VIP still elicited a relaxation response indicative of selective desensitization toward ATP related events [48].

Gastric motility in response to ATP has also been assessed in the rabbit *in vivo* [4,8]. Vagal stimulation caused relaxations (followed by a rebound contraction). ATP (5 - 40 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) produced a similar relaxant response and theophylline had no influence on these responses. However, reactive blue 2 (believed to antagonize P_2-purinoceptors) reduced or blocked the ATP and vagally induced relaxation responses[4].

The evidence reviewed herein shows that ATP is a mediator of NANC relaxations and

that its actions appear to involve P_2-purinoceptors. However, it is not clear by which receptor subclass ATP may be exerting its actions. This will be resolved with the advent of specific antagonists. There is a definite need for antagonists that are specific only to a particular P_2-purinoceptor subclass. A promising antagonist is PPADS (pyridoxalphosphate-6-azophenyl-2'-4'-disulfonic acid) which has been shown to target specifically P_{2x}-purinoceptors in the rabbit urinary bladder and vas deferens [176,178].

Vasoactive Intestinal Polypeptide (VIP)

At the time of the initial studies by Burnstock *et al* that led to the 'purinergic hypothesis', Said and Mutt were isolating from porcine small intestine a 28 amino acid peptide with a structure similar to secretin and glucagon called vasoactive intestinal polypeptide (VIP) [143]. Considered a gut hormone, VIP was shown to be a potent vasodilator, a stimulator of myocardial contractility, glycogenolysis, lipolysis and insulin secretion [23,143]. In the gut VIP is known to: i) promote water and electrolyte secretion by intestinal epithelium and ii) inhibit gastric acid, pepsinogen and gastrin secretion[177].

Since its initial isolation and characterization VIP has been found in high concentrations in nerve cells throughout the mammalian GI tract: (1) including the rat and guinea-pig stomach, small and large intestine, (2) horse jejunum, and, (3) in feline pylorus[52,53,142,149]. Nerve cells demonstrating the presence of VIP were localized to the myenteric and the submucosal networks

Molecular studies show that only one major form of VIP yielding mRNA exists in the human gut [20]. This mRNA occurs only in enteric neurones and colocalizes to VIP

immunoreactive neurones.

In canine ileum and colon VIP positive varicose nerve fibres have been traced in the interconnecting nerve bundles of the myenteric plexus and innervating the circular muscle [11]. VIP immunoreactive nerve fibres innervate the myenteric plexus, circular muscle[11,52,53,149], lamina propria and mucosa [52,53,149,171] in the rat, mouse guinea pig, canine, feline and human GI tract. The circular muscle layer is more densely innervated by VIP immunoreactive fibres than the longitudinal muscle layer. Within the myenteric plexus, VIP positive fibres form dense networks around VIP immunoreactive nerve cell bodies as well as non-VIP cell bodies.

In their study of projections of intrinsic enteric neurones in the guinea-pig small intestine, Furness and Costa observed that VIP immunoreactive myenteric neurones project to the circular muscle layer and project anally providing terminations to other myenteric ganglia [34]. These VIP immunoreactive neurons were not dispersed randomly but were grouped. Some VIP immunoreactive nerve fibres also project from the intestine to prevertebral ganglia.

In the canine gut, VIP receptor binding sites have been localized in all muscle layers in the stomach, whereas in the duodenum, receptors occur only in the mucosa of the duodenum [177].

The topography of VIP positive neurons and nerve fibres from localization studies suggest a possible role as an enteric neurotransmitter. This notion has been bolstered by *in vitro* functional studies which show increases in VIP concentrations as a result of electrically stimulated relaxations of guinea pig[74] and rat[43] gastric fundus muscle strips. The neurotoxin, tetrodotoxin (TTX), prevented this increase in VIP release. In isolated muscle strips from

guinea-pig (gastric fundus and taenia coli), rat (gastric fundus) and cat (gastric fundus) applied VIP produces concentration-dependent relaxations that are insensitive to TTX [12,13,42,44,45,72,79,94].

Additional evidence for VIP to be a transmitter of motor neurones in the gut has been derived from pharmacological studies utilising peptidases, VIP antagonists and VIP antiserum. In the rat and cat gastric fundus strips, peptidases (α-chymotrypsin and trypsin) were able to prevent VIP-induced relaxations and reduce electrically evoked relaxations [42,44,94]. Similar results have been obtained when VIP anti-serum was applied to muscle strips. Both VIP induced relaxations and neurally mediated relaxations in rat gastric fundus and colon, guinea-pig gastric fundus and taenia coli, and cat gastric fundus were inhibited [42,45,72,75,76,79]. Studies using VIP antagonists to date show inconsistent results indicative of the lack of a good antagonist for this peptide. In rat and guinea-pig small intestine the VIP antagonists, VIP_{10-28}, (4Cl-D-Phe^6,Leu^{17})VIP and (Ac-Try^1,D-Phe^2)GRF1-29 (GRF: growth hormone releasing factor) proved to be as effective as VIP antiserum in reducing relaxations induced by electrical stimulation [79]. However, other studies showed (4Cl-D-Phe^6,Leu^{17}) VIP was ineffective against electrically induced relaxations in rat and cat gastric fundus whilst VIP antiserum was effective [42,45]. Similarly in guinea-pig proximal colon (4Cl-D-Phe^6,Leu^{17}) VIP and VIP_{10-28} did not affect the concentration dependent relaxations due to VIP [21]. On this basis, any conclusions for the involvement of VIP in a specific gut function cannot be drawn when only these antagonists are employed.

In an organ bath study of the rat colon, Grider and Makhlouf demonstrated the involvement of VIP in reflex motor activity [73]. An orad stretch of the colon segment resulted

in a descending relaxation occurring while a caudad stretch elicited an ascending contraction. An increase of VIP release was found to accompany only the descending relaxation. Treatment with VIP-antiserum or TTX abolished the descending relaxation. However, whereas TTX abolished the ascending contraction, VIP anti-serum treatment augmented it.

In the dog vagal stimulation produces a gastric relaxation that is accompanied by an increase in plasma VIP concentration [88,89]. Distension of the dog proximal stomach is also accompanied by an increase in portal plasma VIP concentration [32]. Furthermore, intra-arterial infusion of VIP produced a gastric relaxation in both the feline[55] and dog[89].

In the rat intra-arterial injection of VIP was found to cause an increase in gastric volume and inhibit phasic contractions [18]. Contractions were observed 20-40 minutes following the administration of VIP anti-serum. Pre-administration of VIP anti-serum was able to significantly attenuate gastric relaxations induced by VIP injections (30, 60, and 90 minutes later) by 42, 47 and 48 % respectively. Recently, Wechsung and Houvenaghel showed that VIP infused intravenously caused a decrease in porcine antral electrical activity, and in the jejunum, irregular spiking activity or phase two of MMC's were also reduced in response to VIP [172].

Nitric Oxide (NO)

NO was first determined to have a physiological significance when it was identified as a mediator of endothelium derived relaxations in vascular smooth muscles (for review see [155]). This smooth muscle relaxant action spurred investigation of NO actions in the gut. Not only is NO believed to be the elusive endothelium derived relaxing factor (EDRF),

but NO is now proposed to be a transmitter of NANC inhibitory motor neurones of the gut. Furthermore, NO is believed to be involved in: (i) in platelet function, (ii) macrophage and neutrophil action, and, (iii) smooth muscle relaxation function.

NO has some very novel properties - it is a free radical having the form of a colourless, highly diffusible gas with a short half life [148,158]. The nitric oxide synthase (NOS) gene family is responsible for the stereospecific synthesis of NO from the terminal guanidino-nitrogen of L-arginine using the co-substrates O_2 and NADPH [144]. L-citrulline is also co-produced in this reaction. NOS can be classified into 3 types, NOS-I, NOS-II and NOS-III [144] which can fall into two general categories: i) a constitutive form, NOS-I and NOS-III, where NOS interaction with calmodulin is dependent on intracellular free Ca^{2+} concentrations ii) an inducible form, NOS-II, that also binds to calmodulin but is Ca^{2+} independent [148]. The constitutive forms are believed to be localized to the brain (NOS-I), vascular endothelium (NOS-III), the gut neurones (NOS-I) and platelets. The inducible form is suggested to be involved in the immune system [112,153].

Unlike the putative NANC inhibitory neurotransmitters ATP and VIP, there is no receptor on smooth muscle cells for NO. Being a gas, NO diffuses freely into target cells where it binds to the heme moiety of guanylate cyclase [157]. This binding causes an increase in cGMP production, and in smooth muscle cells, results in relaxation. Although the lack of a specific receptor for NO has precluded the use of a receptor antagonist to investigate its action(s), inhibition of NO synthesis has provided a way to functionally investigate the role of NO in the gut. The enzymatic synthesis of NO can be competively inhibited by analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA)

and N^G-nitro-L-arginine methyl ester (L-NAME) [157].

In addition to targeting NO synthesis to provide functional information about NO, the localization of NOS within tissue has afforded insights into its possible cellular function. Localization of NO synthase is usually undertaken through NOS immunoreactivity or using the histochemical localization of NO synthase dependent Diaphorase activity [80]. In canine ileocolonic and pyloric sphincters, pig small intestine, opossum esophagus, cat lower esophagus, guinea pig stomach, small and large intestine, human small intestine and colon, monkey intestine and rat gastrointestinal tract, neurons with NOS activity are found in the myenteric and submucosal nerve networks [2,10,37,40,46,54,59,63,121,130,132,133,135,136,164,168,174].

In the rodent, where it has been most intensively studied, there are many common features in NOS distribution. Of the eight morphologically defined enteric nerve types, morphological types I and II show NOS activity in the rat whereas ganglion cells of types I, II and VI in the guinea pig demonstrate NO synthase activity [132,133]. It has been proposed that morphological type I is indicative of a population of motor neurons that are inhibitory in nature [133]. In both rodents, the highest density of NOS activity was observed in the ileum with the lowest in guinea pig proximal duodenum and rat colon [132,133]. Also, in the rat, guinea pig, cat, monkey, pig and human, NOS containing fibres are present in the myenteric plexus and fibres innervating gut smooth muscle.

Interestingly, Berezin *et al* observed in canine ileum and colon the presence of NOS immunoreactivity, not only in enteric neurons, but sparse localization of NOS within muscle cells of the circular muscle layers [11]. This observation supports a single functional study, albeit in a different species, showing that in isolated guinea pig gastric smooth muscle cells,

VIP caused not only relaxations but the release of NO [78].

Evidence for NO to be released by intrinsic enteric neurones targeting the smooth muscle has been rapidly accumulating since the initial discovery of this substance in the gut in 1990. In 1990, Bult *et al* demonstrated that electrical stimulation of canine ileocolonic junction released a substance that had vasorelaxant activity on endothelialized rabbit aorta [25]. This substance appeared to be non-adrenergic and non-cholinergic in character since neither acetylcholine or noradrenaline could elicit the same vasorelaxant activity. Furthermore, receptor antagonists to these two neurotransmitters could not block the effect of this vasorelaxant substance. However, this vasorelaxant activity could be blocked by the inhibition of NO synthesis and by treating with hemoglobin which is known to avidly trap NO. The release of this substance was sensitive to TTX and frequency dependent indicating that this substance was being released by nerves. On this basis, investigators proposed that NO was being released by NANC gut neurons. But there was a possibility that electrical stimulation of the ileocolonic neurones could be releasing a neurotransmitter that in turn could be causing the release of NO from non-neuronal cells.

Studies have reported that NO donating substances applied exogenously to isolated organ bath preparations of the gut induce relaxations similar to those elicited by NANC nerve stimulation [14,15]. In the canine, intracellular recordings of smooth muscle cells exposed to an NO donor substance displayed membrane hyperpolarization similar to the inhibitory junction potentials evoked by NANC nerve stimulation [156]. In the rat proximal colon, the NO donor, sodium nitroprusside, induced inhibitory junction potentials [150]. Inhibition of NO synthesis by L-arginine analogues have been used as pharmacological tools

to study the role of NO in gut function. As summarized in Table 2, a number of *in vitro* pharmacological studies have shown that NO mediates relaxations in different regions of the gastrointestinal tract in several species including human. In these studies, administration of NO synthase inhibitors (L-NAME, L-NNA or L-NMMA) onto the gut preparations reduced NANC relaxation induced by electrical stimulation [14,15,25]. Enantiomers of these inhibitors (ie. D-NAME) however, did not affect NANC mediated relaxations. The NOS substrate L-arginine but not its enantiomer D-arginine prevented or reversed the inhibitory effect of the NO synthase inhibitors.

Organ bath studies with guinea-pig stomach show that vagal stimulation of the stomach resulted in relaxation that could be inhibited by an NOS inhibitor [49,128]. Pre-incubation with L-arginine prevented the blockade of the induced relaxation by the NOS inhibitor. Furthermore, in one of the studies, the NO donor nitroglycerine produced TTX sensitive relaxations that were similar in profile to those induced by vagal stimulation [128]. Kanada *et al* demonstrated that the neuronal pathway involved in the descending relaxation of the peristaltic reflex in the rat ileum involved NO [96]. Small balloon distension of the gut segment produced a relaxation anal to the point of distension that was L-NNA sensitive and restorable with L-arginine.

The *in vitro* pharmacology of NO related actions coupled with the localization of NO synthesizing neural elements in the myenteric plexus and its innervations of the muscularis, provides strong evidence that NO is also a mediator of NANC induced relaxations. In the human jejunum, intracellular recordings of smooth muscle cells show that NANC stimulated hyperpolarization has two components, an initial fast hyperpolarization followed by a late

TABLE 2

An abridged summary of the species and their gut regions in which *in vitro* pharmacological studies have demonstrated that NO is a mediator of NANC induced relaxations

SPECIES	REGION	REFERENCES
Rat	gastric fundus, small intestine & colon	Li & Rand, *Eur. J. Pharmacol.*, 1990 Kanada *et al*, *Eur. J. Pharmacol.*, 1992 Grider, *Am. J. Physiol.* 1993 Middleton *et al*. *Br. J. Pharmacol.*, 1993 Kanada *et al*, *Eur.J.Pharmacol.*, 1993
Guinea Pig	stomach, duodenum, jejunum, proximal, terminal ileum & colon	Osthaus & Galligan, *J. Pharmacol. Exp. Ther* 1991 Wiklund *et al*, *Neuroscience* , 1993 Lefebvre *et al*, *Br. J. Pharmacol.* 1992 Shuttleworth *et al*, *Neurosci. Lett.*, 1991 Desai *et al*, *Proc.Natl. Acad.*, 1991 Meulemans *et al*, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 1993
Dog	ileum & colon	Boeckxstaens *et al*, *Br. J. Pharmacol.*, 1991 Shuttleworth *et al*, *Neuroscience* , 1993
Opossum	lower esophageal sphincter (LES)	Tottrup *et al*, *Am. J. Physiol.* 1991
Human	LES, jejunum & sigmoid colon	Stark *et al*, *Gastroenterol.*, 1993 McKirdy *et al*, *Exp. Physiol.*, 1992 Burleigh, *Gastroenterol.*, 1992 Boeckxstaens *et al*, *Gastroenterol.*, 1993 Keef et al, *Gastroenterol.* , 1993 McKirdy *et al*, *Digestion*, 1993
Rabbit	distal colon	Ciccocioppo *et al*, *J. Pharmacol. Exp. Ther.*, 1994
Cat	gastric fundus	Barbier & Lefebvre, *J. Pharmacol. Exp. Ther*, 1993

References: [6,15,16,26,49,70,95-97,113,116,124,125,128,129,140,151,152,156,166,173]

sustained hyperpolarization [156]. Recordings with exogenously applied NO show a hyperpolarization that only mimics the late NANC hyperpolarization. This suggests that in the human gut, NO may mediate only one component of NANC mediated relaxations.

Compared to the numerous *in vitro* studies, there are few studies demonstrating NO mediated NANC inhibitory motor activity, *in vivo*. In the anaesthetized dog, the authors showed vagal stimulation and antral field stimulation to induce an inhibitory effect on pyloric motor activity which was blocked by the intravenous administration of the NOS inhibitor L-NAME, and that could be reversed by the administration of L-arginine [3]. Lefebvre *et al* reported that L-NAME (10 $mg \cdot kg^{-1}$ & 30 $mg \cdot kg^{-1}$, i.v.) and L-NNA (10 $mg \cdot kg^{-1}$, i.v.) was able to significantly reduce or abolish vagally induced gastric relaxations in the anaesthetized rat *in vivo* [114]. Pre-treatment with L-arginine (300 $mg \cdot kg^{-1}$, i.v.), but not D-arginine, prevented this effect of L-NAME.

In another *in vivo* study of the rat, Calignano *et al* showed L-NAME (0.5-10 $mg \cdot kg^{-1}$, i.v.) increased phasic contractions and raised basal tone of the jejunum [31]. Compared to control phasic motor activity, responses in the presence of L-NAME were characterized by an increase in both the amplitude and frequency. L-arginine (200 $mg \cdot kg^{-1}$, i.v.) administration reduced the motility responses induced by L-NAME.

Taken together, the results of, *in vitro* and *in vivo* studies to date, demonstrate ATP, VIP and NO are all capable of mediating relaxations of the gut in a variety of mammalian species. Furthermore, these putative inhibitory transmitters may represent distinct NANC motor innervations since inhibitors of NOS were not able to fully block relaxations induced

by electrical stimulation[14], and NOS inhibitors have little effect on relaxations produced by VIP and ATP [14,41,116]. Despite the fact that all regions of the mammalian gut show sensitivity to these inhibitory transmitters, there appears to be some regional differences in the responsiveness to these agents which may reflect some region specificity for the respective NANC innervations.

Apamin (bee venom which blocks voltage dependent potassium channels of smooth muscle cells) distinguishes two types of relaxations in the guinea-pig, one apamin sensitive and the other apamin insensitive [36]. Relaxations caused by the ATP P_{2x}-purinoceptor agonist, α,β-Me ATP were blocked by apamin while VIP induced relaxations were not. Moreover, apamin showed regional differences in its ability to block the stimulation of inhibitory neurons. In the antrum, ileum, taenia coli and distal colon longitudinal muscle, apamin showed great effectiveness. In contrast apamin had little effect in the fundus, proximal colon and distal colon circular muscle. The differentiation of NANC inhibitory innervation into distinct ATP, VIP and NO categories may be too simplistic, since NO and VIP in the rat gastric fundus have been proposed to be dual components of relaxations, with NO mediating the fast component of the relaxation and VIP being responsible for the following slow component [41,116].

In guinea pig ileum, transmural stimulation produced fast inhibitory junction potentials (IJP) that were abolished by apamin, α,β-Me ATP and reactive blue (P_2 purinoceptor antagonist) but not VIP_{10-28} (VIP receptor antagonist) [38]. In the presence of apamin, transmural stimulation produced TTX sensitive IJP's that were blocked by VIP_{10-28} but unaffected by α,β-Me ATP.

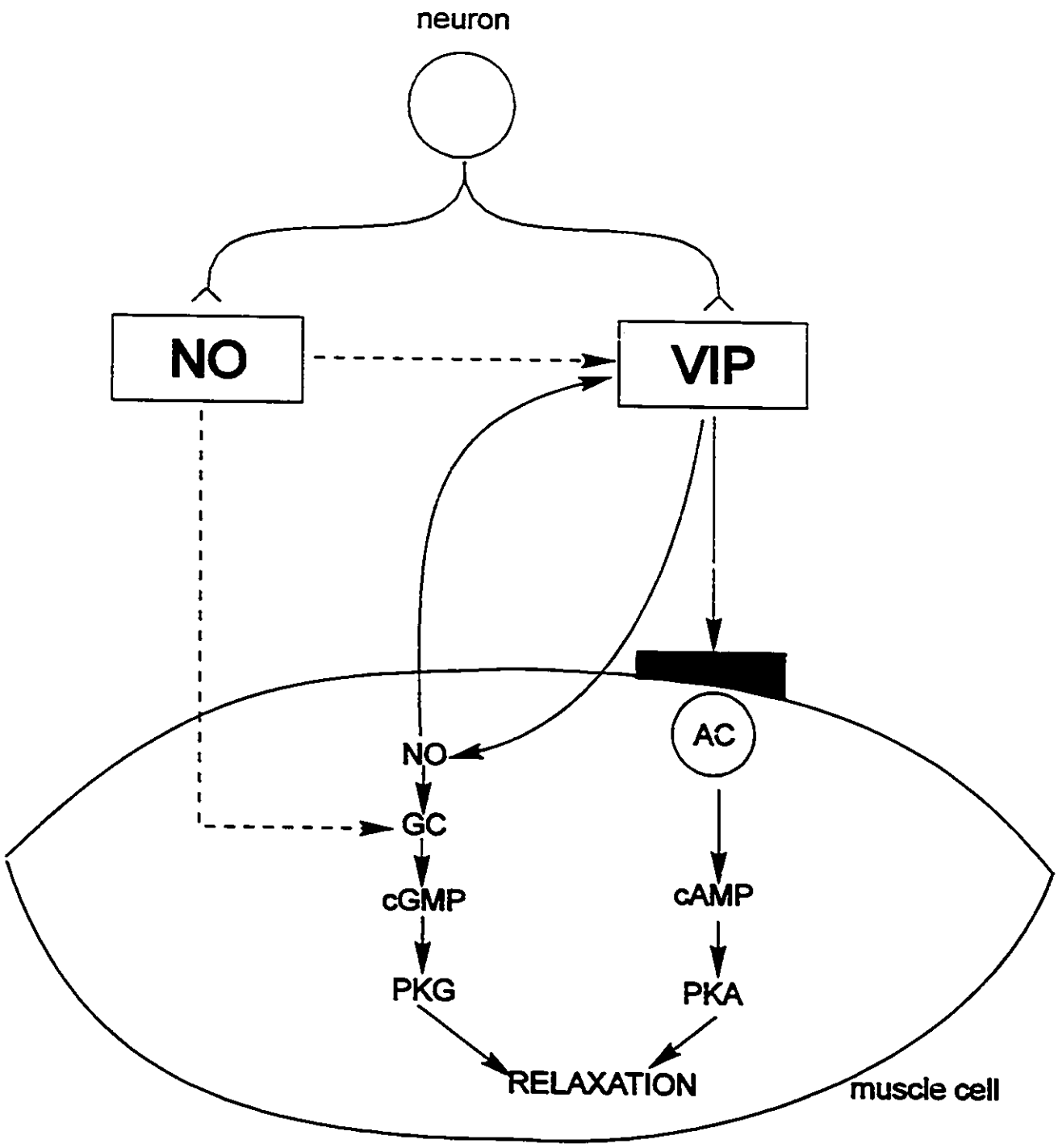
In the human colonic circular muscle, two components involving ATP and NO were demonstrated [97]. Neurally evoked hyperpolarizations revealed two components. Upon a single stimulus the hyperpolarization produced could only be blocked by apamin but not L-NAME. Repeated stimulus revealed a more sustained hyperpolarization that could be antagonized by apamin and L-NAME.

In the ferret, gastric relaxations involve both VIP and NO [81]. Gastric corpus vagal stimulation *in vivo* resulted in a decrease of intracorpal pressure that had two components; an initial rapid component followed by a slower decline for the duration of the stimulation. L-NAME treatment antagonized the initial component leaving the slower component untouched. In ferrets autoimmunized to produce VIP anti-bodies vagal stimulation again produced a two component relaxation but the second was significantly reduced.

In addition to the notion that ATP, VIP and NO mediate separate NANC inhibitory innervations of the gut muscularis, there is accumulating evidence for the colocalization and release of one or more of these agents from the same neurons [9]. A subpopulation of quinacrine-florescent neurones (evidence of ATP localization) in the myenteric plexus of the rat ileum were also reactive for NADPH-diaphorase (demonstrating NOS localization). Whereas almost all quinacrine-fluorescent myenteric neurones in the rat colon were NADPH-diaphorase reactive. In addition, in the canine small intestine and colon, double staining for NOS and VIP revealed that both VIP and NOS were present in the same myenteric nerve varicosities but not in the same organelles [11]. In guinea pig and rabbit gastric fundus smooth muscle cells but not rabbit taenia coli, VIP was shown to not only induce relaxations but stimulate NO production [78,131]. L-NNA was able to abolish all NO production and partially

reduce VIP production and relaxation response. Grider proposes that VIP and NO are both neurally released but that NO is also produced in the smooth muscle cell (Figure 7) [70].

Figure 7 A model illustrating the interplay of VIP and NO during neurally induced relaxation. VIP upon release can stimulate two relaxant pathways i) a cAMP dependent pathway and ii) a cGMP pathway that is activated as a result of VIP stimulating smooth muscle cells to produce NO. NO can also be neurally released to cause relaxations via the cGMP pathway. GC: guanylate cyclase; AC: adenylate cyclase; PKG: protein kinase G; PKA: protein kinase A. Taken from Grider, J., (1993), AMJ [70].


neuron
NO
VIP
NO
AC
GC
cGMP
cAMP
PKG
PKA
RELAXATION
muscle cell

Rationale

Of particular interest to researchers in this laboratory is the study of duodenal ulceration (DU). This complex multifactorial disease involves hypersecretion of gastric acid, impaired mucosal defense mechanisms and alterations of gastroduodenal motor activity [22,159]. In combination with antibiotic treatment to eradicate the gut pathogen *H. Pylori*, therapeutic approaches have focussed on the acid secretory aspects of the disease by using drugs that reduce acid production either through H_2 receptor blockers or the blocking of the parietal cell proton pump. Although acid hypersecretion and duodenal mucosal defense impairment are important to the disease process, these do not explain the location or morphology of lesions in the duodenal bulb [161].

Early studies suggested that gastrointestinal motility changes are important to the pathogenesis of DU, and this may explain the localization of the lesions in the duodenal bulb [161]. The size of acid load in the duodenal bulb is dependent on the rate of entrance to and clearance from the duodenal bulb. This rate of entrance and clearance is related to gastroduodenal motility.

The relationship between gastroduodenal motility and the onset and development of duodenal ulceration is poorly understood. Most of our knowledge about DU pathogenesis has been derived using the cysteamine-HCl rat model [160], where the chemically induced duodenal ulcers have a pathology and morphology similar to the human disease condition. Ulceration is induced quite rapidly (< 24 hrs) in the duodenal bulb without effects in other areas of the gut. Despite a number of *in vivo* studies of gastroduodenal motility following cysteamine-HCL treatment, these lack proper recording and analysis of gut motility patterns. Researchers

in this laboratory have investigated the disruption of gastroduodenal motor activity in response to cysteamine-HCl treatment in awake Sprague Dawley rats utilising serosally implanted foil strain gauges that afford sensitive detection of both contractions and relaxations.

In conscious unrestrained rats cysteamine-HCl treatment immediately disrupts gastroduodenal motility as shown by McKay *et al*[122,123]. In the gastric antrum, contraction amplitude and frequency are significantly increased and antral relaxations are abolished. Similar to the antrum, contractile activity in the proximal duodenum is enhanced while relaxant activity is diminished. Furthermore, disruption of duodenal motor activity was confined to the first 2 cm of the proximal duodenum

In vitro studies of the rat intestine in this laboratory show that the ulcerogen cysteamine-HCl targets three neurogenic sites: i) stimulatory sites on intrinsic NANC inhibitory motor neurons ii) inhibitory sites on NANC neurons and iii) inhibitory sites on cholinergic excitatory neurons [101,109]. In addition, it was demonstrated that cysteamine-HCl can also cause contractions of rat stomach strips via predominantly myogenic actions at muscarinic receptors [101]. Thus the enhanced antral contractility seen with cysteamine-HCl, *in vivo* (observed by McKay and Krantis) may due to the ulcerogen's effect at muscarinic receptors. However, the action of cysteamine-HCl in the duodenum, *in vivo*, does not correlate with any stimulation of NANC inhibitory motor neurons as might be expected from the *in vitro* pharmacology. Duodenal relaxations were diminished following cysteamine-HCl treatment, suggesting that the ulcerogen was in all likelihood causing an inhibition of, or else interfering with, duodenal NANC inhibitory motor neuron activity. Concomitant with

diminished relaxant activity, there was hypercontractility of the duodenum.

The nature of the disruption of gastroduodenal motility in the development of duodenal ulceration is not clear. Studies with the cysteamine-HCl animal model suggest myogenic and/or neurogenic actions dependent upon the gut region. If part or all of the ulcerogen's action is neurogenic, the identity of the neural elements affected may reveal important insights into the development of DU. Although it is accepted that excitatory motor innervation in the mammalian gut is predominantly cholinergic, the NANC inhibitory innervation of the gastroduodenum (which in experimental duodenal ulceration appears to be affected) is not fully understood. Therefore, evaluation of gastroduodenal motility following cysteamine-HCl treatment remains problematic.

Thus, normal gastroduodenal motor activity must be first fully characterized in order to achieve this goal. This forms the underlying rationale for my studies. **More specifically, I sought to examine the nature of the inhibitory innervation of the gastroduodenum by examining spontaneous and evoked motor activity in anaesthetized Sprague Dawley rats.** This work was undertaken in two parts and is presented as such in this thesis.

A. **In the first part, I describe the refinement of methodology**. The method developed in this laboratory for motor activity recordings in the conscious rat was adapted here for use in anaesthetized rats. In addition, the data acquisition system was converted to a direct IBM based online system and software was refined to allow calibration of recorded responses. In addition *ex vivo* preparations of the intact gut were used to validate the sensitivity of extraluminal foil strain gauges to record activity from the gastric antrum and the proximal duodenum following direct application of drugs onto the gut wall. Further validation of

the recording/analysis software was undertaken by examining spontaneous contractions and relaxations of the gut *in vivo* pre and post treatment with the ulcerogen cysteamine-HCL. Cysteamine-HCl injection was used to test for the ability of the recording/analysis technique to detect and quantitate alterations in motility.

B. **In the second part, I describe an investigation of the roles of nitric oxide, adenosine triphosphate and vasoactive intestinal peptide in relaxations of the stomach and proximal duodenum, in order to determine the nature of intrinsic inhibitory motor innervation of the rat gastroduodenum.** More specifically I examined whether NO, ATP or VIP are involved in spontaneous relaxant motor activity in the gastroduodenum, and whether specific patterns of relaxations in the gastroduodenum *in vivo* can be distinguished based on their sensitivity to manipulations either of these transmitters, or of their target sites. In addition, the neural pathways controlling gastroduodenal relaxations were investigated with particular attention paid to the involvement of GABAergic interneurons.

CHAPTER 1

REFINEMENT AND VALIDATION OF METHODS

Reflex relaxation is a key component of peristalsis and as such, determining the nature of this motor activity under different physiological conditions can provide valuable information about normal gut motor function. Although many *in vitro* studies have investigated reflex relaxation, *in vivo* studies have paid little attention to this response, due in part to the techniques employed which, although sensitive for recording contractile activity did not adequately detect contractions and relaxations together.

During her PhD research in this laboratory Dr. Allison McKay sought to develop a reliable, sensitive and inexpensive method for simultaneous recording of gastrointestinal relaxations and contraction, *in vivo*. This work was successful and the findings were presented to the Canadian Physiological Society (1991)[122]. Essentially, the method involved the attachment of foil strain gauges to the serosal surface of the stomach and duodenum and the monitoring of motor activity in conscious unrestrained Sprague Dawley rats. Data was recorded directly on polygraph and onto video tape for later input to an IBM PC for analysis. Also developed was a computer based analysis technique that would allow the differentiation and quantification of these motor activities. These studies formed the baseline for the initial phase of my research which sought to adapt the method to record gastroduodenal motor activity from the anaesthetized rat, *in vivo*. In addition to establishing the profile of motor activity under resting condition in anaesthetized rats, I also sought to characterize the pharmacology of relaxant activity in the rat gastroduodenum, *in vivo*. In particular, I

examined the role of ATP, NO and VIP in relaxations of the stomach and proximal duodenum, in order to determine which, if any, of these putative inhibitory transmitters are involved in intrinsic inhibitory motor innervation of the rat gastroduodenum.

The first section of this chapter describes the methodology adapted from *in vivo* conscious animal studies for use in anaesthetized rats *in vivo* using a direct online data acquisition system.

The aims of this study were:

A. **To convert from an indirect online data acquisition system to a IBM based direct online data acquisition system.** This was achieved in two part i) In the first part of this work the existing recording and analysis system was further refined with the collaboration of Dr. F. Johnson (Institute of Medical Engineering, University of Ottawa). Refinement of the recording method utilising foil strain gauges involved the following: Previously, data was recorded onto a converted VCR capable of recording data onto VHS video tapes and then later down loaded onto a computer. With the direct online system, the computer was now capable of directly acquiring and storing the data. The data recorder/VCR based system was susceptible to loss of data when motor activity that drifted out of range had to be brought back into range through baseline changes; likewise for large responses which moved off scale. In addition, the sensitivity could be decreased to increase range but not without the penalty of decreased sensitivity to recording some motor events. These problems were circumvented by the direct online system which provided increased recording range and resolution. Therefore, the need to change the baseline was all but eliminated. Furthermore, this allowed for the evaluation of relative changes in smooth

muscle tone (baseline). The increased range also allowed for the capture, in detail, of large changes due to drug application without a loss of sensitivity. Overall, the direct online system allowed for an accurate, precise uninterrupted real time recording of gastroduodenal motor activity.

ii) Another shortcoming, of *in vivo* recording of motor activity was the likelihood of recording artifacts. This was addressed by implementing in the software, a method to filter out artifacts without hampering computer analysis of motor events.

B. **To validate the reliability of using foil strain gauges in recording both contractions and relaxations in the gastroduodenum of the anaesthetized rat using an *ex vivo* adaptation of this method.** These *ex vivo* experiments tested the ability of the foil strain gauges to respond correctly to both types of motor events in the gastric antrum and the proximal duodenum.

C. **To determine the normal patterns of contractile and relaxant gastroduodenal motor activity in the anaesthetized rat *in vivo*.**

METHODOLOGY

The methodology used was modified after the method of McKay, A. (1992: PhD thesis, U of Ottawa). An illustration of the experimental setup is shown in Figure 8.

Surgery

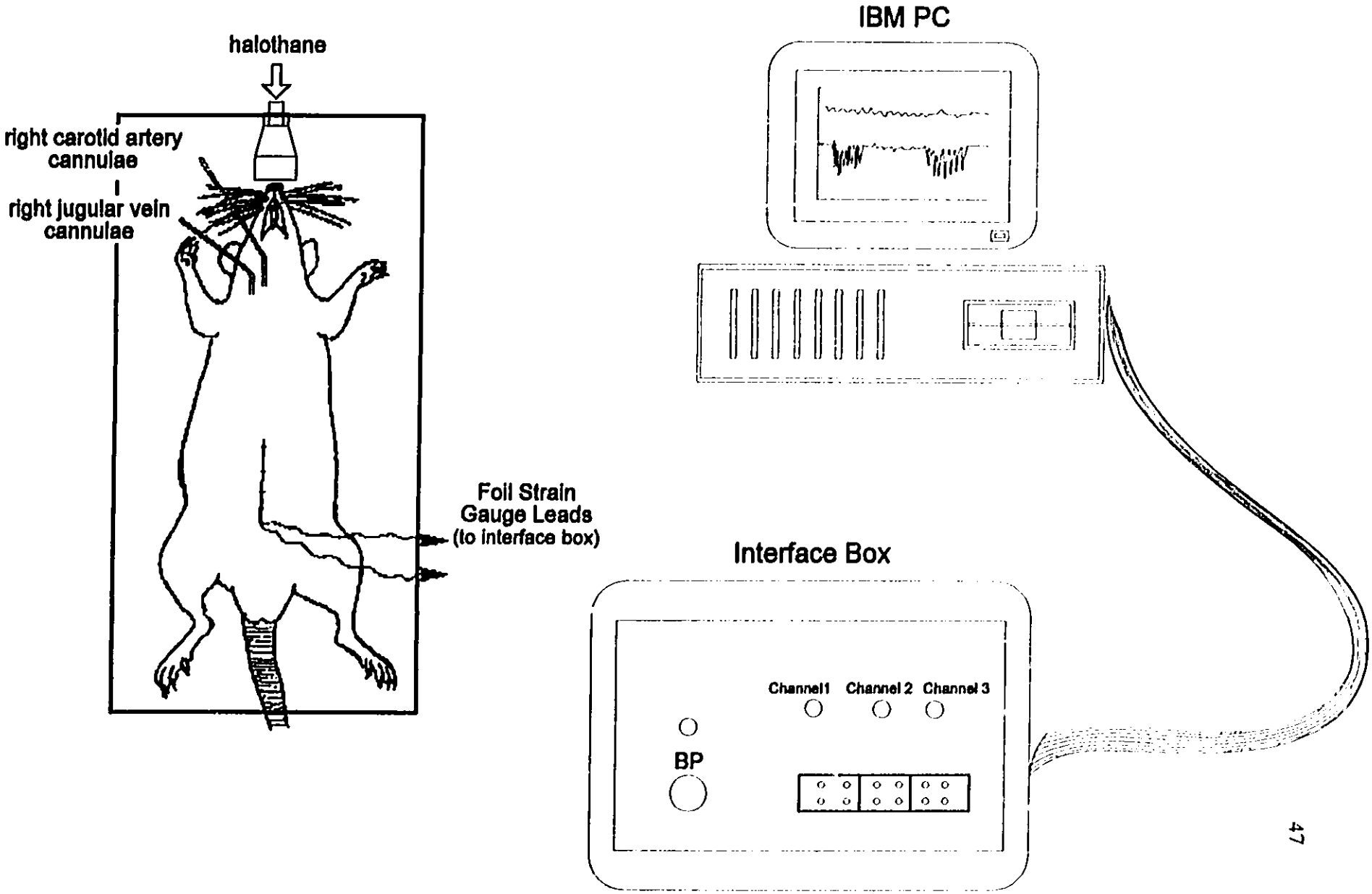
Male, Sprague-Dawley rats (250-400g) were fasted for 24 hours with free access to water prior to surgery. Rats were placed into an induction box and anaesthetized with halothane gas (3.0-4.0%, O_2 flow rate: 500 ml·min^{-1}). The proper plane (surgical anaesthesia)

of unconsciousness was determined using a tail or toe pinch test. The absence of a reflex response by the animal to the pinch indicated that the animal was under the proper level of anaesthesia. The rat was then transferred onto an aluminum scavenging table that was maintained at a temperature of 37°C with a water-circulating heating pad. The level of halothane was then reduced and maintained at 2.0 - 3.0% for the remainder of the surgery.

Blood pressure (mean BP) was monitored continuously by means of a pressure transducer (Gould Statham, USA, P23 ID) connected to a cannula (PE50 polyethylene tubing, Intramedic) inserted into the right carotid artery. The blood pressure transducer was subsequently attached to an IBM PC data acquisition system via an interface box (channel 4). Cannulation of the right jugular vein allowed for i.v. drug infusion. The midline neck incision made for the cannulation of these two vessels was closed with stainless steel wound clips (9 mm, Clay Adams, USA).

Following a mid-line laparotomy the abdomen was exposed using two hemostat clamps. Using two saline soaked cotton-tip applicators the stomach was gently exteriorized along with a 5-8 cm segment of the proximal duodenum onto saline soaked gauze pads. Two foil strain gauges were then glued using Vet Bond glue (tissue adhesive, #1469, 3M Company) to the serosal surface of the stomach and the proximal duodenum (Fig. 9). The first foil strain gauge (S_1) was glued onto the gastric antrum approximately 2 cm proximal to the pyloric sphincter,while the second gauge (D_1) was glued onto the anti-mesenteric border of the duodenum, 2 cm distal to the sphincter. Both foil strain gauges were glued parallel to the axis of the longitudinal muscle layer. Some experiments required the use of a third foil strain gauge which was also located on the gastric antrum (S_2). However, the S_2

Figure 8 Diagram illustrating the experimental setup for recording motor activity from the rat gastroduodenum using a IBM based data acquisition system


halothane
right carotid artery cannulae
right jugular vein cannulae
Foil Strain Gauge Leads (to interface box)
IBM PC
Interface Box
Channel1
Channel 2
Channel 3
BP

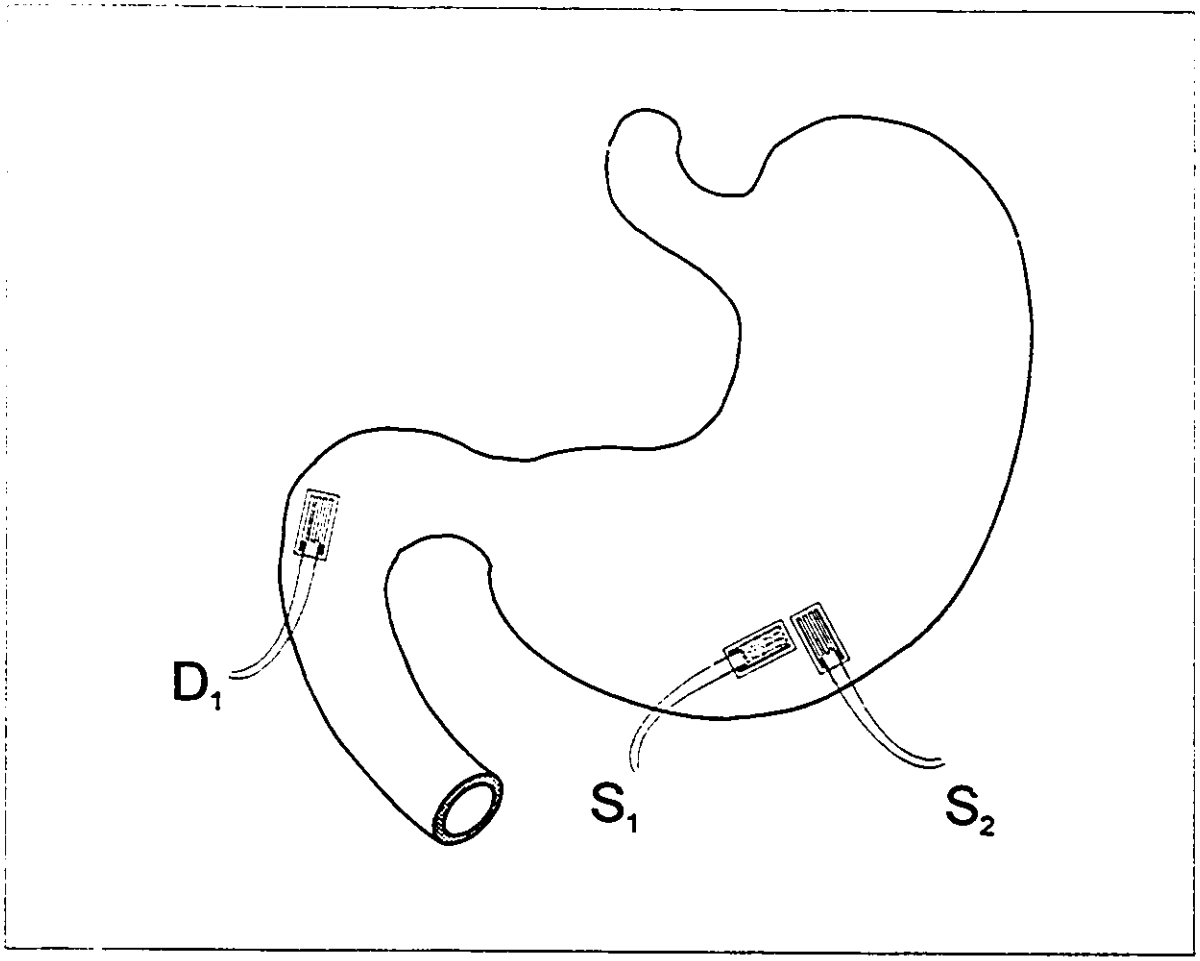


Figure 9 A schematic illustration of the placement of the foil strain gauges on the serosal surface of the rat gastroduodenum for *in vivo* recording of motility. S_1 and S_2 represents the antral foil strain gauges, positioned approximately 2 cm proximal to the pylorus. D_1 represents the strain gauge positioned on the anti-mesenteric border of the proximal duodenum, approximately 2 cm distal to the pylorus. The S_1 and D_1 foil strain gauges were attached oriented to the longitudinal muscle layer. The S_2 foil strain gauge was attached juxtaposed to the S_1 foil strain gauge but oriented to the circular muscle layer.

foil strain gauge was glued juxtaposed and with its orientation perpendicular to the S_1 foil strain gauge. At the distal end of the closure the wire leads attached to the foil strain gauge were exteriorized so that the leads could be attached to the IBM data acquisition system via an interface box (channel 1: S_1; channel 2: D_1; channel 3: S_2) (Fig. 8). Closure of the abdominal incision was accomplished using a simple continuous stitch suture (3-0 or 4-0 coated vicryl suture, round needle) for the fascia and stainless steel wound clips for skin. The animal was then placed in a prone position, covered with a blanket to help maintain body temperature, and the percentage of halothane reduced to 1% and maintained at this level until the end of the experiment.

After surgery the animal was allowed a one hour period of stabilization. A blood pressure level between 70 to 100 mmHg was deemed acceptable. All animals that were subjected to drug treatment had a one hour control period recording before treatment was administered. Post-treatment recordings were carried out for up to 4 hours.

Analysis

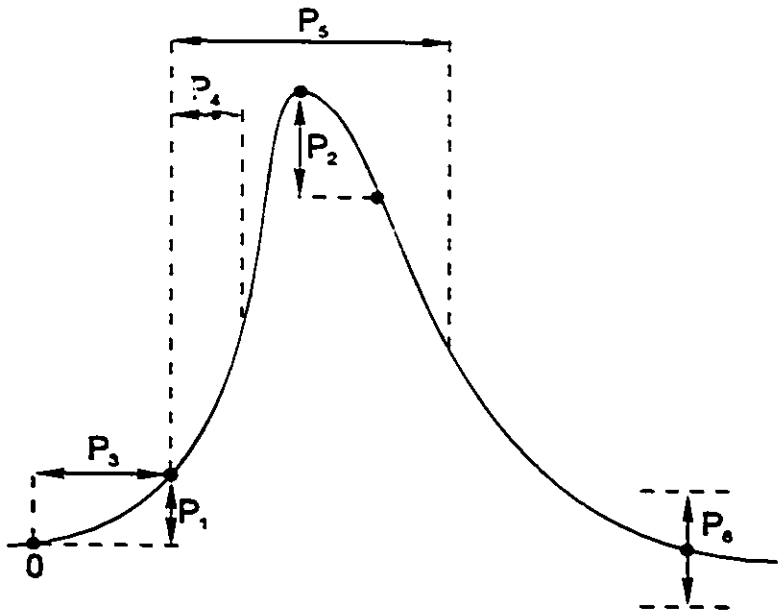
During the experiment an IBM data acquisition system (IBM 486 with AD1000 analog to digital conversion card, Real Time Devices, Inc.; acquisition software: AQ, Dr. Frank Johnson, Medical Engineering, University of Ottawa) acquired, digitized and stored the motility data. The digitized files (motility recordings) were then analyzed with a proprietary software program (GI-Analysis: CGIQTRANS, Dr. F Johnson & C. Wood, Med. Eng., U. of Ottawa). This program afforded us the ability to simultaneously analyze the files for contractions and relaxations. The CGIQTRANS program was able to accomplish this using

an algorithm that required the utilization of six parameters. The parameters (which are definable) detected an event on the basis of thresholds being all satisfied within certain time parameters. There are two identical sets of six parameters, six to identify contractions (P_1-P_6), six for relaxations (N_1-N_6). The method of analysis is described below with reference to Figure 10 which illustrates the parameters for a contraction but the algorithm for analysis is the same for relaxations. The algorithm sequence is the following:

1. A baseline is assumed and can be any starting point.
2. A deviation from the baseline of a value of P_1 within the time period of P_3 satisfies the initiation of a contraction event. The program will begin to summate the area under the event. Once the computer has found the initiation of an event no other event can be searched for until the end of P_5.
3. After the time of P_4 and before the time of P_5 a fall of P_2 from the maximum value in that time window is searched for. If this is satisfied the program realizes a contraction peak and records the amplitude and the time to peak.
4. The program now examines whether the return portion or the descending part of the event returns to within the P_6 of the initial baseline, if so all parameters have been satisfied and a contraction event is scored. The time of the event, duration and the final area can be computed and stored.

When all of these parameters are satisfied a contraction response was recorded as a separate event.

Each channel (channel 1: S_1 and channel 2: D_1) for a particular experiment was analyzed separately. Analysis consisted of two iterations. The first involved going through the



0 - Baseline

P_1- Threshold

P_2- Threshold for peak maximum

P_3- Time(s) for peak minimum

P_4- Slope minimum

P_5- Slope maximum

P_6- Return to baseline

Figure 10 Schematic representation of a typical response (contraction) illustrating the threshold and time parameters (P_1- P_6) used in the detection of motor activity.

entire channel in two minute segments to determine which numerical value assigned to each parameter would correctly score motor events within the segment. (Two minute segments were chosen because motor events were more easily identified hence allowed for accurate identification of motor events.) For each two minute segment any or all of the parameters could be redefined to ensure that analysis accuracy remained high (90 - 100%). For each two minute segment, the parameters (P_1-P_6 for contractions and N_1-N_6 for relaxations) deemed acceptable were recorded on paper for the second iteration. The second iteration also involved going through the entire channel in two minute segments chronologically. However, for each segment the parameters recorded for that segment were keyed into the CGIQTRANS program and the program was then requested to analyze that segment and store the information on the scored motor events into an output file.

The CGIQTRANS during analysis of a file was able to separate relaxation and contraction information into different output files. The CGIQTRANS program outputs three files per channel for each experiment. One file contained information about frequency (events per minute) of both contractions and relaxations. The second file stored information on the time a scored event occurred, the area, amplitude, time to peak (tpk) and duration of each contraction that occurred. Likewise, the third file contained the same information, but for relaxations.

This information was then imported into a spreadsheet (Quattro Pro, version 3.0) with a macro that collated the data and stored the information in tabular form for export to a statistical software program.

Identification of an Event

For proper analysis of motility recordings certain criteria was used in aiding the determination of (i) what was an even and (ii) if it was a contraction or a relaxation. All motility recordings have a baseline level of tonic and phasic activity. In a majority of the experiments baseline tone was stable (there was no drift). Two criteria were used for event identification:

i) A contraction was identified as a positive deflection above the baseline whereas a relaxation was a negative deflection below the baseline. These deflections be it a contraction or relaxation must return to within 90% of baseline activity level.

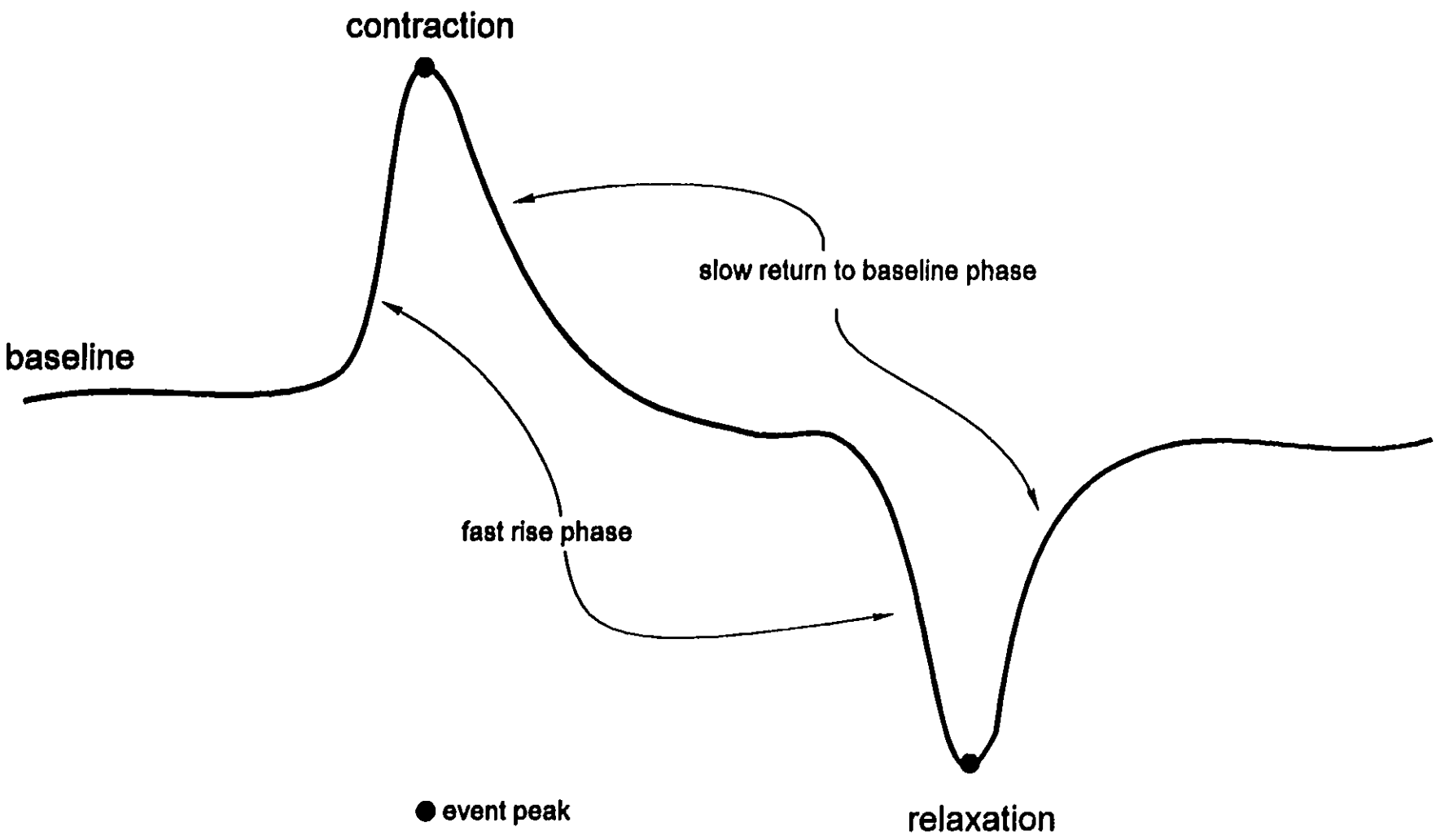
ii) The profile of the event also aids in event identification. For example, contraction is characterized by a fast ascent followed by a slower descent or return to baseline (Fig. 11). Likewise the relaxation profile follows a fast descent followed by a slower ascent or return to baseline.

Dealing with Artifacts

With recording of motor activity in the anaesthetized rat there are no movement artifacts with which to contend. However, there is a respiratory component that is recorded. This artifacts was more prevalent at the S_1 recording site than at the D_1 recording site. Although, respiratory artefact did not hamper the qualitative observations of motor activity, it did reduce the accuracy of the software in identifying motor events. As a refinement, the analysis software allowed the investigator, as an option, to choose a filter as an option. Filtering was achieved using a simple moving average smoothing procedure. This filter calculated a moving average for a time series by averaging a selected number of points on

Figure 11 A schematic diagram illustrating the typical profile of a contraction (a positive deflection) and a relaxation (a negative deflection). The profiles for the contraction and relaxation both show the characteristic fast rise to peak followed by a slower return to baseline.

Typical profile of a Contraction and Relaxation



either side of a target value. The number of points was definable. It was critical that the number chosen filtered out the respiratory artefact and not distort the motility data. Since the respiratory frequency was much higher than any frequency of motor events there was no difficulty in defining a number to filter out the respiratory artifact.

Statistical Analysis

Spreadsheet software (Quattro Pro) prepared the data for statistical analysis. The 3 output files for a particular channel of an experiment were imported into three separate Quattro Pro files. In each file a macro stripped the imported data of all textual information and grouped the data into control and treatment periods. Motor activity in the proximal duodenum (D_1) consisted of two distinct patterns which were termed 'Grouped' and 'Intergroup'. Therefore, experimental data from channel 2 (D_1) was further separated into 'Grouped' and 'Intergroup' events to test if drug treatment differential affected the events within these distinct duodenal motor patterns. In order to do this the experiment from which the data files came from were observed using the CGIQTRANS program. Channel 2 was observed and the times and the duration of 'Grouped' and 'Intergroup' periods were visual determined and recorded. With this information and the fact that all events in the data imported into Quattro Pro had a time of occurrence label, separation of the data into 'Grouped' and 'Intergroup' periods was achievable.

For this study the parameters analyzed were; i) the amplitude (μm) of the event and ii) the frequency of the events (events per minute). An ANOVA with a Tukey multiple comparison test (Statsgraphics Plus, version 5.2) was used to determine significance between

the treatment period and the control period data. A $p<0.05$ was considered statistically significant. All tabulated results are expressed as a average of the mean ± SEM (μm) from each experiments.

Chemicals

Halothane was obtained from Animal Care Services at the University of Ottawa. The drugs that were used were: adenosine triphosphate (ATP), carbachol, cysteamine-HCl and papaverine (Sigma Chemical Company, St. Louis, MO, USA).

Foil Strain Gauge Preparation

For the motility recordings Showa foil strain gauges (N112-FA-1-120-11, Durham Instruments, Pickering, ON, Can.) were used. Before use, the foil strain gauges were prepared as follows. The wire leads from the foil strain gauges were cut to approximately 0.5-0.8 cm in length. Two plastic insulated (Alpha Wire Co., 32 AWG,) wires were cut to 30 cm in length. Both ends of the wire were stripped to expose approximately 0.5 cm of bare wire. Each stripped end of a wire were dipped in soldering paste, tinned with solder and then each end was soldered to one of the foil strain gauge leads. The foil strain gauges were then coated once with Gagekote #8 liquid acrylic (Durham Instruments) to prevent the foil strain gauge leads unit from causing electrical shorts and allow for reuse. The whole strain gauge, the soldered joint, and approximately 0.5 cm of insulated wire at the soldered end are coated with the liquid acrylic. To confirm that the strain gauge was still patent, the resistance of the strain gauge assembly was measured to ensure that it was approximately 120 Ω. The plastic

insulation of the wires are colour coded for proper identification during and after surgery (S_1 strain gauge: red; D_1 strain gauge: green and S_2 strain gauge: yellow).

After use, the foil strain gauges were recycled for future use in the following way. Under a dissecting microscope with the use of a scalpel and two fine tipped forceps the Gagekote was very carefully peeled off the used foil strain gauge. The foil strain gauge was then examined under the microscope for any damage and then the resistance was measured (refer to above for acceptable resistance). Any foil strain gauge with the proper resistance and no damage was recoated with Gagekote for future use. A foil strain gauge was used for up to three experiments before being discarded.

Calibration of Foil Strain Gauge

In essence, any motility event recorded is observed by the data acquisition system as a change in voltage. These changes in voltage are a result of a change in displacement of the foil strain gauge (which causes a change in resistance). The total displacement range of the showa foil strain gauges prepared with Gagekote is represented by a -5 to +5 volt range. This range is be applied to all motility recordings during acquisition. During acquisition for computer storage and manipulation, the recordings must go through an analog to digital process. This process takes the -5 to +5 volt range and converts it to an -2048 to 2048 digital unit (A/D units) range. Calibration studies (with C. Wood, Medical Engineering, University of Ottawa) with strain gauges set up in a displacement jig and subsequently attached to the data acquisition system have shown that a 64 μm displacement of the strain gauge results in a 38.07 change in A/D units on the acquisition software.

Validation Experiments

Ex vivo experiments were carried out to validate that serosally placed strain gauges were indeed capable of recording both contractions and relaxations. This was accomplished by recording the responses to known contractile and relaxant agents applied directly onto the serosa of the stomach and proximal duodenum. Male Sprague Dawley rats were surgically prepared as they were for the *in vivo* studies except the animals were stabilized for 60 minutes in a supine position. After the stabilization period, the abdominal cavity was reopened and exposed using 2 hemostat clamps The stomach and a segment of proximal duodenum with their serosally glued strain gauges were very gently exteriorized onto saline soaked gauze pads. Care was taken to ensure that the strain gauges were orientated parallel to the longitudinal axis of the gut. The gut was kept constantly moist with saline (37°C).

At the S_1 site, 100 µl of the cholinergic muscarinic agonist, carbachol (a contractile agent) was serosally applied close to the strain gauge. Excess drug was soaked up using gauze pads and cotton tip applicators, thus preventing the drug from entering the abdominal cavity. Responses were recorded by the attached IBM data acquisition system. The same protocol was used for the D_1 recording site. To test for relaxation responses, the smooth muscle relaxants papaverine, and ATP were used. When testing papaverine in the proximal duodenum the tissue was first precontracted with carbachol and then papaverine was applied. ATP was applied with no pre-contraction sequence. All agents were dissolved in saline heated to 37°C so as not to induce a temperature response upon application.

EXPERIMENTAL PROTOCOL

All experiments had an initial 60 minutes of control period of continuous recording. This allowed each rat to be its own control so pre and post treatment regions could be compared. Rats were divided into treatment groups as follows:

Chapter 1

Group 1: Control experiments determined normal motor activity in the gastric antrum and proximal duodenum under anaesthetized conditions.

Group 2: Saline (bolus injection of 0.9% saline i.v.) treated rats allowed for the assessment of vehicle effects on motor activity.

Group 3: The ulcerogen cysteamine-HCl (560 $mg \cdot kg^{-1}$) was injected subcutaneously after 60 minutes of control recordings. This drug tested the ability of the recording/analysis technique to detect and quantitate alterations in motility. This protocol was adapted from that used by Dr. McKay in her PhD thesis (University of Ottawa, 1992).

Chapter 2

Group 4: After 60 minutes of control recording rats were injected with L-NAME (10 $mg \cdot kg^{-1}$, i.v.). This experiment tested the effects of nitric oxide synthase inhibition on gastroduodenal motor activity.

Group 5: Animals were prepared for *ex vivo* experiments as described in this chapter. After 60 minutes of control recordings, L-NAME (3.7 mM, 100µl) was directly applied close to the D_1 recording site to test for the local effects of nitric oxide synthase

inhibition on duodenal motor activity. The effects of this *ex vivo* experiment were compared to the effects of intravenously applied L-NAME experiments.

Group 6: L-arginine (300 $mg \cdot kg^{-1}$, i.v.) was injected after 60 minutes of control recording to test the effect of L-arginine, a NO synthase substrate, on gastroduodenal motor activity.

Group 7: Rats were pre-treated with (after 60 minutes of control recording) with L-NAME (10 $mg \cdot kg^{-1}$, i.v.) followed by an injection of L-arginine (300 $mg \cdot kg^{-1}$, i.v.) 60 minutes later. This experiment tested the ability of L-arginine to reverse the effects of L-NAME on motor activity of the gastroduodenum.

Group 8: Rats received intravenously (i.v.) an infusion of ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$) for 1 min after 60 minutes of control motility recording. This treatment group determined the effects of ATP (a putative NANA inhibitory neurotransmitter) on gastroduodenal motor activity. The infusion protocol and doses were derived from those used in ferret *in vivo* studies done by Baccari *et al* (1990)[4].

Group 9: Suramin (30 & 60 $mg \cdot kg^{-1}$, i.v.) was administered after 60 minutes of recording to test the effects of P_2-purinoceptor antagonism on gastroduodenal motor activity. The initial dose of 30 $mg \cdot kg^{-1}$ represents a dose that is slightly higher than effective dose used in humans against parasites[85,126].

Chapter 3

Group 10: Animals were infused with α,β methylene ATP (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 1 min, i.v.) after 60 minutes of control recording. α,β methylene ATP has been shown to be a specific agonist to the P_{2x} receptor, a P_2 receptor subtype shown to functional

present on gut smooth muscle [36,119]. Therefore, this experiment investigated the effects of P_{2x} receptor desensitization using this ATP analogue on proximal duodenal motor activity. This protocol followed that used by Delbro and Fandriks (1984)[48].

Group 11: Rats received an injection of 2 methylthio ATP (360 $\mu g \cdot kg^{-1}$, i.v.) after 60 minutes of control recording. This experiment tested the effects of a P_{2y}-purinoceptor agonist on relaxant motor activity in the proximal duodenum.

Group 12: Animals after 60 minutes of control recording were injected with L-NAME (10 $mg \cdot kg^{-1}$, i.v.) followed by injection of the P_{2y} receptor agonist 2 methylthio ATP (360 $\mu g \cdot kg^{-1}$, i.v.) 60 minutes later. This experiment tested whether the effects (agonist effects) of 2 methylthio ATP involved NO.

Chapter 4

Group 13: After 60 minutes of control recording rats were administered with 3-APS (100 $mg \cdot kg^{-1}$, s.c). This experiment tested the effects a $GABA_A$ agonist of gastroduodenal motor activity.

Group 14: Bicuculline (350 $\mu g \cdot kg^{-1}$, s.c.) was administered after 60 minutes of control recording to test the effects of $GABA_A$ receptor antagonism on gastroduodenal motor activity.

Chapter 5

Group 15: Rats after 60 minutes of control recording were injected with VIP (6 $\mu g \cdot kg^{-1}$, i.v.). The doses used in these studies followed those used in rat in vivo studies by Bojo et al (1993)[18]. This experiment determined the VIP's role as a proposed inhibitory

neurotransmitter on gastroduodenal motor activity.

Group 16: Rats first received a VIP (6 $\mu g \cdot kg^{-1}$, i.v.) injection after 60 minutes of control recording, 60 minutes later L-NAME (10 $mg \cdot kg^{-1}$, i.v.) was administered followed by a second VIP (6 $\mu g \cdot kg^{-1}$, i.v.) injection 60 minutes later. This group of experiments were with an additional antral foil strain gauge (S_2) which was attached juxtaposed to the S_1 foil strain gauge but oriented to the circular muscle direction. This experiment tested whether VIP induced effects in the gastric antrum observed in Group 16 experiments were specific to the longitudinal layer and if these effects within the gastric antrum involved nitrergic systems.

Group 17: Treatment of rats followed the same experimental protocol as Group 16 rats except L-NAME treatment was replaced with bicuculline (350 $\mu g \cdot kg^{-1}$, s.c.) treatment. This experiment also involved an additional antral foil strain gauge (S_2) This experiment tested whether VIP induced effects with the gastric antrum involved GABAergic neurons acting via $GABA_A$ receptors.

Group 18: Rats received a intra-arterial injection of VIP (6 $\mu g \cdot kg^{-1}$) after 60 minutes of control recording. The effects of VIP (i.a.) were compared to those observed with intravenous administration of VIP.

RESULTS

Ex Vivo Validation of Foil Strain Gauges

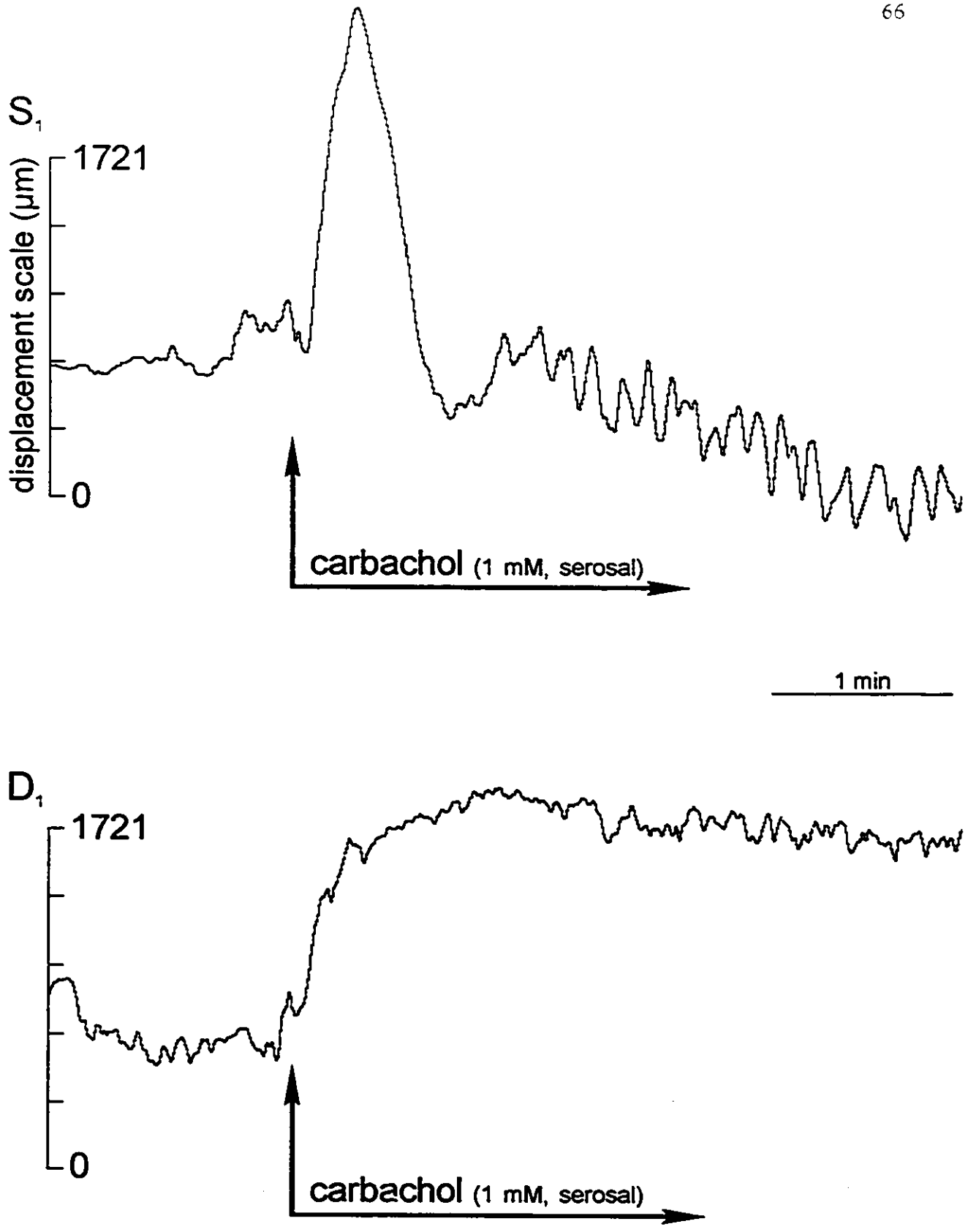
These experiments were designed to test whether the foil strain gauges were capable of properly recording both contractions and relaxations. To test for the response to contractions, carbachol (a muscarinic agonist) was directly applied close to both strain gauge sites to test the recorded response to contractions. Both the S_1 and D_1 strain gauges were capable of recording carbachol induced contractions (Fig. 12). The contractions induced in the gastric antrum had a duration of approximately 1 minute, while those in the proximal duodenum were longer in duration, lasting for as long as 10 minutes. The sensitivity of the foil strain gauges is evident from the dose dependent carbachol actions presented in Figure 13.

To test for relaxation responses to induced relaxations the relaxant papaverine and the putative enteric non-adrenergic non-cholinergic inhibitory neurotransmitter, ATP were used. Papaverine induced dose dependent relaxations at both recording sites (Fig. 13 & Fig. 14). Likewise, relaxations were recorded upon application of ATP to both gut regions (Fig. 15).

Control Motor Activity

Under control conditions spontaneous patterns of gastroduodenal motility in the anaesthetized rat (n = 4) included non-propagating single and grouped motor events. Also observed were events that occurred simultaneously at recording sites S_1 and D_1 which have been termed 'coupled events'. No propagating activity between the stomach and the proximal duodenum was observed in control recordings.

Figure 12 Typical recording of motor activity following direct application (under *ex vivo* conditions) of the muscarinic agonist, carbachol at the gastric antrum (S_1) and the proximal duodenum (D_1). Carbachol induced contractions at both recording sites. The displacement scale on the y axis can be used to compare the alteration in tone and amplitude of the contraction response. The time bar represents 1 minute.


S1
displacement scale (µm)
1721
0
carbachol (1 mM, serosal)
1 min
D1
1721
0
carbachol (1 mM, serosal)

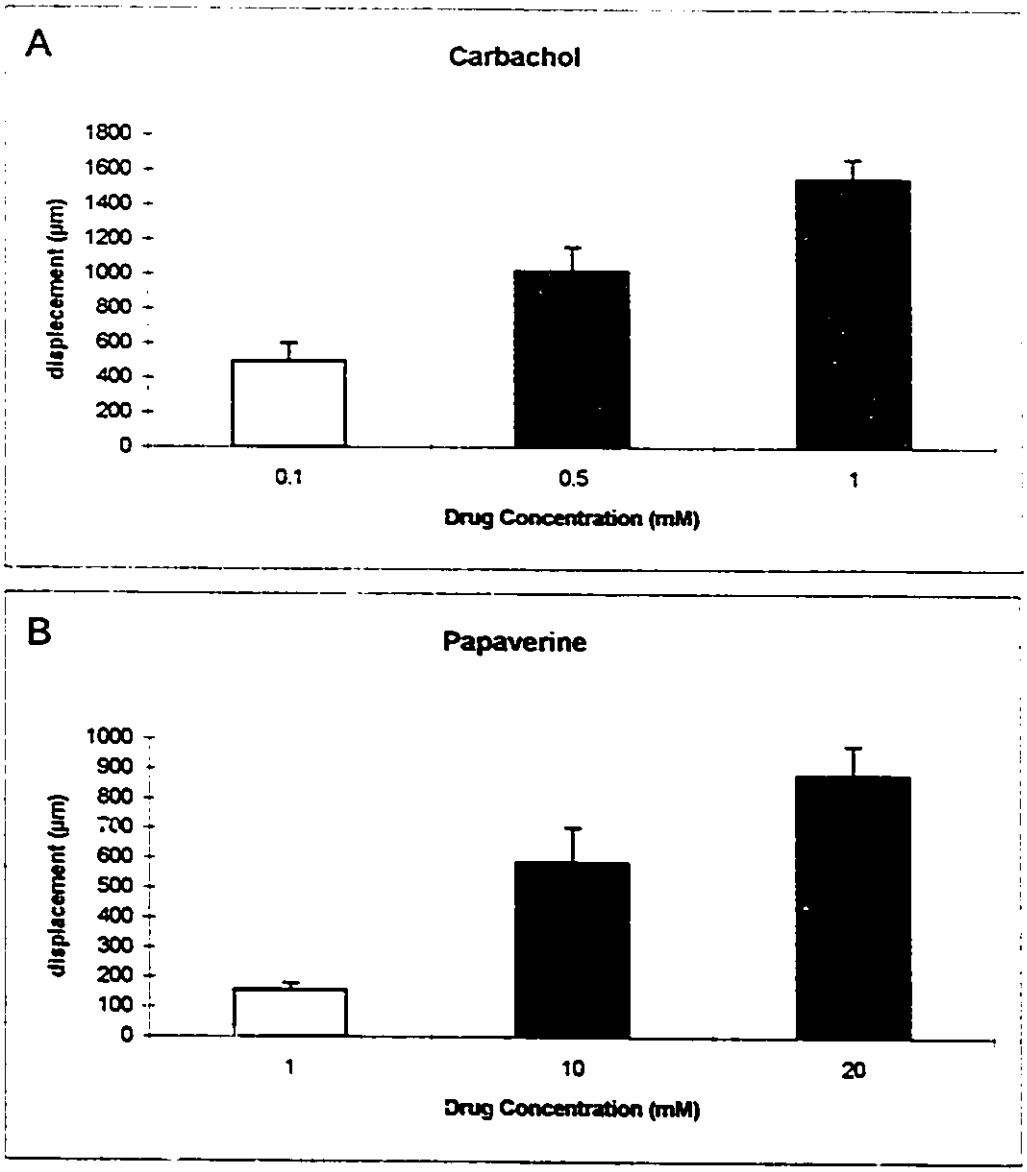
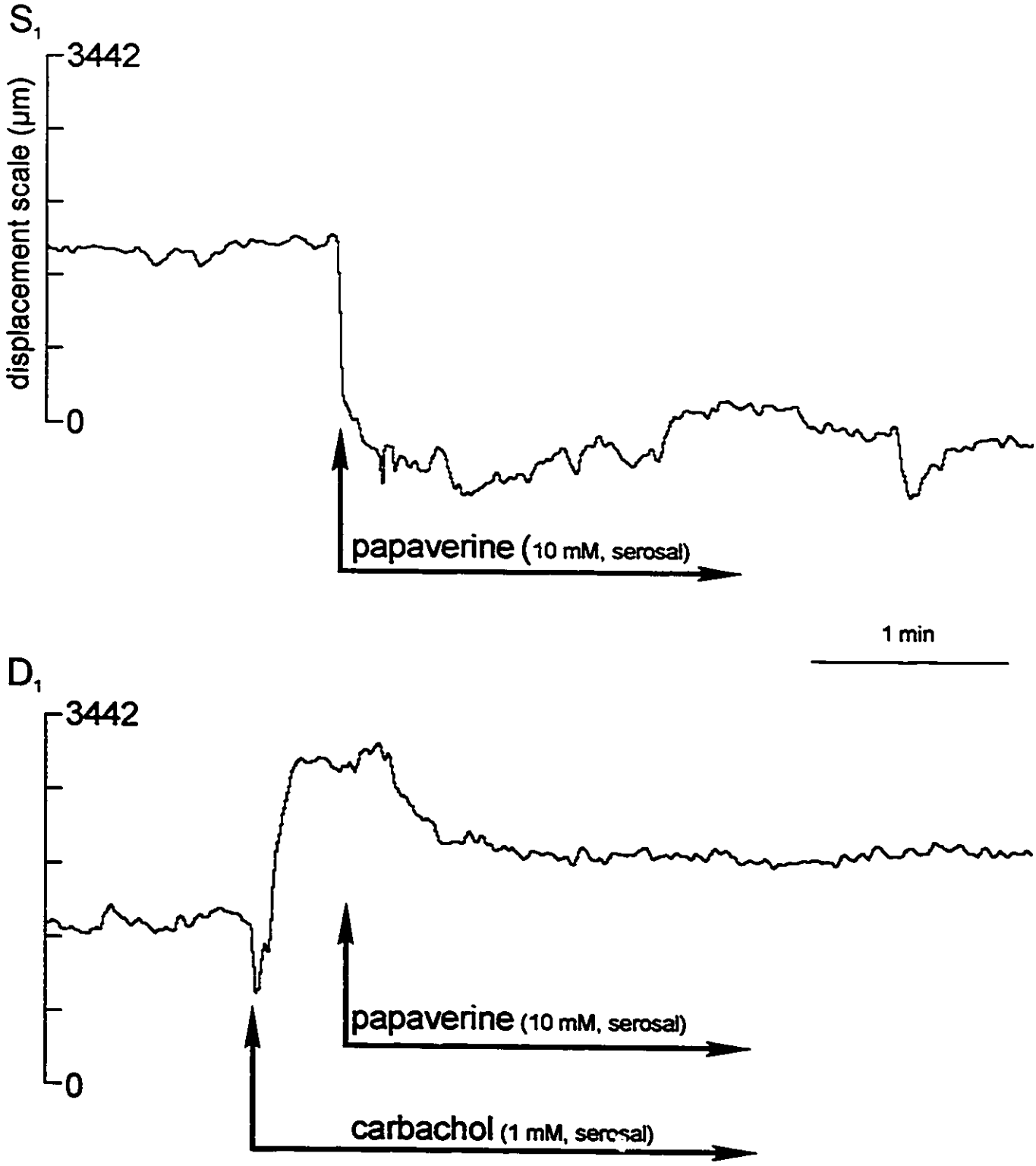


Figure 13 The concentration dependent response of carbachol induced contraction (panel A) and the papaverine induced relaxation (panel B) in the rat proximal duodenum, *ex vivo*. Both contraction and relaxation responses are expressed as displacement in µm of the foil strain gauge. 100 µl of the agent was applied directly to the area surrounding the foil strain gauge.

Figure 14 An typical recording of the response to the direct *ex vivo* application of the smooth muscle relaxant, papaverine (10 mM) at the gastric antrum (S_1) and the proximal duodenum (D_1). The proximal duodenum was precontracted with carbachol (1 mM) before application of papaverine. Papaverine always induced a relaxation (a negative deflection) at both recording sites. The displacement scale on the y axis can be used to compare the alteration in tone and amplitude of the induced relaxation. The time bar represents 1 minute.


S1
3442
displacement scale (µm)
0
papaverine (10 mM, serosal)
1 min
D1
3442
0
papaverine (10 mM, serosal)
carbachol (1 mM, serosal)

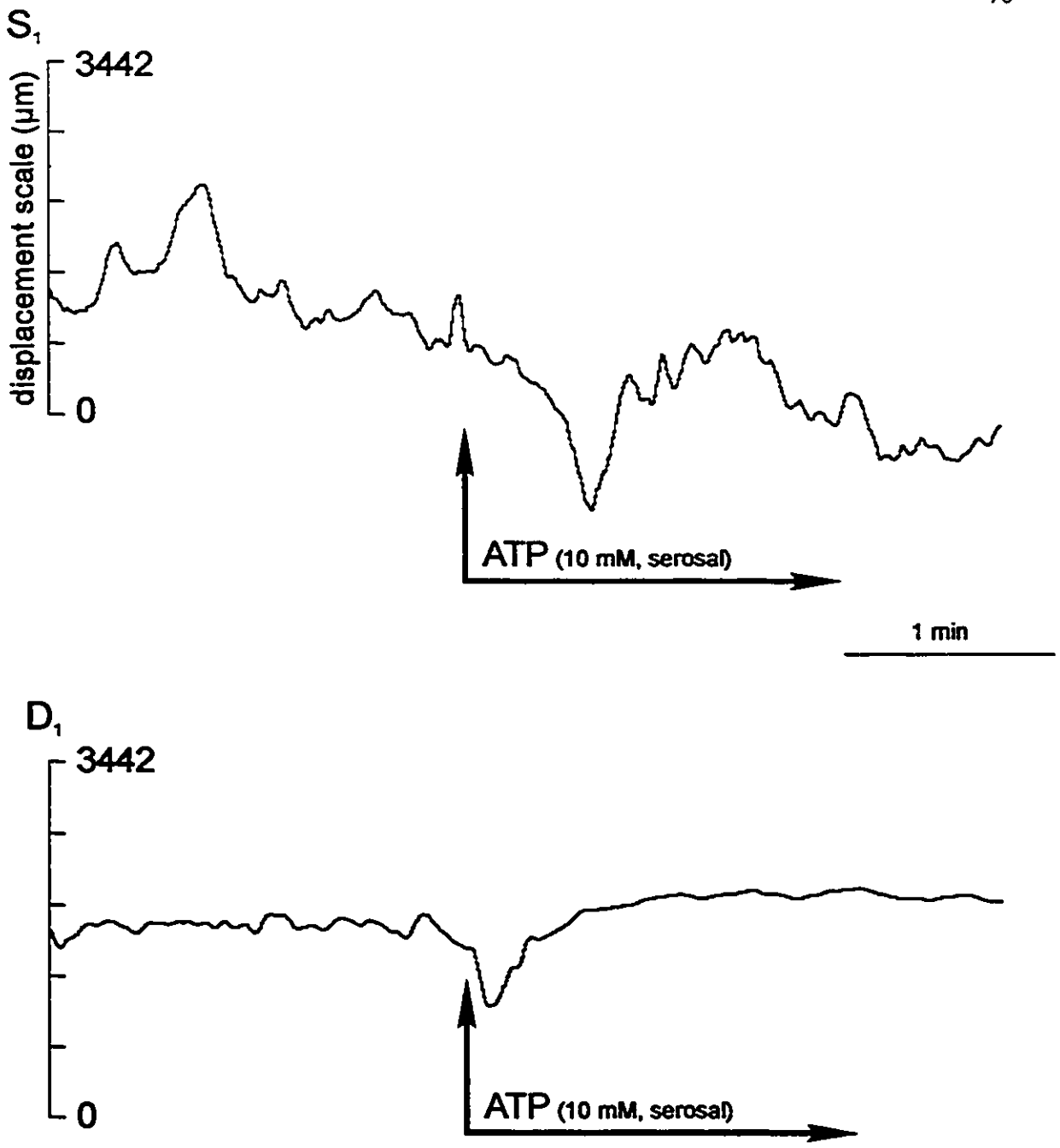


Figure 15 A typical recording of the response of to the direct *ex vivo* application of ATP (10 mM) at the gastric antrum (S_1) and the proximal duodenum (D_1). ATP always induced a transient relaxation (a negative deflection) at both recording sites. The displacement scale on the y axis can be used to compare the alteration in tone and amplitude of the induced relaxation response. The time bar represents 1 minute.

In the gastric antrum (S_1), motor activity consisted primarily of periodic relaxations that often appeared oscillatory in appearance (Fig 16). These relaxations in general were similar in amplitude and occurred with a frequency of approximately 8 min^{-1}. In contrast contractions randomly appeared less frequently with varying amplitude.

In the proximal duodenum (D_1), motor activity consisted of two distinct patterns which have been termed 'Grouped' and 'Intergroup' as shown in Figure 16. 'Grouped activity' was characterized by distinct groups of large amplitude, high frequency relaxations together with contractions. Each 'Grouped' activity period lasted 0.5-3.0 minutes and was periodic occurring approximately every 10-15 minutes. Within this period of 'Grouped' activity, it was sometimes possible to distinguish two phases. In the first phase responses appeared to build in intensity; this was followed by a phase of more regular intense activity, reminiscent of migrating myoelectric complexes (MMC's), [24,30]. During the *ex vivo* series of experiments, it was possible to observe distinct regions of contraction/relaxations to propagate caudally down the proximal duodenum. These occurred with the same frequency as the 'Grouped activity'. Motor activity occurring in between the episodes of 'Grouped' activity, herein termed 'Intergroup', comprised both contractions and relaxations. Compared to relaxations within the 'Grouped' activity, 'Intergroup' relaxations occurred with a lower frequency and the majority of these relaxations were smaller in amplitude, approximately one third that of 'Grouped' relaxation amplitude. Injection of saline vehicle (0.9%, i.v.) had no effect on gastroduodenal motor activity

Occasionally 'coupled' events (as referred to above) occurred during control recordings. A random large amplitude event would occur in the gastric antrum that was always coupled

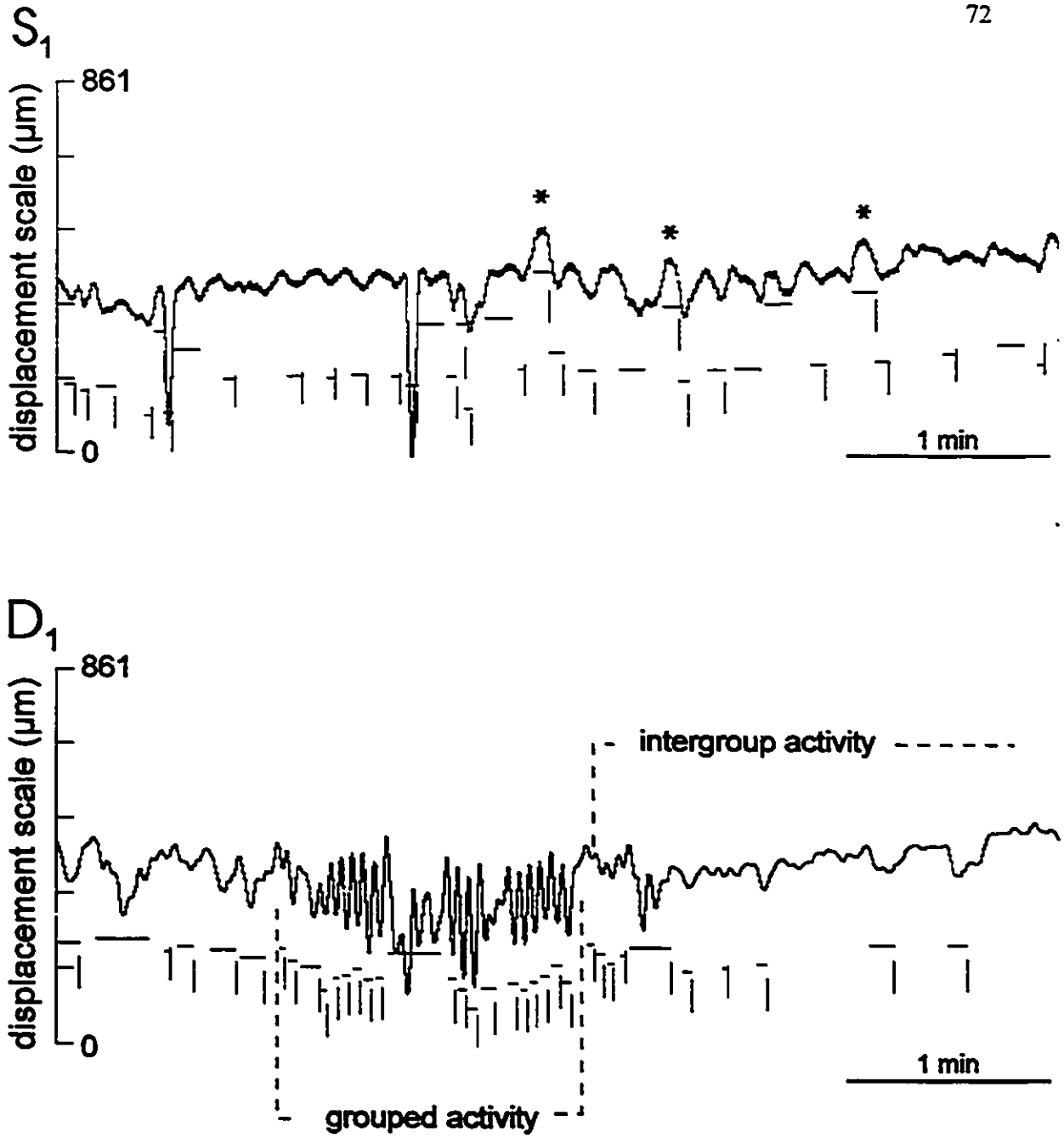
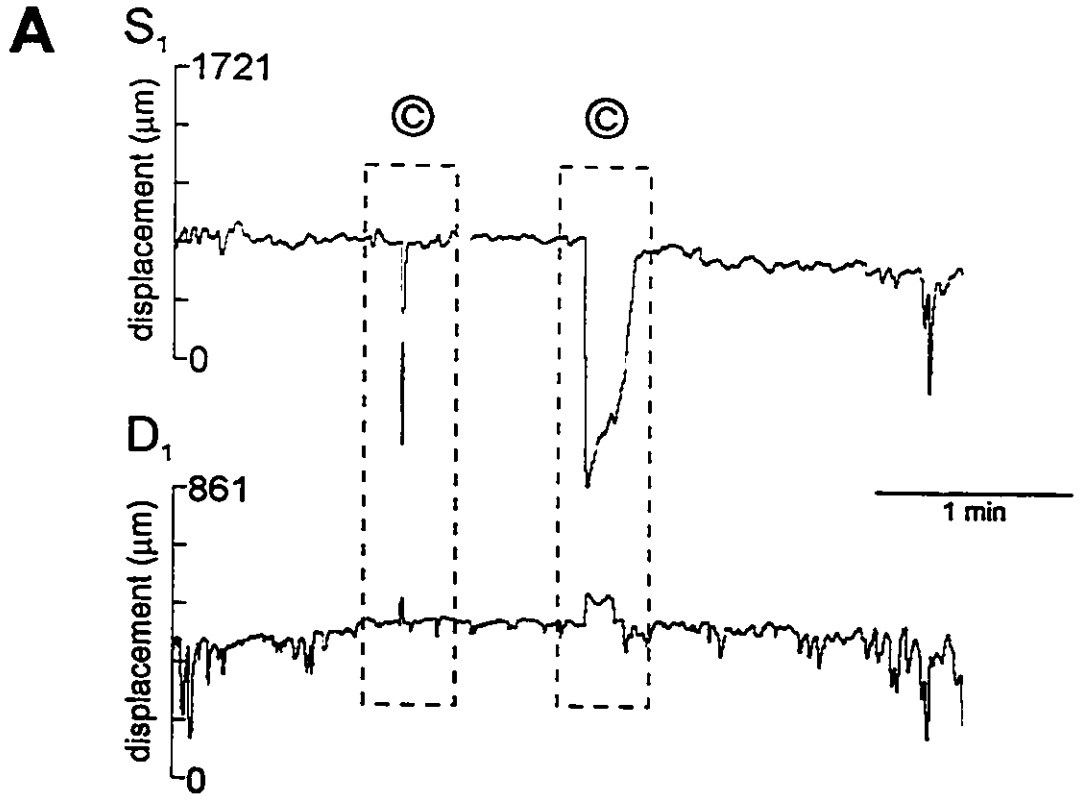
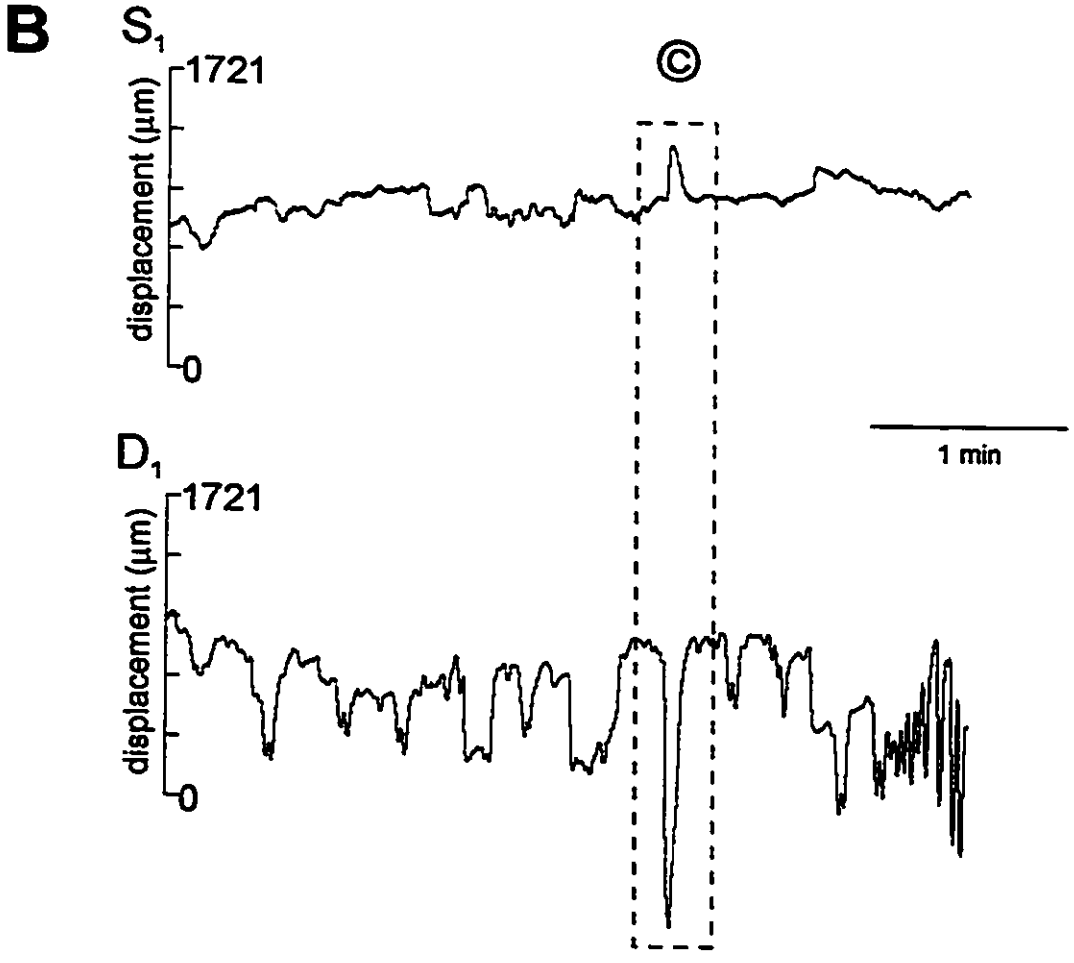


Figure 16 Motor activity recordings of control of spontaneous gastroduodenal motor events of the anaesthetized rat as recorded from two different animals: a) the gastric antrum (S_1) and b) the proximal duodenum (D_1). In the gastric antrum, contractions (*) and relaxations typically appearing oscillatory and periodic. In the proximal duodenum, contractions and relaxations also occurred within two distinct patterns: 'Intergroup' and 'Grouped'. The time bar represents 1 minute.

Figure 17 Simultaneous recording of motor activity from the antrum (S_1) and the proximal duodenum (D_1) of an anaesthetized rat. Typical coupled responses (©) are enclosed by hatched boxes. The time bar represents 1 minute.


A
S1
1721
displacement (µm)
0
D1
861
displacement (µm)
0
1 min


B
S1
1721
displacement (µm)
0
1 min
D1
1721
displacement (µm)
0

with a simultaneous large amplitude response in the proximal duodenum as shown in Figure 17. These responses displayed identical profile in onset and offset and in a majority of cases their profiles were opposite. The large amplitude was many orders of magnitude greater than any other forementioned control motor pattern.

The Effects of Cysteamine-HCl

To test the ability of the recording/analysis technique to detect and quantitate alterations in motility the ulcerogen cysteamine-HCl (560 $mg \cdot kg^{-1}$, s.c.) was injected after 60 minutes of control recordings. In the antrum, motor activity was unaffected by treatment with cysteamine-HCl. However, in the proximal duodenum (D_1) cysteamine had a dramatic effect on motor activity as shown in Figure 18. Under control conditions, the duodenum displayed motor activity that was identical to the patterns described in control recordings, that is 'Grouped' and 'Intergroup' activities were present. Within 5 minutes of cysteamine-HCl treatment and for the remaining duration of the experiment (up to 3 hours), it was no longer possible to distinguish the two types of activity. Therefore, it was not possible to discern the effects of the two types of activity. Nevertheless, contractions and relaxations were differentially altered as shown in Table 3. Contraction amplitude and frequency were significantly increased (161% and 210% of control respectively). In contrast, relaxation amplitude was significantly decreased (to 73% of control) whereas, relaxation frequency was not affected.

Figure 18 A typical recording of duodenal (D_1) motor activity before and after the treatment of cysteamine-HCl (final dose 560 $mg \cdot kg^{-1}$). The panels represent recordings from t = 35 min to t = 70 min. Cysteamine was injected at t = 60 min. In the top panel 'Grouped' and 'Intergroup' motor patterns are highlighted. Following Cysteamine-HCl injection, these patterns were abolished and replaced with high frequency motor activity. The displacement scale represented in the y axis can be used to compare the alteration in tone and amplitude of the responses.

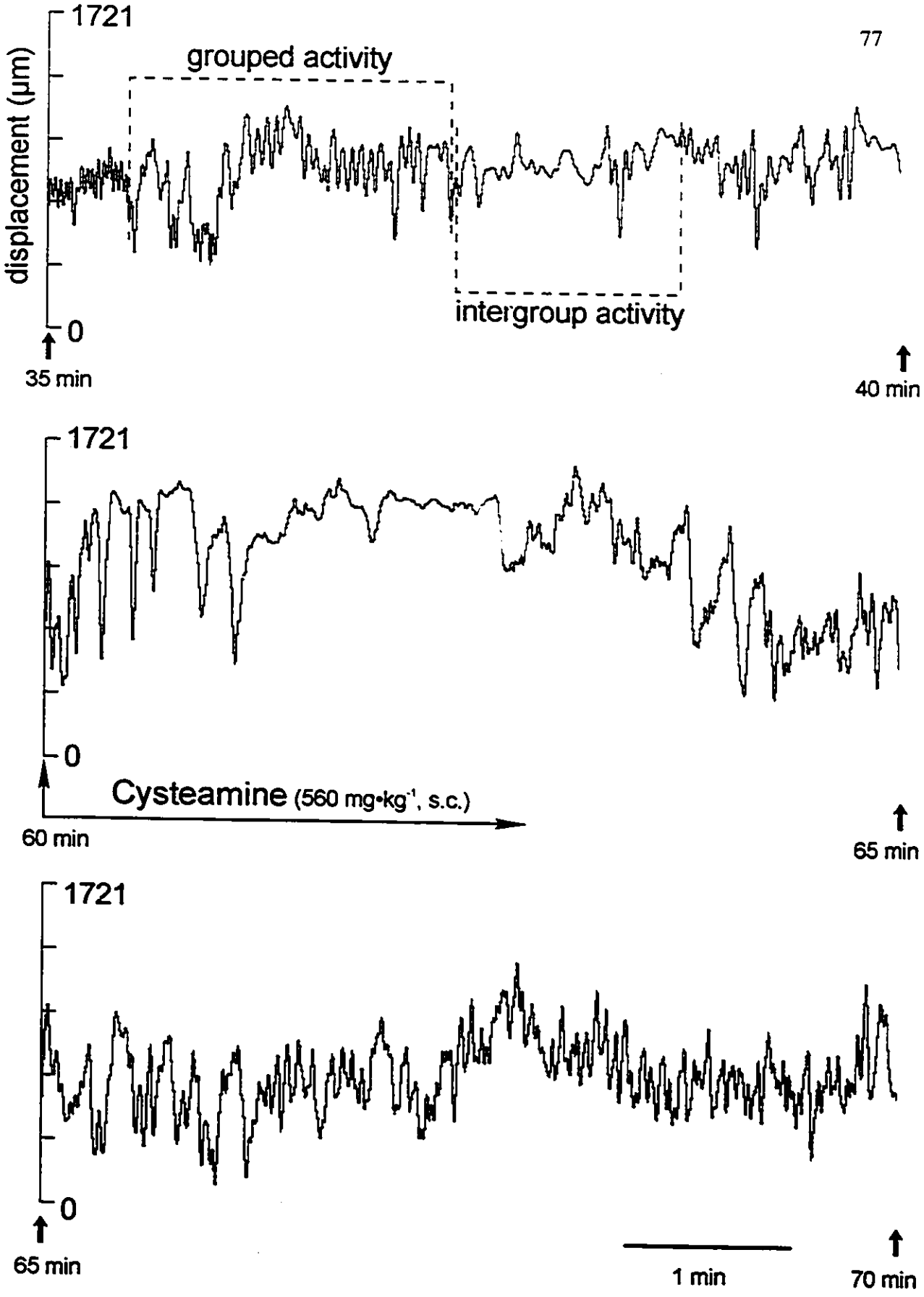

1721
displacement (µm)
grouped activity
intergroup activity
0
35 min
40 min
1721
0
Cysteamine (560 mg•kg⁻¹, s.c.)
60 min
65 min
1721
0
65 min
1 min
70 min

TABLE 3

Effects of Cysteamine-HCl on Duodenal Relaxation Amplitude and Frequency

Region + Event Type	Amplitude		Frequency	
	control	CYS	control	CYS
D_1 contractions	78±12	126±9.1*	0.91±0.2	1.91±0.1*
D_1 relaxations	126±11	92±4.2*	7.8±0.45	8.0±0.2

* denotes statistical significance $p < 0.05$; $n = 4$

CYS = cysteamine-HCl (final dose: 560 $mg \cdot kg^{-1}$)

All tabulated data is presented as mean ± SEM

amplitude = displacement (μm); frequency = $events \cdot min^{-1}$

DISCUSSION

Recording systems employed to record and measure motility, *in vivo*, must be able to detect not only contractions of the gut, but relaxation events as well. This is important, since both reflex excitatory and inhibitory motor activity form the basis for gut motility patterns. Because the technique I was employing represented a modification of existing methodology it was essential to validate the technique by evaluating drugs with known actions in the gut, as well as confirming the ability of this technique to detect altered motor patterns. The ability of our recordings system to achieve these criteria was first assessed in *ex vivo* experiments, where the responses of the foil strain gauges to direct application of known contractile and relaxant agents could be observed. The results demonstrated conclusively that the foil strain gauges attached to the serosa of either the stomach or duodenum were capable of recording contractions and relaxations. Since the *ex vivo* experimental protocol, except for the abdomen being left open for the direct application of drugs, is identical to the *in vivo* arrangement of gauges and connections, the results can be extrapolated to the *in vivo* recording circumstance.

Under control *in vivo* conditions, the gastroduodenum of the fasted anaesthetized rat shows region specific spontaneous motor patterns. In the gastric antrum, motor activity consisted of both contractions and relaxations of which the relaxations were predominant. In the proximal duodenum, contractions and relaxations were also recorded but two distinct patterns were observed; referred to as 'Intergroup' and 'Grouped' activity. The 'Grouped' activity consisted of high frequency, large amplitude responses which were primarily relaxations. This 'Grouped' activity was interspersed with periods of generally small, random

and low frequency responses, 'Intergroup' activity.

'Grouped' activity occurred with a frequency and profile reminiscent of migrating myoelectric complexes (MMC's)[62]. MMC's can be divided into three phases: phase 1 is a period of no activity, phase 2 is a period of random activity, followed by a period of regular intense activity (phase 3)[68]. In this study, 'Grouped' activity was found to occur approximately every 10 to 15 minutes and lasted for 0.5 - 3.0 minutes. In this regard Bueno *et al* observed in the jejunum of the fasted rat, that electrical spiking activity was regularly interspersed at 15 minute intervals with a period of relative quiescence [24]. These investigators also observed that the spiking activity had two phases: the first was irregular followed by a phase of regular spiking activity. Likewise, Caenepeel *et al* observed that the period of MMC's in the rat proximal small bowel was 13±1 minutes with phase 3 of the MMC's lasting 2.9±0.57 minutes [30]. Therefore, the periodicity and duration of the 'Grouped' activity observed in this study coincides with that of migrating motor and/or associated electrical activity observed in other studies. Furthermore, like MMC's, 'Grouped' activity responses appeared to build in intensity similar to phase 2 and 3 of MMC's.

Another salient feature of MMC's is that they propagate to the caudad end of the gut. During *ex vivo* evaluation of the gastroduodenum, visual inspection of the exposed intestine revealed a wave of contraction/relaxation coinciding with 'Grouped" activity (as recorded at D_1) that propagated caudally down the duodenum. The pattern, timing and propagatory nature of 'Grouped' activity in the proximal duodenum should be further examined utilising a second foil strain gauge (D_2) implanted distal to the D_1 foil strain gauge. This will then allow for the comparison of migration velocity of 'Grouped' activity with other studies and under different

treatment regimens.

A significant feature of this recording system is its sensitivity, which not only allowed assessment of independent contractile and relaxant motor responses, but also revealed motor activity in the antrum that appeared temporally coupled to duodenal motor activity. To my knowledge, this type of motor activity has not been previously reported. The nature of these coupled responses is unclear. The type and quality of the responses that occurred at the different recording sites varied and could not be accounted for by any obvious perturbations within the experimental protocol. Indeed, the temporal coupling but lack of correlation in the response type strongly suggest some common neural pathway projecting between the gastric antrum and proximal duodenum. Long projecting intrinsic neurons also occur in the gut. However it is interesting that we never observed propagating activity between the stomach and duodenum. The only evidence of propagation was the visual observations of propagatory 'Grouped' activity. Motor responses originating in the gastric antrum have been observed by A. McKay (PhD thesis, 1992, University of Ottawa) to propagate into the duodenum in the conscious rat.

Two differences exist between Dr. McKay's experiments and the experiments herein: i) the animals in these studies are anaesthetized and ii) in the unrestrained conscious rat model the animals are allowed to recover for 5 days before motility recordings. One or both of these differences could be responsible for the absence/disruption of propagatory activity between regions. In a similar study, Pascaud *et al* utilised strain gauges to record gut motility in unrestrained conscious rats[141]. The recordings presented and described in this chapter show similarities to the tracings shown by Pascaud *et al*, in that there was oscillatory activity in the

gastric antrum and in the duodenum, motor activity was patterned into intense activity interspersed with more quiescent activity. Interestingly, Pascaud *et al* allowed animals a 45 minute recovery period after surgery before recordings began. Therefore, it is possible that the duration of the recovery period may affect the amount of propagatory activity observed. Future studies need to test for this by recording for a longer duration (eg. > 10 hours as opposed to 4 hours).

In this study, subcutaneous injection of the ulcerogen cysteamine-HCl did not affect antral motor activity, but caused differential effects on contractions and relaxations in the proximal duodenum. Cysteamine-HCl treatment stimulated large amplitude contractile activity while exerting inhibitory actions on relaxant activity, without altering the frequency of relaxations. In addition, patterned motor activity was disrupted. Previous *in vivo* studies have reported the effect of cysteamine-HCl on contractile activity in the gut, but no findings were reported regarding possible inhibition of relaxations. This is attributed to the lack of attention paid to either the recording and/or the analysis of relaxant motor activity in the gastroduodenum. In this laboratory, *in vitro* pharmacological studies show that cysteamine-HCl interacts with NANC inhibitory motor neurons in rat isolated intestine [109]. My *in vivo* results confirm and extend this observation.

In conclusion, the results from these experiments indicate that the action(s) of cysteamine-HCl, are markedly different when considering relaxation and contraction of the gut muscle, *in vivo*. Not only did cysteamine-HCl affect contractions and relaxations, but pattern activity was disrupted, suggesting an interference with the integration of peristaltic activity. These findings provide insight into possible sites or pathways for inhibitory action

in vivo. Therefore, the results using cysteamine-HCl demonstrate that the recording technique and method of analysis is capable of simultaneously recording and analyzing contractions and relaxations of the gut *in vivo*. The usefulness of such a recording technique is appreciated in light of the differential actions of cysteamine-HCl.

CHAPTER 2

INVOLVEMENT OF NITRIC OXIDE (NO) AND ADENOSINE TRIPHOSPHATE (ATP) IN RAT GASTRODUODENAL MOTILITY *in vivo*

Substantial pharmacological and physiological evidence exists demonstrating that the mammalian gut wall contains a distinct class of intrinsic inhibitory motor neurons, the so called non-adrenergic and non-cholinergic (NANC) neurons (for review see [62]). These neurons mediate functional relaxation of the gut smooth muscle [28,29,62]. However, chemical characterization of this inhibitory innervation is complicated by the existence of more than one candidate putative NANC inhibitory neurotransmitter, including ATP, VIP and NO. Each of these putative inhibitory neurotransmitters is capable of mediating NANC relaxation of the gut in a variety of species (see Introduction), and there is accumulating evidence for more than one population of intrinsic inhibitory motor neurons in the mammalian gut. In Chapter 1, I have presented a description of normal motor activity patterns observed in the gastroduodenum of the anaesthetized rat. Antral motor patterns displayed a predominance of periodic relaxations. The proximal duodenum consisted of two obvious patterns, termed by us as 'Intergroup' and 'Grouped' activity, both involving relaxations. The existence of distinct patterns of spontaneous motor activity may be indicative of different intrinsic innervations. The aims of the research described in this chapter were to: i) examine the inhibitory innervations and neurotransmitters involved in spontaneous gut relaxant activity, and, ii) determine whether patterned relaxant activity can be differentiated on the basis of the

involvement of different neurotransmitters. **More specifically, I sought to investigate the involvement of nitrergic and purinergic mechanisms in the pathways controlling the relaxant activity in the rat gastroduodenum *in vivo*.**

METHODS

In vivo Experiments

Sprague Dawley rats were surgically implanted with two foil strain gauges (S_1 & D_1) as described in the methods section of Chapter 1. After surgery, the animal was allowed a one hour period of stabilization before motility recordings began. Experiments consisted of a 60 minute control period of recording, followed by administration of drug treatment and then motility recordings for up to 4 hours. For all drugs except suramin, the treatment period started at the time of injection. Suramin is a slowly equilibrating competitive P_{2x}-purinoceptor antagonist, with its maximum effect *in vitro* evident within 90 minutes [1,119]. On this basis, the treatment period for animals injected with suramin in this study, started at t = 120 min post suramin application.

Ex vivo Experiments

Intravenous administration of various drugs does not permit conclusions regarding sites of actions. *Ex vivo* experiments were therefore carried out to compare the effects of L-NAME previously tested intravenously. Animals were prepared for *ex vivo* experiments as described in Chapter 1. In these animals, L-NAME (3.7 mM, 100 µl) was directly applied close to the D_1 recording site to test the local effects of this NOS inhibitor.

Chemicals

Adenosine triphosphate, L-arginine and N^G-Nitro-L-arginine Methyl Ester (L-NAME) were obtained from Sigma Chemical (St. Louis, MO, USA). Suramin was obtained from CBChemicals (Woodbury, CT, USA)

RESULTS

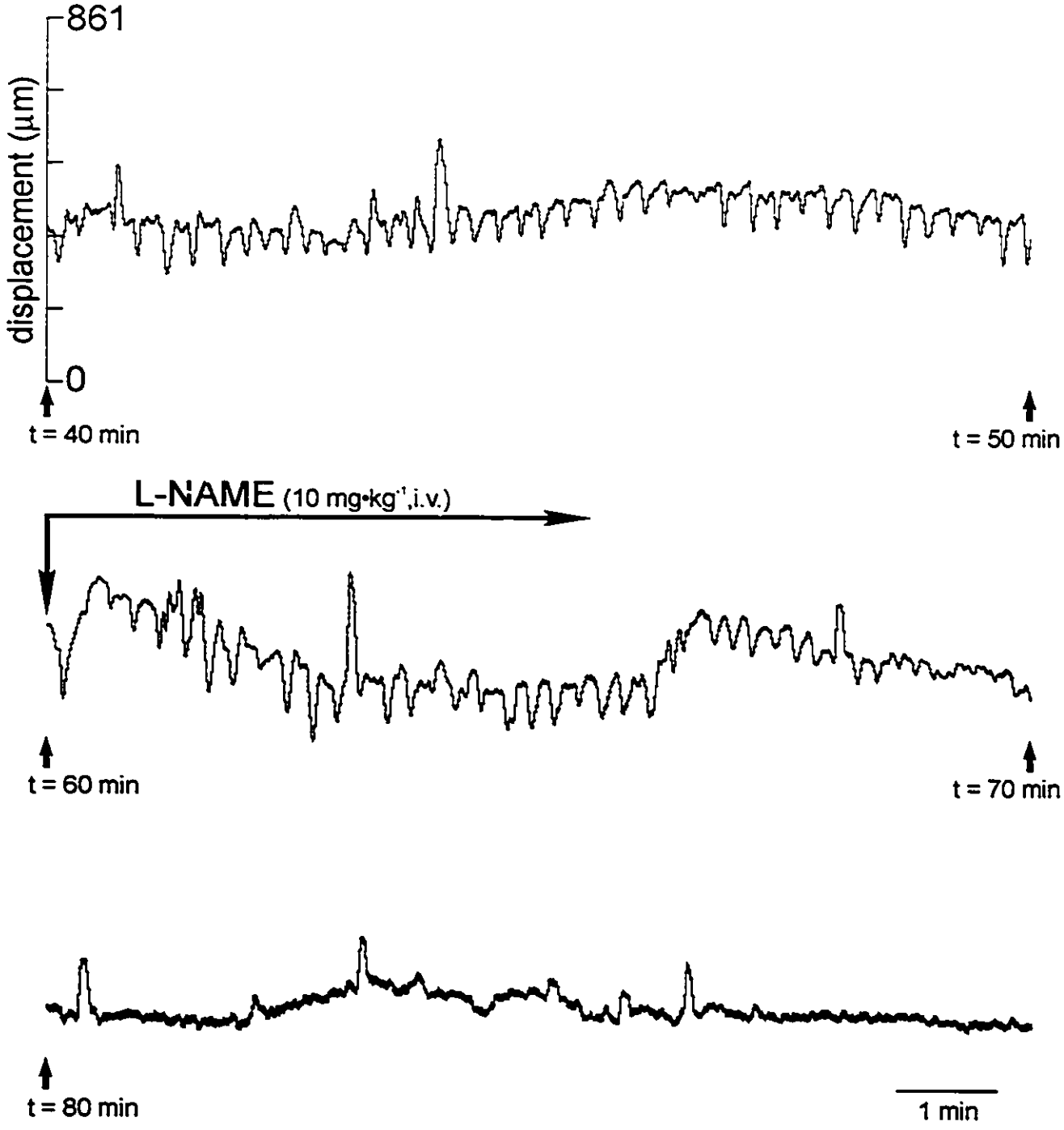
Effects of Treatment with the NO Synthase Inhibitor L-NAME

L-NAME (10 $mg \cdot kg^{-1}$, i.v.) was administered as a single bolus after 60 minutes of control recording. Typical motor recordings of antral (S_1) and duodenal (D_1) motor activity pre and post L-NAME are presented in Figures 19 and 20. Contractions in the gastric antrum showed no significant ($p>0.05$) change in either amplitude or frequency following intravenous L-NAME treatment (Table 4). However, within 10-15 minutes of L-NAME injection, spontaneous antral relaxations were significantly ($p<0.05$) reduced in both amplitude (to 57 % of control) and frequency (to 53% of control, Table 4). The effects of L-NAME lasted for the duration of the experiment (up to 3 hours post injection) with no recovery of motility patterns to those observed in the control recording period.

In the proximal duodenum, the two types of control motor activity patterns ('Intergroup' and 'Grouped' as described in Chapter 1) were differentially affected by L-NAME. 'Grouped' relaxations were enhanced by L-NAME (Fig 20) characterized by a significant ($p<0.05$) increase in amplitude (to 266% of control) and frequency (to 115% of control), Table 4. In contrast, 'Intergroup' relaxations were either reduced or abolished upon intravenous L-NAME treatment (Fig. 20). This is reflected in the significant reduction in 'Intergroup' relaxation amplitude and frequency, to 38% and 29% of control respectively (Table 4).

Duodenal contractions were also significantly altered by intravenously administered L-NAME. 'Grouped' contraction were increased to 203% of control and 'Intergroup' contractions increased to 199% of control (Table 4).

Figure 19 Effects of L-NAME at S_1. A typical recording of antral motor activity before and after the injection of L-NAME (10 $mg \cdot kg^{-1}$, i.v.). The three panels represent 10 minute segments taken from a continuous recording. L-NAME was injected as a single bolus at t = 60 min. Control activity consisted predominantly of relaxations with few contractions. Following L-NAME treatment, relaxations were lost within 10 minutes. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.


861
displacement (µm)
0
t = 40 min
t = 50 min
L-NAME (10 mg•kg⁻¹,i.v.)
t = 60 min
t = 70 min
t = 80 min
1 min

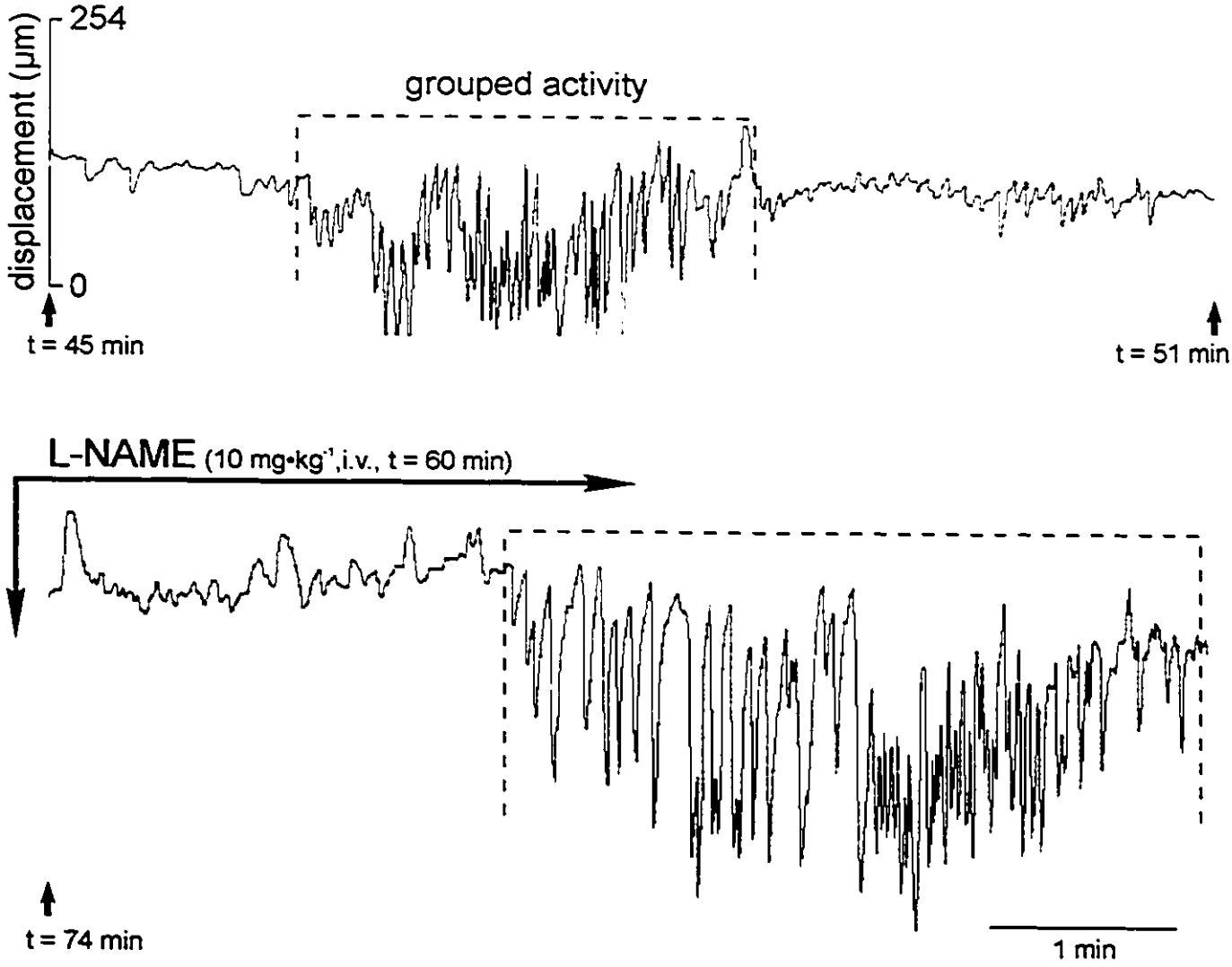


Figure 20 Effects of L-NAME at D_1. A typical recording of duodenal motor activity pre and post the injection of L-NAME (10 mg·kg^{-1}, i.v.). The three panels represent 10 minute segments of continuous recording. L-NAME was injected as a single bolus at t = 60 min. Following L-NAME treatment, 'Intergroup' relaxations were reduced or abolished while 'Grouped' activity was enhanced. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

TABLE 4

Effects of L-NAME on Amplitude and Frequency of Contractions and Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	L-NAME	control	L-NAME
S_1 contractions	18.6 ±3.9	10.4 ±5.9	1.56±0.15	1.49±0.1
S_1 relaxations	17.0 ±1.7	7.3 ±1.0*	0.96±0.13	0.45±0.15 *
D_1 Grouped contractions	25.9 ±1.2	52.6 ±16.2*	0.14 ±0.08	0.2 ±0.06
D_1 Grouped relaxations	8.7±0.7	23.1±1.0 *	5.1±0.3	5.9±0.4*
D_1 Intergroup contractions	11.0 ±2.2	21.9±0.44*	0.06 ±.05	0.004 ±0.004
D_1 Intergroup relaxations	10.1±1.5	3.8±0.7 *	1.4±0.3	0.4±0.1 *

* denotes significance ($p < 0.05$) compared to control; n = 6

L-NAME = L-NAME (10 $mg \cdot kg^{-1}$, i.v.)

amplitude = displacement (μm); frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

Ex Vivo **Recordings**

To compare the systemic (i.v.) with local administration of L-NAME, the effect of direct application of L-NAME (3.7 mM, 100µl) close to the D_1 foil strain gauge site was recorded in an *ex vivo* preparation. The effects of L-NAME under these conditions were comparable to that observed with intravenous L-NAME treatment. 'Grouped' activity was enhanced while 'Intergroup' relaxations were reduced (Figure 21).

Effects of Combined L-NAME + L-arginine Treatment

Two experiments were carried out involving the use of the NO synthase substrate, L-arginine. The first experiment tested the effect of L-arginine as a NOS substrate on gastroduodenal relaxations administered alone. The second experiment tested the ability of L-arginine to reverse the effects of L-NAME. If L-NAME's effects on motor activity were due to competitive inhibition of NO synthase then L-arginine should result in effects that are opposite to those of L-NAME treatment.

When administered alone, the effects of L-arginine (300 $mg \cdot kg^{-1}$, i.v.) on antral and duodenal relaxations, were opposite to those observed when L-NAME was administered alone. In the gastric antrum, L-arginine significantly increased relaxation amplitude (to 156% of control) and frequency (to 170% of control), Table 5. In contrast to the antrum, duodenal 'Grouped' relaxation amplitudes were significantly reduced to 71% control with no significant change in frequency. The action of L-arginine in the duodenum was confined to the 'Grouped' activity and all effects of L-arginine were transient, lasting 4 to 10 minutes before motor activity recovered to within 90% of control levels. L-arginine did not affect gastroduodenal

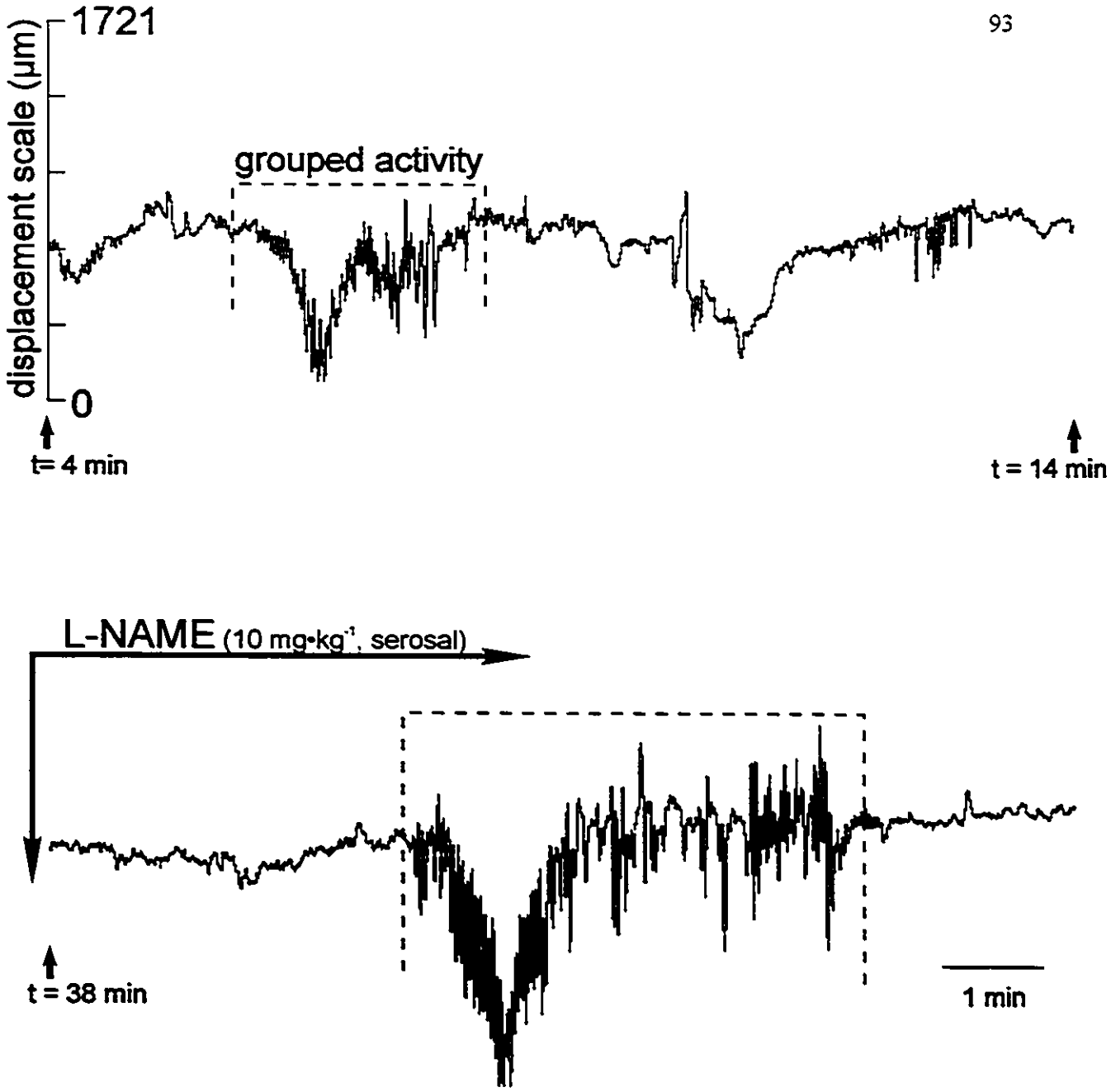


Figure 21 Effects of L-NAME at D_1, *ex vivo*. A typical recording of duodenal motor activity pre and post the direct application of L-NAME onto the serosa of the duodenum around the foil strain gauge (10 mg·kg^{-1}, 100 µl). L-NAME application was at t = 60 min. Serosal application of L-NAME mimicked the effects of systemic L-NAME treatment. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

TABLE 5

Effects of L-arginine on Amplitude and Frequency of Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	**L-arg**	**control**	**L-arg**
S_1 relaxations	20.3±2	31.7±2.5 *	1.61±0.16	2.75±1.0 *
D_1 Grouped relaxations	9.4±0.6	6.7±0.8 *	4.0±0.4	4.3±0.3
D_1 intergroup relaxations	6.2 ±0.7	8.8 ±0.7	4.2 ±0.5	5.5 ±0.7

* denotes significance ($p < 0.05$) compared to control; n = 6

L-arg = L-arginine (300 $mg \cdot kg^{-1}$, i.v.)

amplitude = displacement (μm); frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

TABLE 6

Effects of L-NAME + L-arginine on Amplitude of Relaxations at S_1 and D_1

Region + event type	Amplitude		
	control	L-NAME	L-arg
S_1 relaxations	51.2±2.3	37.2±3.8*	82.8±3.6*
D_1 intergroup relaxations	8.0±1.2	5.3±1.8*	10.8±2.3
D_1 grouped relaxations	10.9 ±1.4	31.1 ±1.3*	1.6 ±0.6*

* denotes significance ($p < 0.05$) compared to control; n = 4

L-arg = L-arginine (300 $mg \cdot kg^{-1}$, i.v.)

amplitude = displacement (μm)

All tabulated data is presented as mean ± SEM

Figure 22 The effects of combined L-NAME + L-arginine at S_1. L-NAME (10 $mg \cdot kg^{-1}$, i.v.) was injected as a single bolus at t = 60 min. The first panel represents a 5 minute segment of control recording. With L-NAME treatment (panel 2), antral relaxations were reduced. L-arginine (300 $mg \cdot kg^{-1}$, i.v.) was injected at t = 120 min. L-arginine was able to transiently reverse the effects of L-NAME, panel 3 and 4. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

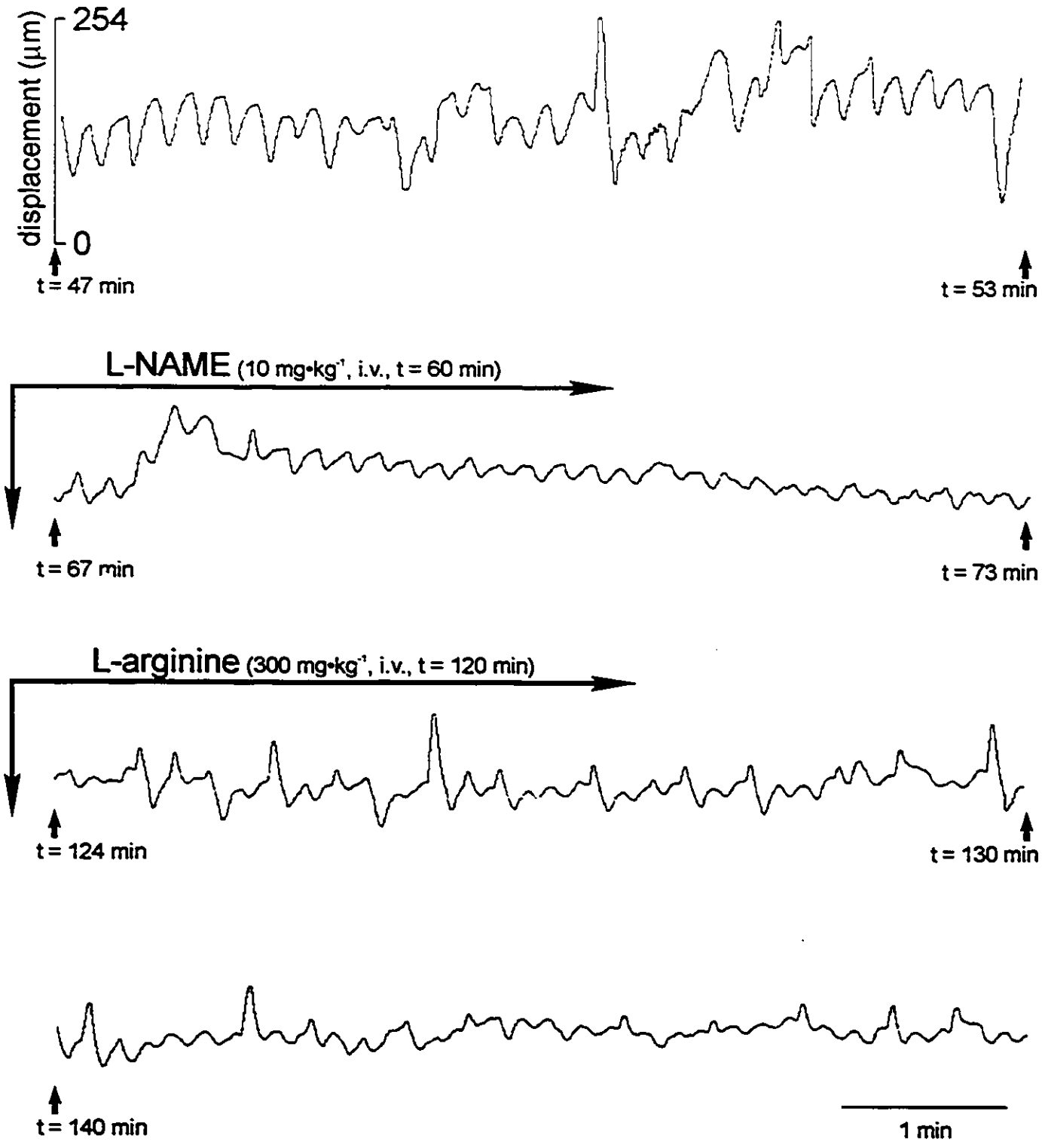
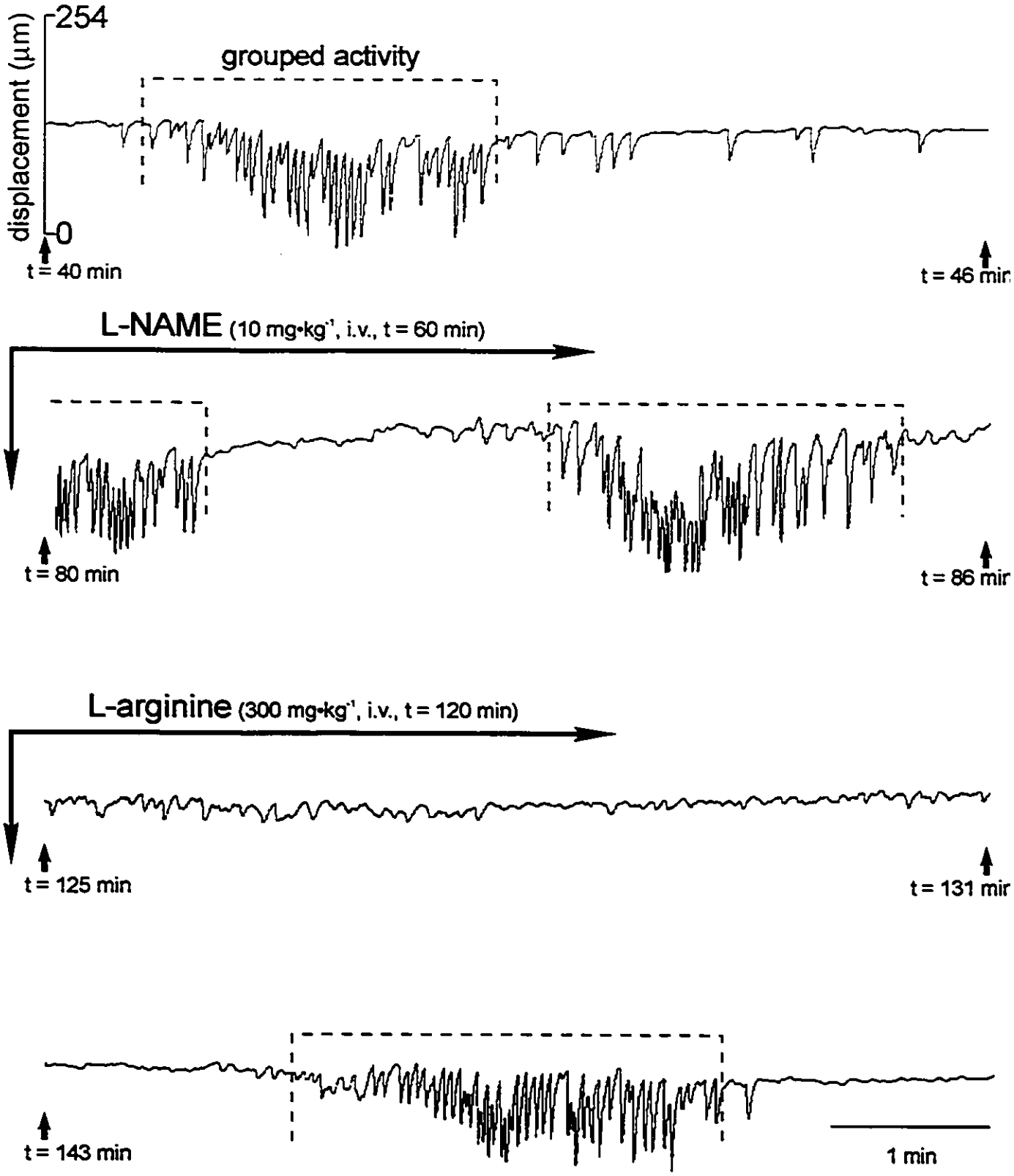

displacement (µm)
254
0
t = 47 min
t = 53 min
L-NAME (10 mg•kg⁻¹, i.v., t = 60 min)
t = 67 min
t = 73 min
L-arginine (300 mg•kg⁻¹, i.v., t = 120 min)
t = 124 min
t = 130 min
t = 140 min
1 min

Figure 23 The effects of combined L-NAME + L-arginine at D_1. L-NAME (10 mg·kg^{-1}, i.v.) was injected as a single bolus at t = 60 min. The first panel represents a 6 min segment of control recording showing 'Grouped' and 'Intergroup' activity. After L-NAME treatment (panel 2), 'Grouped' relaxations were enhanced while 'Intergroup' relaxations were abolished. L-arginine (300 mg·kg^{-1}, i.v.) was injected at t = 120 min. L-arginine was able to transiently reverse the effects of L-NAME (panel 3) before a return to pre-L-arginine, post-L-NAME effects (panel 4). The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.


254
displacement (µm)
0
grouped activity
t = 40 min
t = 46 min
L-NAME (10 mg•kg⁻¹, i.v., t = 60 min)
t = 80 min
t = 86 min
L-arginine (300 mg•kg⁻¹, i.v., t = 120 min)
t = 125 min
t = 131 min
t = 143 min
1 min

contractions.

When injected 60 minutes after the administration of L-NAME (10 $mg \cdot kg^{-1}$, i.v.), L-arginine (300 $mg \cdot kg^{-1}$, i.v.) transiently reversed (for up to 10 minutes) the effects of L-NAME (Fig. 22 and Fig. 23). Antral relaxation amplitudes which were attenuated (to 73% of control, $p<0.05$) by L-NAME treatment, were significantly increased (to 162% of control) following injection with L-arginine, Table 6. The magnitude of this effect of L-arginine was comparable to that seen when L-arginine was administered alone. A consistent feature of gut motor activity of rats injected with L-NAME followed by L-arginine, was the increase in antral relaxation frequency (to 171% of control), Table 7.

In the proximal duodenum, L-NAME significantly enhanced 'Grouped' relaxations corresponding to an increase in both amplitude(to 285% of control) and frequency (to 241% of control), Tables 5 & 6. L-arginine reversed the effects of L-NAME on 'Grouped' and 'Intergroup' motor activity (Fig. 22 and 23) and further reduced the 'Grouped' relaxations to 15% of control amplitude and to 2.5% of control frequency.

The Effects of ATP and the P_2-Purinoceptor Antagonist, Suramin

The effects of ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$, for 1 min i.v.) on gastroduodenal motor activity, injected after a 60 minute control recording period, are shown in Figure 24. In the gastric antrum, ATP infusion caused large amplitude (215% of control) relaxations, with a frequency of 118% of control (Table 8). These easily distinguishable actions lasted for approximately 10-15 min, before a return of relaxant motor activity to control patterns. Antral contraction amplitude and frequency were also significantly increased within the first 20

TABLE 7

Effects of L-NAME + L-arginine on Frequency of Relaxations at S_1 and D_1

Region + event type	Frequency		
	control	L-NAME	L-arg
S_1 relaxations	2.38±0.2	2.8±0.2	4.07±0.27*
D_1 intergroup relaxations	1.48±0.2	0.56±0.2 *	2.6±0.5 *
D_1 grouped relaxations	3.9 ±0.6	9.4 ±0.8 *	0.1 ±0 *

* denotes significance ($p < 0.05$) compared to control; n = 4

L-arg = L-arginine (300 $mg \cdot kg^{-1}$, i.v.)

frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

Figure 24 The effects of ATP at S_1. The three panels represent 10 min segments from a continuous recording taken from t = 50 to t = 80. ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$, i.v.) was infused for 1 min at t = 60 min. Large amplitude relaxations were induced (panel 2) in the gastric antrum for 10-15 min before a return to control conditions. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

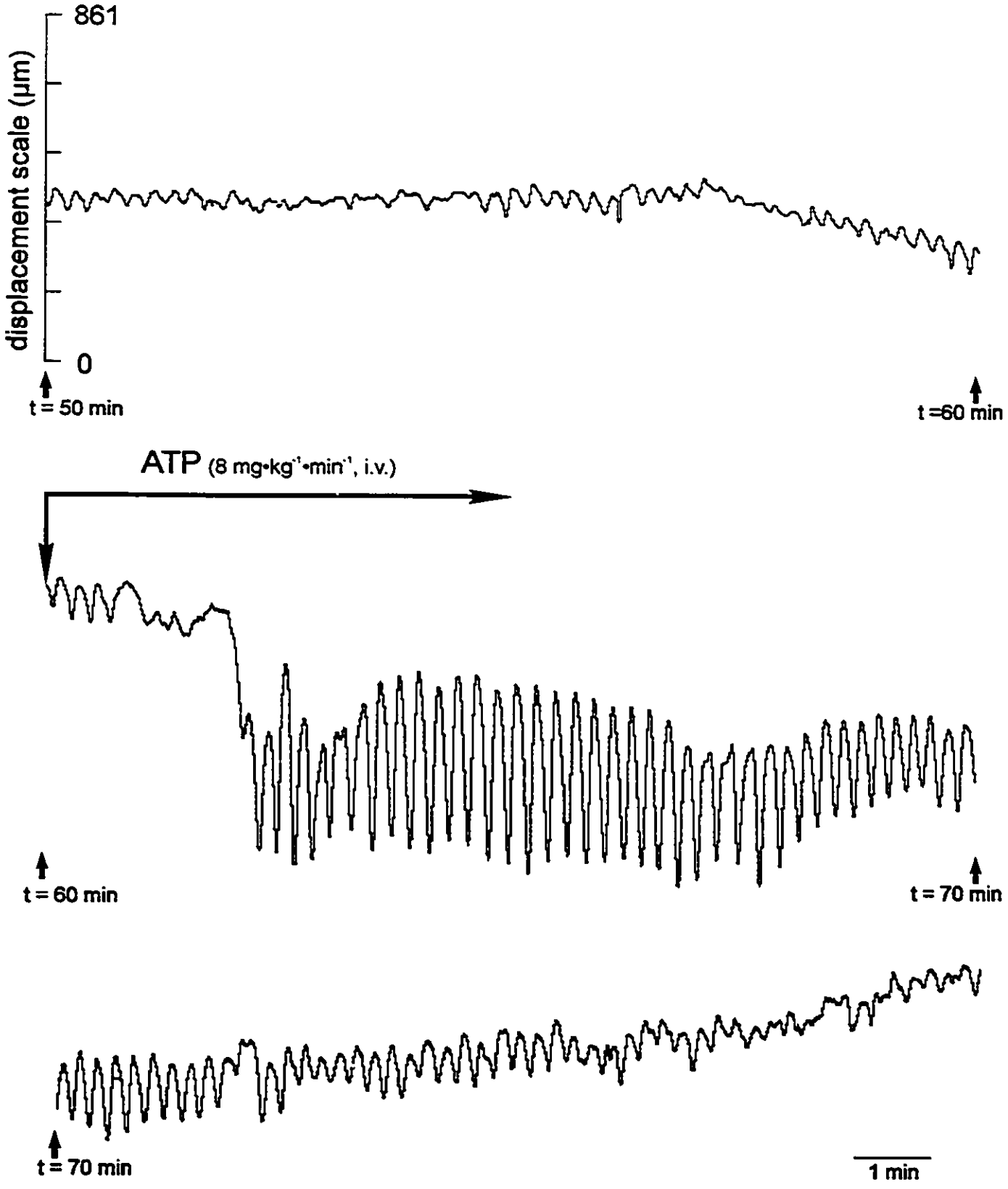
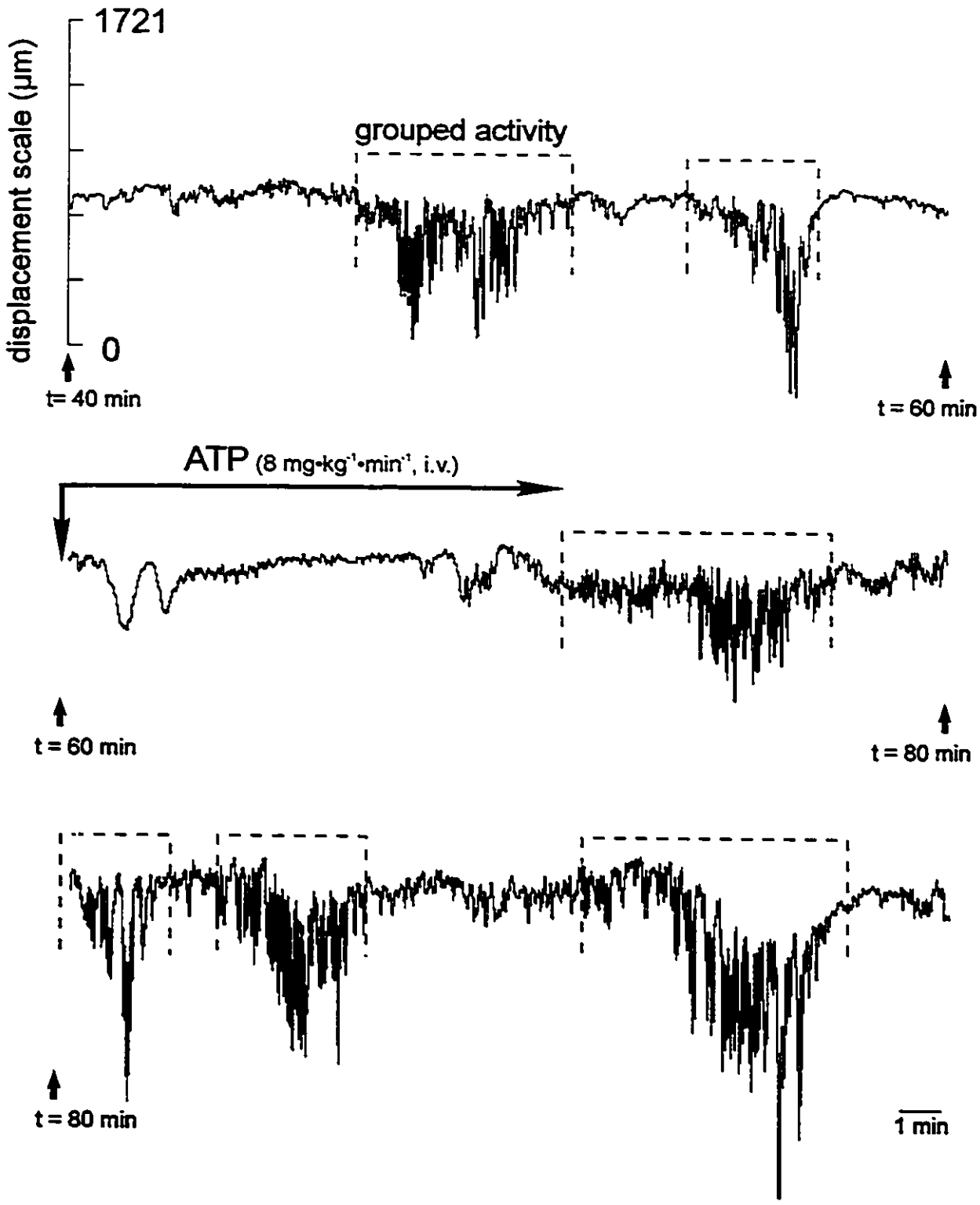

861
displacement scale (µm)
0
t = 50 min
t =60 min
ATP (8 mg•kg⁻¹•min⁻¹, i.v.)
t = 60 min
t = 70 min
t = 70 min
1 min

Figure 25 The effects of ATP at D_1. Shown is a typical recording pre and post ATP infusion (8 $mg \cdot kg^{-1} \cdot min^{-1}$, for 1 min i.v., at t = 60 min) from t = 40 min to t = 90 min presented. The duodenum, usually responded with a single and sometimes double transient relaxation (panel 2) within 1 min of ATP infusion. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.


1721
displacement scale (µm)
0
grouped activity
t= 40 min
t = 60 min
ATP (8 mg·kg⁻¹·min⁻¹, i.v.)
t = 60 min
t = 80 min
t = 80 min
1 min

minutes of ATP treatment, Table 8.

In contrast to the antrum, the duodenum usually only responded with a single, transient relaxation within 1 minute of ATP injection, Fig. 25. However, this response occurred or else was visible only if, at the time of the injection, 'Intergroup' activity was occurring at D_1. This relaxation could easily be distinguished from the random spontaneous relaxa[illegible] during 'Intergroup' activity by its long duration and large amplitude. ATP did not affec[illegible]s activity of either the 'Grouped' or 'Intergroup' periods.

To [illegible]idate the involvement of the purine ATP in relaxant activity of the gastroduoden[illegible], parallel experiments were conducted using the P_2-purinoceptor antagonist suramin. Pharmacological studies have shown that ATP induces relaxations of enteric smooth muscle via P_2-purinoceptors in the muscle. If any of the gastroduodenal relaxations observed in this study involved P_2-purinoceptors, suramin should antagonize these relaxations. Preliminary *in vivo* experiments which employed a dose of 30 $mg \cdot kg^{-1}$ (represents a dose that is slightly higher than the effective dosage used in humans against parasites[85,126]), found that the effects of suramin were not consistent. However, suramin at dose of 60 $mg \cdot kg^{-1}$ i.v. was consistently effective without altering vital signs in the test animals. Therefore, suramin at the higher dose was employed in this series of experiments. The effects of suramin 60 $mg \cdot kg^{-1}$ administered as a single i.v. bolus injection after a 60 minute control recording period, are shown in Figure 26. Motor activity of the antrum was not affected (Table 9). However, in the duodenum, suramin caused changes in 'Grouped' motor activity evident from 90 min and maximal at 120 min post drug injection. This long equilibration time for suramin action is comparable to that observed in other studies [115,119]. Suramin actions were characterized by

an increase in the average duration of 'Grouped' activity periods (from 4.63 to 7.12 min, $p<0.05$), reduction of 'Grouped' relaxation amplitude and frequency, and significantly increased ($p<0.05$) 'Grouped' contraction amplitude and frequency (Table 9).

TABLE 8

Effects of ATP on Amplitude and Frequency of Contractions and Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	ATP	control	ATP
S_1 contractions	75.3±12.4	81.6±4.3*	1.49±0.22	3.0±0.4*
S_1 relaxations	58±4.2	124.9±21*	1.71±0.1	2.02±0.4*

* denotes significance ($p < 0.05$) compared to control; n = 6

ATP = ATP (8 $mg \cdot kg^{-1} \cdot min^{-1}$, for 1 min i.v.)

amplitude = amplitude (μm); frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

Figure 26 The effects of Suramin at D_1. Suramin (60 $mg \cdot kg^{-1}$, i.v.) was injected as a single bolus at t = 60 min. The maximal effect was evident at 120 min post injection. 'Grouped' relaxations were significantly inhibited while contractions were significantly increased after suramin treatment. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

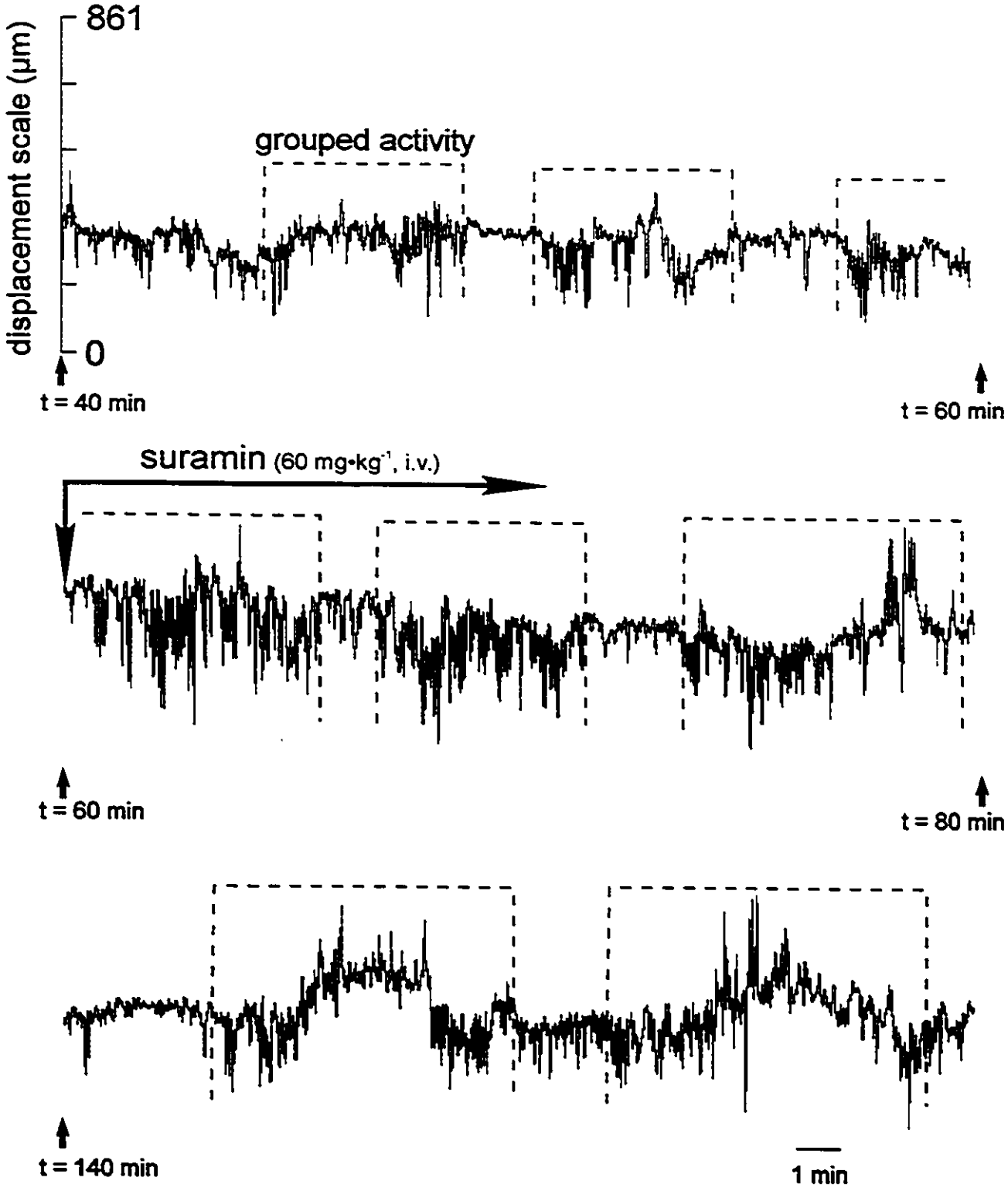

861
displacement scale (µm)
0
grouped activity
t = 40 min
t = 60 min
suramin (60 mg•kg⁻¹, i.v.)
t = 60 min
t = 80 min
t = 140 min
1 min

TABLE 9

Effects of Suramin on Amplitude and Frequency of Contractions and Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	suramin	control	suramin
S_1 contractions	29±5.0	28.1±4.2	0.73±0.9	0.78±0.2
S_1 relaxations	19.8±1.7	23.9±3.0	1.24±0.1	1.37±0.1
D_1 Grouped contractions	39.5±5.9	47.2±2.4*	0.74 ±0.23	3.19±0.3*
D_1 Grouped relaxations	56.5±2.4	36.9±1.4*	9.1±0.4	5.5±0.65*
D_1 Intergroup contractions	41.9±8.6	37.3±3.1	1.05±0.3	1.59±0.2
D_1 Intergroup relaxations	39.0±3.5	33.4±1.7	4.5±0.6	4.6±0.5

* denotes significance ($p < 0.05$) compared to control; n = 4

amplitude = displacement (μm); frequency = events·min^{-1}

All tabulated data is presented as mean ± SEM

DISCUSSION

The results obtained in these experiments show that intravenous injection of the NO synthase inhibitor L-NAME differentially alters spontaneous motor activity in the gastric antrum and the proximal duodenum *in vivo*. These actions were reversed by the NO synthase substrate L-arginine. All relaxations in the gastric antrum were either significantly reduced or else abolished by L-NAME whilst antral contractions appeared unaffected. However, in the duodenum, only 'Intergroup' relaxations were inhibited, and 'Grouped' relaxations were significantly enhanced. These results indicate that NO is not the primary transmitter of the pathway controlling relaxations occurring within the 'Grouped' activity period. However, the results clearly show that NO has a primary role in relaxations of the antrum and within duodenal 'Intergroup' activity. The effect of L-NAME on 'Grouped' relaxations was unexpected and contrary to the notion that NO functions only as a transmitter of NANC inhibitory motor neurones, or as a relaxant factor within the smooth muscle cells[70]. These findings are discussed further on.

The involvement of NO in relaxations of the rodent stomach *in vivo* has been previously reported by Lefebvre *et al* who demonstrated that L-NAME (10 & 30 $mg \cdot kg^{-1}$ i.v.) either abolished or greatly reduce vagally stimulated relaxations of the gastric fundus in anaesthetized rats [114]. Interestingly these investigators also observed that L-NAME treatment enhanced vagally stimulated gastric contractions. However, in the present study no significant change in spontaneous antral contractions with local or intravenous L-NAME (10 $mg \cdot kg^{-1}$) treatment was observed. The possibility exists, therefore, that the contractions observed by Lefebvre *et al* to be evoked by vagal stimulation involve pathways distinct from those

mediating spontaneous contractile activity described in this study, and that vagally stimulated contractions are in some way modulated by NO.

Although the inhibition of all antral relaxations by L-NAME treatment is strongly suggestive of the involvement of NO in the generation of these responses, NO related elements may not be the final component in the pathway mediating these responses. NO could be stimulating the release of ATP, since intravenous ATP injection was also able to cause relaxations in the gastric antrum, and ATP is known to have direct actions on gastrointestinal smooth muscle [28]. In this study, intravenous injection of ATP caused relaxations in the gastric antrum, without affecting spontaneous antral relaxations. In addition, treatment with the P_2-purinoceptor antagonist suramin at doses that inhibited relaxations to intravenous ATP, had no effect on spontaneous antral relaxations. Recently, Grider *et al* sought to identify the NANC inhibitory neurotransmitter(s) in guinea pig stomach muscle strips by using selective ligands to protect postjunctional receptors combined with the blockade of all other postjunctional receptors using N-ethylmaleimide (NEM) [69]. When only ATP receptors were protected (by ATP) from NEM blockade, ATP, but not electrical field stimulation, was able to induce relaxations. Grider and coworkers concluded that ATP actions were not neurogenic, but rather ATP acted directly on the gastric smooth muscle. Moreover, their results showed that ATP could not be the transmitter released by the electrically stimulated inhibitory motor neurons. Taken together, it would appear that NO and not ATP is the transmitter of the inhibitory (presumably NANC) motor nerves mediating spontaneous relaxation of the rodent stomach. This is further supported by a recent *in vitro* study of rat gastric fundus muscle strips which showed that pre-treatment with the NO synthase inhibitor,

N^G-nitro-L-arginine, had no effect on ATP evoked relaxations[41].

In an *in vivo* study of the rat jejunum, Calignano *et al* reported an increase in motility following the intravenous administration of NO synthase inhibitor[31], and, on the basis of their observation, proposed that NO modulates the release of certain contractile mediators from local neuronal or intestinal cellular sources. Likewise, studies of anaesthetized rat and cat small intestine also show that inhibition of NO synthesis causes an increase in phasic motor activity[82,83], indicative of a neuromodulatory role for NO in the small intestine. Enhancement of 'Grouped' relaxations following L-NAME treatment observed in the present study is further evidence for NO to have a neuromodulatory role in the rat duodenum. The simplest possible mechanism for this enhancement of duodenal responses by L-NAME treatment is that NO is released by inhibitory interneurons targeting neurons in the pathway controlling 'Grouped' relaxations. Therefore, treatment with this NO synthase inhibitor would reduce the constitutive production of NO effectively disinhibiting this pathway. This may also explain how L-NAME treatment also substantially increased the magnitude but not the quantity of contractions within both 'Grouped' and 'Intergroup' duodenal motor activities.

The localization of NO related cells and fibres in the rat, guinea-pig, porcine and human myenteric and submucosal nerve layers, together with the rich investment of nitrergic nerve fibres in the muscularis supports a motor neurotransmitter role for NO. However, the extensive distribution and dense localization of NO synthesizing varicose fibres within almost all intestinal ganglia of these species, strongly suggest that NO may also be a transmitter of enteric interneurons[2,40,132,133,164]. Functional proof of this has been provided by Young *et al* [175], who showed that NO stimulates cGMP production within both myenteric and submucosal

ganglion cells. This notion is further strengthened by the results of anatomical studies in this laboratory where, a subpopulation of intestinal GABAergic ganglion cells and therefore by definition enteric interneurons[57,64,103], were found to display Ca^{2+} dependent NO synthase activity and hence the capacity to constitutively synthesize NO [135]. Thus, it is highly unlikely that NO functions only as a transmitter of intrinsic inhibitory motor neurons.

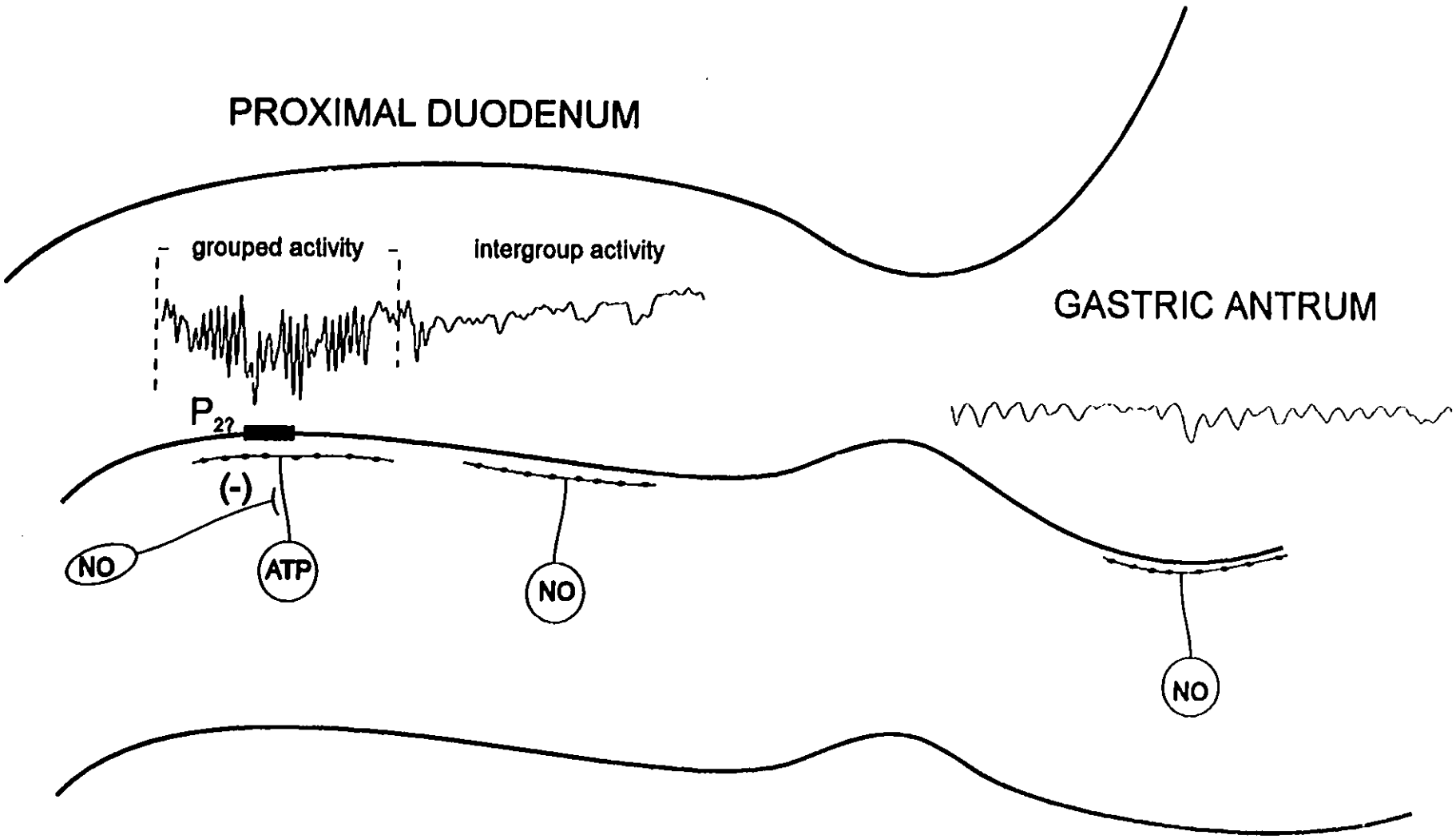
The results of *ex vivo* experiments in this study where L-NAME was applied directly onto the gut wall, not only support the notion that NO has a neuromodulatory role but provides evidence that this action resides within the wall of the proximal duodenum.

The identity of the neurotransmitter mediating the duodenal 'Grouped' relaxations observed, still has to be addressed. For the rat, at least two different inhibitory motor systems have been proposed to occur in the small intestine, with ATP being the major NANC inhibitory transmitter in the duodenum [65]. Results from the present experiments using L-NAME treatment show that the inhibitory transmitter responsible for 'Grouped' relaxations is not NO. Rather, the transmitter mediating these relaxations is ATP since treatment with the slowly equilibrating P_2-purinocepter antagonist suramin [115,58], blocked 'Grouped' relaxations. An interesting aspect of the effects of suramin was the almost two-fold increase in duration of the Grouped activity period together with the increase in contraction amplitudes. This phenomenon has been observed *in vitro* in the guinea-pig stomach, where applied suramin not only inhibited NANC inhibitory junction potentials but also enhance cholinergic excitatory junction potentials [137]. On this basis, it is proposed that 'Grouped' relaxations are mediated (in part or in whole) by ATP and involve its P_2-purinoceptors. The identity of the P_2-purinoceptor subtype [98] mediating these ATP actions was investigated in a separate series of experiments

described further on in Chapter 3.

In conclusion, the results presented in this chapter provide compelling evidence that NO is the primary inhibitory neurotransmitter of pathway(s) controlling spontaneous relaxant motor activity in the gastric antrum of the anaesthetized rat (as summarized in Figure 27). In the proximal duodenum, there appears to be more than one inhibitory motor innervation. These can now be functionally separated with respect to the distinct patterns of motor activity within the gastroduodenum: 'Intergroup' relaxations, like the antral relaxations are mediated by NO while 'Grouped' relaxations are mediated by ATP. In addition to having a role as an inhibitory neurotransmitter within the rat gastroduodenum, NO may also be involved in the neuromodulatory control of the ATP-related pathway mediating relaxations within 'Grouped' motor activity of the proximal duodenum. Moreover, NO may be released by interneurons modulating excitatory motor innervation of the duodenum.

Figure 27 A schematic representation of the inhibitory innervation of the rat gastroduodenum. All relaxations in the gastric antrum are mediated by nitrergic neurons. In the proximal duodenum, two NANC neuronal populations exist and correspond to mediating relaxations within the two distinct patterns of motility, 'Grouped' and 'Intergroup'. A nitrergic inhibitory population (NO) is responsible for mediating 'Intergroup' relaxations while 'Grouped' relaxations are mediated by ATP via a purinoceptor, possibly the P_2 type. 'Grouped' relaxations are also modulated by NO.


PROXIMAL DUODENUM
grouped activity
intergroup activity
GASTRIC ANTRUM
P2?
(-)
NO
ATP
NO
NO

CHAPTER 3

INVOLVEMENT OF P_2-PURINOCEPTORS IN SPONTANEOUS MOTOR ACTIVITY OF THE RAT GASTRODUODENUM

Studies of the spontaneous motor activity of the gastroduodenum in the anaesthetized rat (presented in Chapter 2) showed that NO and ATP mediated different patterns of motor relaxant activity. NO mediated antral relaxations and duodenal 'Intergroup' relaxations, whilst the duodenal 'Grouped' relaxations were mediated by ATP, acting at suramin sensitive P_2-purinoceptors. Since ATP actions in the gut have been characterized to involve a family of receptor types which, of themselves, help characterize the nature of ATP mediated events in the gut wall, I sought to the identify the purinoceptor subtype involved in 'Grouped' relaxations.

In the mammalian gut, the P_2-purinoceptors are classified into two subtypes, types P_{2x} and P_{2y}. While certain investigators consider P_{2y}-purinoceptors to be the receptor by which ATP mediates NANC relaxation of the intestine[27,98], there is also evidence for ATP to induce relaxations via P_{2x}-purinoceptors [36,119].

Characterizing the nature of the P_2-purinoceptor subtype involvement in ATP related responses of the gut is made difficult by the lack of readily available specific antagonists (see introduction & Table 1). Suramin may be suitable for antagonizing P_2-purinoceptors but it is limited in its ability to distinguish between the specific subtypes. PPADS (pyridoxalphosphate-6-azophenyl-2'-4'-disulfonic acid) has been shown to selectively antagonize P_{2x}-purinoceptor mediated responses in the rabbit urinary bladder [176]. However, this

antagonist is a proprietary synthesized product and as such is not readily available. An alternative approach has been to examine these subtypes by their rank order potency for several ATP analogues (summarized in Table 1). P_{2x}-purinoceptors are more responsive to α,β-methylene ATP and functional studies show that this receptor subtype can be readily desensitized with continuous exposure to this ATP analogue. Fortunately, 2-methylthio-ATP is more potent at the P_{2y}-purinoceptor. In order to examine the identity of the P_2-purinoceptor subtype responsible for ATP mediated 'Grouped' relaxations in the duodenum of anaesthetized rats *in vivo*, desensitization with α,β-methylene ATP and treatment with 2-methylthio-ATP were employed.

METHODS

In vivo

Sprague Dawley rats were surgically implanted with two foil strain gauges (S_1 & D_1) as described in the methodology of Chapter 1. After surgery, the animal was allowed a one hour period of stabilization before motility recordings began. Experiments consisted of a 60 minute control period of recording at which point drug treatment was administered followed by motility recordings for up to 4 hours. For all drugs except α,β-Methylene ATP, the treatment period started at the time of injection. For experiments where α,β-Methylene ATP was administered, the treatment period started after the development of desensitization.

Chemicals

α,β-Methylene adenosine triphosphate (α,β-Me-ATP), N^G-Nitro-L-arginine Methyl Ester (L-NAME) and tetrodotoxin (TTX) were obtained from Sigma Chemical (St. Louis, MO, USA). 2-methylthio-adenosine triphosphate (2-Me-S-ATP) was obtained from Research Biochemicals International (MA, USA).

RESULTS

Effects of α,β-Methylene ATP

α,β-Me ATP (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 1 min, i.v.) was infused after 60 minutes of control recording, (Figure 28). Initially, 'Grouped' relaxant activity was significantly augmented in amplitude (to 211% of control) for 2-3 minutes. This increase in relaxation amplitude was then followed by a reduction of both amplitude and frequency of 'Grouped' relaxations, to 24% and 52% respectively (see Table 10). This effect persisted to the end of the experiment, indicative of a persistent desensitization. At a lower dosage (150 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 1 min, i.v.), α,β-Me ATP exerted similar effects but these did not persist, and in some cases there was recovery as early as 30 minutes post injection. Although there was an increase in 'Intergroup' relaxation activity, these changes were not significant. There were no significant changes in contraction activity.

Effects of 2-Methyl-Thio-ATP

2-methyl-thio-ATP (2-Me-S-ATP, 360 $\mu g \cdot kg^{-1}$, i.v.) was administered after 60 minutes of control recording. Upon injection, a single large relaxation was observed that was easily distinguishable from all other spontaneous activity, as is apparent in Figure 29. This relaxation response had a mean amplitude of 1288±71 μm with a duration of approximately 1.5 minutes. No significant effects on spontaneous 'Grouped' relaxation amplitude and frequency were found (Table 11). However, 2-Me-S-ATP significantly reduced 'Intergroup' relaxation amplitude (80% of control) and frequency (78% of control), Table 11.

Figure 28 Effects of α,B methylene ATP at D_1. A typical recording of duodenal motor activity pre and post α,B Methylene ATP (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 1 min, i.v.). 'Grouped' activity was transiently augmented followed by a reduction, due to P_{2x} purinoceptor desensitization. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 min.

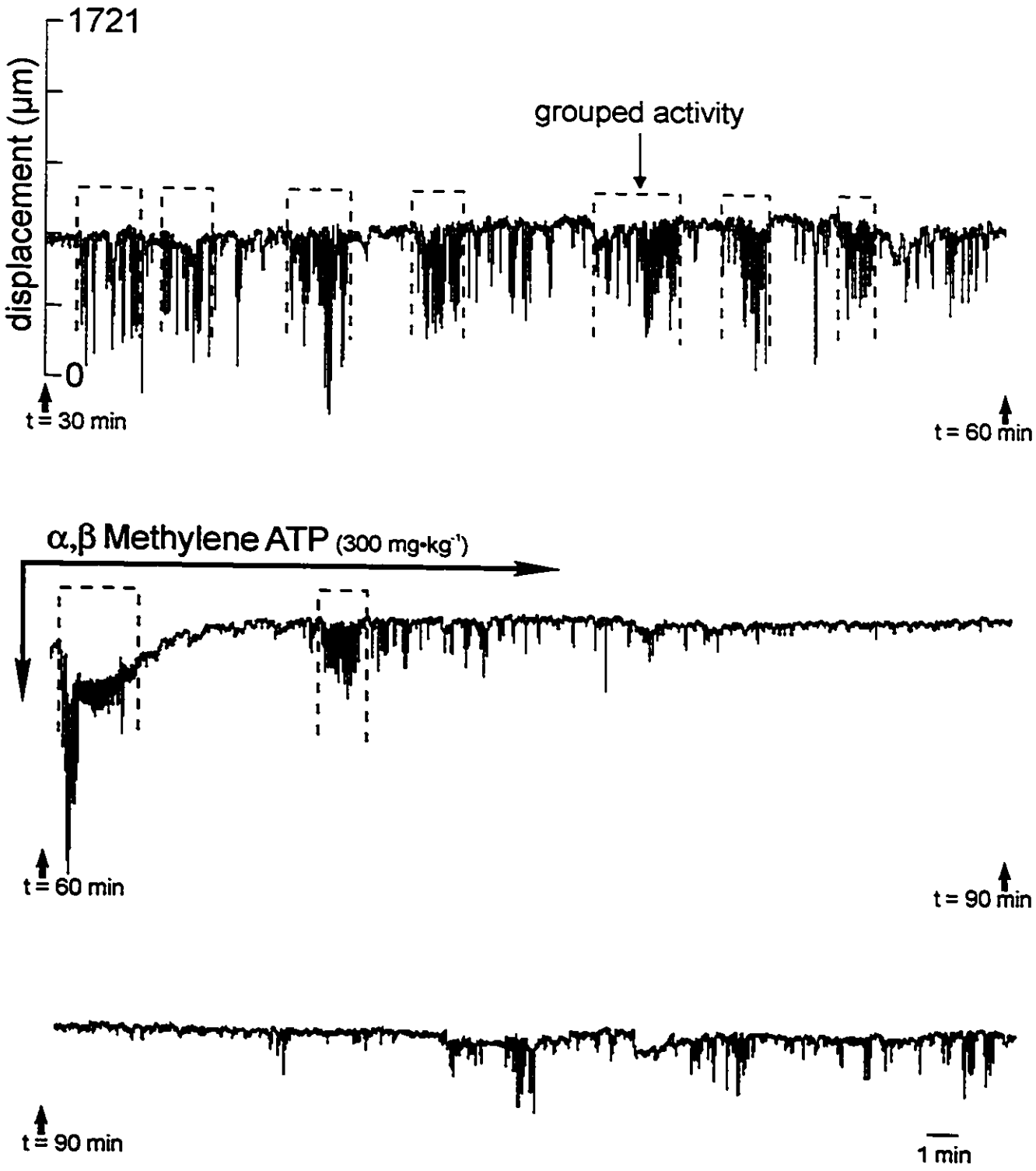

1721
displacement (µm)
0
grouped activity
t = 30 min
t = 60 min
α,β Methylene ATP (300 mg·kg⁻¹)
t = 60 min
t = 90 min
t = 90 min
1 min

TABLE 10

Effects of α,β-Methylene ATP on Relaxation Amplitude and Frequency at D_1

Region + Event type	Amplitude		Frequency	
	control	α,β-Me ATP	control	α,β-Me ATP
D_1 Grouped relaxations	152±8.7	37±3.9*	9.95±1.0	5.27±0.8*
D_1 Intergroup relaxations	38.2±2.9	48.4±4.3	4.43±0.4	3.1±0.2
D_1 Grouped contractions	59.7±8.7	47.9±5.5	0.99±0.4	1.32±0.2
D_1 Intergroup contractions	61±9.1	42±12	0.44±0.2	0.44±0.1

* denotes significance ($p < 0.05$) compared to control; n = 4

α,β-Me-ATP = α,β-methylene-ATP (300 $\mu g \cdot kg^{-1}$, i.v. over 1 min.)

amplitude = amplitude (μm); frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

To test whether the large 2-Me-S-ATP induced relaxation involved NO, this P_{2y} agonist was applied (360 $\mu g \cdot kg^{-1} \cdot min^{-1}$, i.v.) to a separate group of animals 30 minutes post L-NAME (10 $mg \cdot kg^{-1}$, i.v.). In experiments with L-NAME pre-treatment, 2-Me-S-ATP induced relaxations were significantly reduced or abolished when compared to the amplitude of the 2-Me-S-ATP evoked relaxation response in experiments without L-NAME pre-treatment, Table 12.

Figure 29 The effects of 2-Me-S-ATP at D_1. Shown is a typical recording pre and post the injection of 2-ME-S-ATP (360 $\mu g \cdot kg^{-1}$, i.v.) at t = 60 min. The first panel shows typical duodenal control motor activity. Upon injection of 2-ME-S-ATP a single large relaxation was induced as shown in the second panel. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

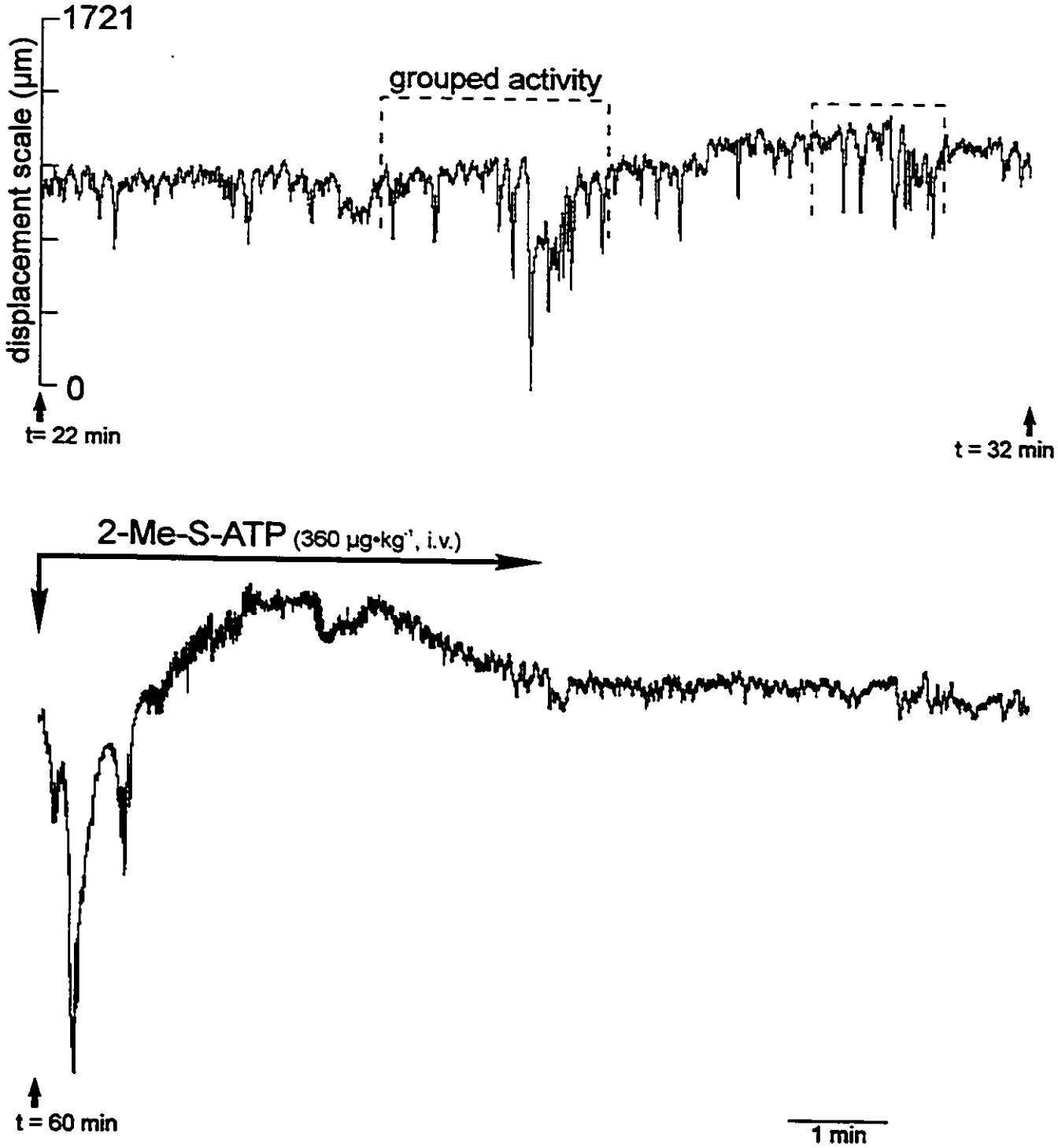

1721
displacement scale (µm)
0
grouped activity
t = 22 min
t = 32 min
2-Me-S-ATP (360 µg•kg⁻¹, i.v.)
t = 60 min
1 min

TABLE 11

Effects of 2-Methythio ATP on Relaxation Amplitude and Frequency of at D_1

Region + Event type	Amplitude		Frequency	
	control	2-Me-S-ATP	control	2-Me-S-ATP
D_1 Grouped relaxations	156 ±8.4	150 ±8.7	8.1 ±0.6	9.2 ±0.8
D_1 Intergroup relaxations	87 ±6.2	70 ±3.9*	4.1 ±0.4	3.2 ±0.3*

* denotes significance ($p < 0.05$) compared to control; n = 4

2-Me-S-ATP = 2-methyl-thio-ATP (360 $\mu g.kg^{-1}$, i.v.)

amplitude = amplitude (μm); frequency = events·min^{-1}

All tabulated data is presented as mean ± SEM

TABLE 12

Effects of 2-Methythio ATP, pre/post L-NAME, on Relaxation Amplitude at D_1

Region + event type	Amplitude	
	2-Me-S-ATP (control)	post L-NAME 2-Me-S-ATP
D_1 relaxation	1134±10	12±10*

* denotes significance ($p < 0.05$) compared to control; n = 4

2-Me-S-ATP = 2-Methyl-thio-ATP (360 $\mu g \cdot kg^{-1}$, i.v.)

L-NAME = L-NAME (10 $mg \cdot kg^{-1}$, i.v.)

amplitude = displacement (μm)

All tabulated data is presented as mean ± SEM

DISCUSSION

The results presented here show that the P_{2x}-purinoceptor agonist, α,β-Me-ATP was able to block duodenal 'Grouped' relaxations by desensitization after an initial, but transient, augmentation of 'Grouped' relaxation amplitude. Unexpectedly, 2-Me-S-ATP induced a large relaxation in the proximal duodenum that was found to be sensitive to L-NAME. Like α,β-Me-ATP, prolonged exposure to 2-Me-S-ATP was also found to cause a reduction in motor activity; however, this was apparent only for 'Intergroup' relaxant activity.

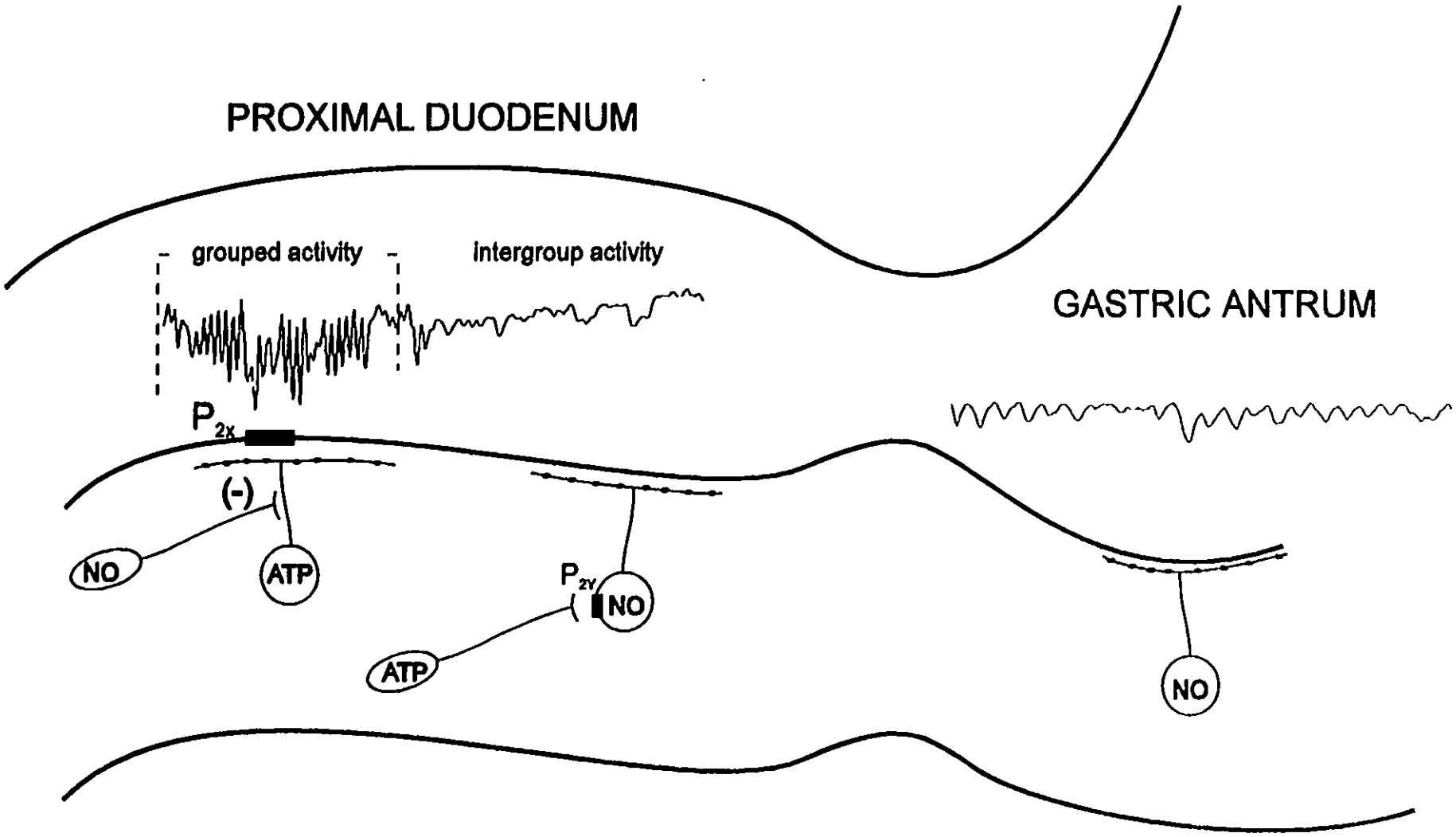
α,β-Me-ATP has been used previously as an agonist to induce relaxations in rat gastric fundus, guinea pig taenia coli and the circular muscle of guinea pig antrum, ileum and distal colon [36,71,119]. Intracellular electrophysiological recordings from guinea pig ileum smooth muscle cells show that ATP induced hyperpolarizations are antagonized by α,β-Me-ATP [38]. Similarly, in rat gastric fundus, relaxations induced by ATP are completely blocked by α,β-Me-ATP desensitization [119]. As discussed in the Introduction, α,β-Me-ATP is a potent agonist of P_{2x}-purinoceptors. However, in only one of these previous studies has the potential for P_{2x}-purinoceptors involvement in mediating gut relaxations been discussed. Mathura and Hollingsworth employed α,β-Me-ATP both as an agonist and a desensitization tool and, on the basis of their findings, proposed that ATP induced relaxations of rat gastric fundus are mediated via P_{2x}-purinoceptors [119]. This was the first study to contradict the generally accepted notion that purinergic gut relaxations are all mediated by P_{2y}-purinoceptors. Using the α,β-Me-ATP desensitization protocol, the results of the present study show that α,β-Me-ATP desensitization specifically blocks duodenal 'Grouped' relaxations, and is evidence that the receptor involved in the ATP mediated 'Grouped' relaxant activity is the P_{2x}-

purinoceptor. This proposal is further strengthened by the observation that treatment with the P_{2y}-purinoceptor agonist, 2-Me-S-ATP had no effect on the 'Grouped' relaxant activity. Rather, 2-Me-S-ATP induced a relaxation that was sensitive to L-NAME. This may be indicative of the presence of P_{2y}-purinoceptors on enteric neurons presumably releasing NO, or else an interneuron within the pathway targeting the NO motor neurons. Alternatively, 2-Me-S-ATP may be exerting its effects directly on the smooth muscle via a myogenic NOS system. An *ex vivo/in vivo* experiment where TTX is applied to the serosa of the proximal duodenum prior to the intravenous administration of 2-Me-S-ATP would determine if the action of 2-Me-S-ATP is nerve mediated. In the canine ileum it has been shown that NO was the final inhibitory mediator of ATP induced relaxations [15]. However, the purinoceptor targeting the nitrergic inhibitory motor neuron was not identified in that study. The second effect of 2-Me-S-ATP in the present study was the reduction of 'Intergroup' relaxant activity. In Chapter 2 it has already been proposed that 'Intergroup' activity is mediated by NO. Like α,B Me ATP, 2-Me-S-ATP administration results in biphasic actions. Following an initial phase where 2-Me-S-ATP evokes relaxations, tachyphylaxis develops and P_{2y} receptor mediated relaxation responses are desensitized. Since all 'Intergroup' relaxations of the duodenum are mediated by NO, and P_{2y}-purinoceptor related desensitization blocks 'Intergroup' relaxations, it would appear that ATP via P_{2y} sites represents a functional input (directly or indirectly) of NO related motor innervation in the proximal duodenum. Evidence for 2-Me-S-ATP desensitization has been shown in the guinea pig caeci where, after the initial 2-Me-S-ATP induced relaxation, repeated addition of 2-Me-S-ATP did not elicit a relaxation response [50]. Figure 30 illustrates the results of the present study utilising a model for nitrergic and

purinergic innervations in the proximal duodenum.

Definitive studies to confirm that P_{2x}-purinoceptors are involved in the mediation of 'Grouped' relaxations are still needed. Recent studies with guinea-pig taenia caeci show that the P_{2x} receptor antagonist DIDS (4,4'-Diisothio-cyanotostilbene-2,2'-disulphonate) could not attenuate relaxations by ATP or the P_{2y} agonist 2-Me-S-ATP, but blocked α,β-Me-ATP induced relaxations [50]. DIDS is readily available and may afford differentiation between the P_{2x} and P_{2y} receptor subtypes.

Figure 30 Inhibitory innervation of the rat gastroduodenum. In the proximal duodenum, two distinct patterns of motor activity exist, 'Grouped' and 'Intergroup'. 'Grouped' activity is mediated by ATP via P_{2x}-purinoceptors. NO mediates relaxations in the 'Intergroup' period. P_{2y}-purinoceptors must be present in the pathway controlling the 'Intergroup' NO-related relaxations. These purinoceptors may be present on NO motor neurones (as shown) of P_{2y}-purinoceptors may be on interneurons belonging to a pathway stimulating these NO neurones.


PROXIMAL DUODENUM
grouped activity
intergroup activity
GASTRIC ANTRUM
P2x
(-)
NO
ATP
P2Y
NO
ATP
NO

CHAPTER 4

γ-AMINOBUTYRIC ACID (GABA) INVOLVEMENT IN RELAXANT ACTIVITY OF THE RAT GASTRODUODENUM

The previous chapters have dealt with the identification of the neurotransmitters responsible for the spontaneous gastroduodenal relaxant activity in the rat and by extrapolation, the NANC inhibitory motor nerve innervation of the gastric antrum and proximal duodenum in this species. The question then of the nature of neural pathways associated with these different innervations, remains to be answered. This is addressed here, in a study designed to determine whether GABAergic interneurons mediate stimulation of these spontaneous relaxant gastroduodenal motor activities.

Studies show that there are at least five neurochemically distinct populations of interneurons (as identified in the guinea pig, for review see [19,33]). The populations include one class of ascending interneurons and four classes of descending interneurons. The ascending interneurons are cholinergic and target nicotinic neural receptors. Of the four classes of descending interneurons, three are cholinergic, and these can be further distinguished based on their colocalizing with 5-hydroxytryptamine (5-HT), somatostatin or VIP. The fourth class of descending interneuron is non-cholinergic, and is proposed to contain VIP, NO and gastrin releasing peptide. Unfortunately, not mentioned or else ignored by investigators, is the role of γ-aminobutyric acid (GABA) as a neurotransmitter of enteric interneurons and hence the involvement of GABAergic interneurons in the pathways controlling gut motility.

GABA is recognized as a major inhibitory neurotransmitter in the mammalian central

nervous system[57], and it is well documented that GABA is present in neurones and endocrine cells of the mammalian gut. Initial evidence for an involvement of GABA in actions relating to gut motility was provided in the late 50's and early 60's by Hobbiger[57] and Inouye *et al*[57], who showed that GABA applied onto isolated gut bath preparations of the guinea-pig small intestine induced both contractions and relaxations. In addition, acetylcholine induced contractions could be reduced by applied GABA. Although GABA was not seriously considered a candidate enteric neurotransmitter for many years following initial studies, these studies had essentially demonstrated all the present known motor actions of GABA. The nature of these actions is now well understood and GABA has fulfilled all of the criteria to be recognized as a transmitter of enteric neurons in the rodent. Like in the CNS, two pharmacologically and functionally distinct GABA receptor types exist in the mammalian gut: bicuculline sensitive $GABA_A$ sites, and $GABA_B$ sites which are bicuculline insensitive and selectively activated by baclofen and antagonized by phosphonic analogues of baclofen [57,100]. Enteric $GABA_A$ receptors, similar to central $GABA_A$ receptors, are coupled to chloride channels and, together, form a supramolecular complex with benzodiazepine and barbiturate binding sites [90,110]. However, unlike the central $GABA_A$ receptors which mediate hyperpolarization of neurons, activation of enteric $GABA_A$ receptor sites results in the depolarization of myenteric neurons [110]. Several selective $GABA_A$ agonists exist, and in addition, barbiturate and benzodiazepine potentiate enteric $GABA_A$ receptor mediated responses [57]. In contrast, $GABA_B$ receptors are associated with the cAMP second messenger system. Furthermore, $GABA_B$ receptors are not influenced by barbiturates or benzodiazepines [57].

Several studies provide evidence that neurons in the myenteric plexus release GABA upon depolarization. In the cat colon, and in guinea pig ileum and colon, experiments have shown that muscle strips preloaded with [^{3}H]$GABA_A$, upon electrical stimulation, results in a significant increase in [^{3}H]$GABA_A$ efflux [91,99,162,163]. Furthermore, in the guinea pig intestine, the use of a neuronal high affinity uptake inhibitor reduced the increase of GABA efflux upon stimulation [99]. Likewise, in explant cultures of myenteric neurons from guinea pig taenia coli, induced depolarization showed similar results [91]. Furthermore, this release of GABA was shown to be sensitive to the neurotoxin TTX.

Early pharmacological studies with GABA implied that GABA may have multiple neural sites of action in the mammalian intestine [57]. However, not evident from these studies was the identity of theses sites, in particular, whether GABA evoked relaxations represented direct or indirect actions of this amino acid transmitter on enteric motor neurons. More recent studies have provided insight to these questions.

GABA applied onto gut muscle strips from the guinea pig ileum and colon, and rat duodenum and ileum has been shown to induce contractions [67,105,107,139]. In addition, GABA has also been shown to induce relaxations in guinea pig ileum and colon, rat small intestine and colon, as well as in canine ileum and ileocolonic junction [17,77,105,107,117]. In nerve-free strips of guinea pig ileal muscle and rat colonic smooth muscle cells, GABA was ineffective [77,105]. Furthermore, GABA induced responses in intact gut segments were blocked by TTX. These contraction and relaxation responses could be mimicked with the application of the $GABA_A$ receptor agonist 3-APS (3-amino-1-propanesulphonic acid) and were sensitive to the $GABA_A$ antagonist bicuculline. Contractions induced by GABA were blocked by atropine; evidence

that supports electrophysiological data[110] for $GABA_A$ receptors to be localized to cholinergic neurons. GABA induced relaxations were found to be non-adrenergic and non-cholinergic in nature, insensitive to the $GABA_B$ agonist baclofen and not affected by the $GABA_B$ antagonist phaclofen. As to the identity of the mediator of the NANC inhibitory neuron, Maggi *et al* [117] observed that desensitization with ATP blocked GABA induced neurogenic relaxations in the rat duodenum. However, in the canine terminal ileum and ileocolonic junction, ATP desensitization did not affect relaxations induced by GABA [17]. Rather, NO was shown to be the mediator of these GABA induced relaxations [15]. In addition to targeting both ATP and NO mediated relaxations, experiments with rat colon muscle strips showed that GABA induced relaxations were accompanied by release of VIP [77]. This neurogenic relaxation was inhibited by the VIP receptor antagonist VIP_{10-28}. Taken together, it appears that GABA has excitatory actions via $GABA_A$ sites that exist on cholinergic excitatory motor neurons, and that $GABA_A$ mediates stimulation of a variety of NANC relaxations.

In the guinea pig ileum and colon, GABA applied to the muscle strips has also been found to induce a neurogenic relaxation that was insensitive to bicuculline [66,67]. Similar results were achieved with the $GABA_B$ agonist baclofen but not with $GABA_A$ agonists. It was proposed that these GABA and baclofen relaxations were a result of prejunctional inhibition (modulation) of cholinergic motor neurons the via $GABA_B$ receptor. The modulatory actions of GABA on cholinergic neurons have been demonstrated in the guinea pig ileum and the rat small intestine, where electrically stimulated cholinergic contractions were reduced by GABA and baclofen and these actions were insensitive to bicuculline, and could not be mimicked by the $GABA_A$ receptor agonist 3-APS [107,139]. Similar results were also observed with

spontaneous contractions in the guinea pig colon [139].

This targeting of neurones by GABA in the ENS appears to be important in the reflex control of gut motility. In the rat colon, radial stretch of the caudad end of the colonic segment caused a cholinergic ascending contraction that was inhibited by bicuculline [77]. Similarly, the descending relaxation (mediated by VIP) induced by a radial stretch of the oral end, was blocked by bicuculline. In contrast, GABA application augmented both the ascending contraction and descending relaxation. In faecal expulsion and induced propulsion experiments using guinea pig and rabbit colon, the relative contribution of $GABA_A$ and $GABA_B$ receptors has been described [108,139,165]. $GABA_A$ receptor antagonism using picrotoxinin or bicuculline slowed the rate of propulsion. Likewise, baclofen inhibited propulsion velocity, while 3-APS was ineffective demonstrating the inhibitory modulation effect of GABA via $GABA_B$ receptors on peristalsis. Tachyphylaxis to the $GABA_B$ receptor agonist baclofen also resulted in the slowing of propulsion. The dual inactivation of both $GABA_A$ (using picrotoxinin) and $GABA_B$ (tachyphylaxis with baclofen) sites resulted in a rapid abolishment of peristalsis (stopped pellet expulsion). Investigators proposed that $GABA_B$-related tachyphylaxis leads to a disruption of this $GABA_B$ receptor modulation, leading to a loss of coordination of peristaltic activity, and the reduction in propulsion velocity. It was also suggested that $GABA_A$ receptor inactivation leading to a reduction in propulsion velocity was evidence that GABAergic interneurons (acting via $GABA_A$ sites) are involved in the reflex pathways of peristalsis. Hence, $GABA_A$ blockade also disrupts coordination of peristaltic activity causing a decrease in propulsion velocity.

Therefore, at least three distinct functional GABAergic innervations of the mammalian

gut occurs. These include $GABA_A$ related (A-GABAergic) innervation of cholinergic motor neurons; $GABA_A$ related (A-GABAergic) innervation of NANC motor neurones and $GABA_B$ related (B-GABAergic) prejunctional innervation of cholinergic motor neurones. (The nomenclature 'A-GABAergic' and 'B-GABAergic' has been adopted here from the work of Krantis *et al* [110]) This correlates with the now well characterized GABA nerve cell and fibre innervation of the mammalian gut wall. GABAergic nerve cell bodies with shapes and projections characteristic of Dogiel Type I and II enteric ganglion cells are localized in the myenteric and submucosal plexi[64,92,104,106,111] and GABAergic nerve fibres are present throughout all the enteric nerve fibre networks [64,111]. In the submucosa, not only are there GABAergic neurons in Henle's and Meissners plexus, but also in the human colon, within the Intermediate plexus[135]. Furthermore, in the human colon, GABA has been localized in the nerve fibres bundles in the longitudinal muscle layers[135], and GABAergic fibres innervate the muscularis mucosae and mucosa[102-104]. In the rodent and human, GABAergic nerve fibres occur in the paravascular nerve bundles, and mucosal endocrine cells also localize GABA [56,104]. Anatomical studies of the rat large intestine conducted in this laboratory showed that 50% of myenteric ganglion cells were immunopositive for the $GABA_A$ receptor while the proportion of NO synthase positive ganglion cells was approximately 14% [110]. A subpopulation (30-35%) of $GABA_A$ immunoreactive ganglion cells were also NO synthase positive. In comparison, $GABA_A$ receptors were found to be present on 90% of all NO synthase positive neurons. Furthermore, there is preliminary evidence for a similar innervation of the rat ileum (A. Krantis, personal communication).

In Chapters 2 & 3 of this thesis, the results showed that, in the rat gastroduodenum,

the relaxant motor activity can be differentiated on the basis of regional occurrence, and that within the duodenum, these occur within temporally distinct motor patterns which show dependency upon either NO or ATP. It was concluded that the nitrergic system is responsible for antral and 'Intergroup' relaxant activity while 'Grouped' relaxant activity is mediated by the purinergic system via P_{2x}-purinoceptors. Since these motor activities are independent, then their control must be via distinct neural pathways. Since A-GABAergic innervation of NANC inhibitory motor neurones in the large intestine is now well established, **I sought to test whether any of the NANC relaxant motor activities in the rat gastroduodenum involve an 'A-GABAergic' component.** We hypothesize that $GABA_A$ receptors are functionally involved in the pathway controlling spontaneous nitrergic gastric and 'Intergroup' relaxant activity.

METHODS

The experimental protocol was followed as described in Chapter 1. However, after 60 minutes of control recordings, the drug was injected subcutaneously in the back of the neck.

Chemicals

The drugs used were 3-Amino-1-propanesulphonic acid (3-APS) and bicuculline methiodide (Sigma Chemical Company, St. Louis, MO, USA).

RESULTS

Effects of 3-Amino-1-propanesulphonic acid (3-APS)

Animals were injected with the $GABA_A$ agonist 3-APS (100 $mg \cdot kg^{-1}$, s.c.) after 60 minutes of control recording. Typical recordings of antral (S_1) and duodenal (D_1) motor activity during pre and post 3-APS administration are presented in figures 31 and 32. Within 1 minutes of 3-APS administration, antral motor activity was altered, and within 30 minutes, spontaneous antral contractions showed a significant increase in amplitude (243% of control), but a significant decrease in frequency to 17% of control (Table 13). This profile of action was also evident for relaxant activity, with a significant increase in antral relaxation amplitude (235% of control), and a significant decrease in relaxation frequency (50% of control), Table 13. These effects of 3-APS on antral activity persisted for up to 70 minutes after drug administration.

In the proximal duodenum, of the two types of control motility patterns observed only the relaxant events in the 'Intergroup' period were affected by 3-APS. Both amplitude and frequency were enhanced (144% and 558% of control, respectively). Although a reduction in 'Intergroup' contraction activity was observed, insufficient 'Intergroup' contractions were monitored to allow proper analysis. The increased 'Intergroup' relaxant activity persisted for up 90 minutes post injection with the maximal effect observable within the first 30 minutes post injection. Prolonged exposure to 3-APS (> 90 minutes), systemically, resulted in a permanent disappearance of 'Intergroup' activity.

Effects of $GABA_A$ receptor antagonism

Experiments were carried out using the $GABA_A$ antagonist bicuculline (350 $\mu g \cdot kg^{-1}$). This was administered subcutaneously after 60 minutes of control recording. In the gastric antrum, bicuculline did not alter relaxation amplitude but significantly decreased relaxation frequency to 61% of control frequency (Table 14, Fig 33). In contrast to the results with 3-APS, bicuculline significantly increased both contraction amplitude (127% of control) and frequency (625% of control).

In the proximal duodenum, 'Grouped' activity was unaffected by bicuculline (Fig. 34). However, 'Intergroup' relaxations were inhibited by bicuculline, with a significant decrease in both relaxation amplitude (58% of control) and frequency (30% of control), Table 14. Unlike in the 3-APS experiments, a significant number of contractions were present in the 'Intergroup' period and, therefore, it was possible to assess the effect of bicuculline effect on duodenal contractions. Bicuculline abolished 'Intergroup' contractions, Table 14.

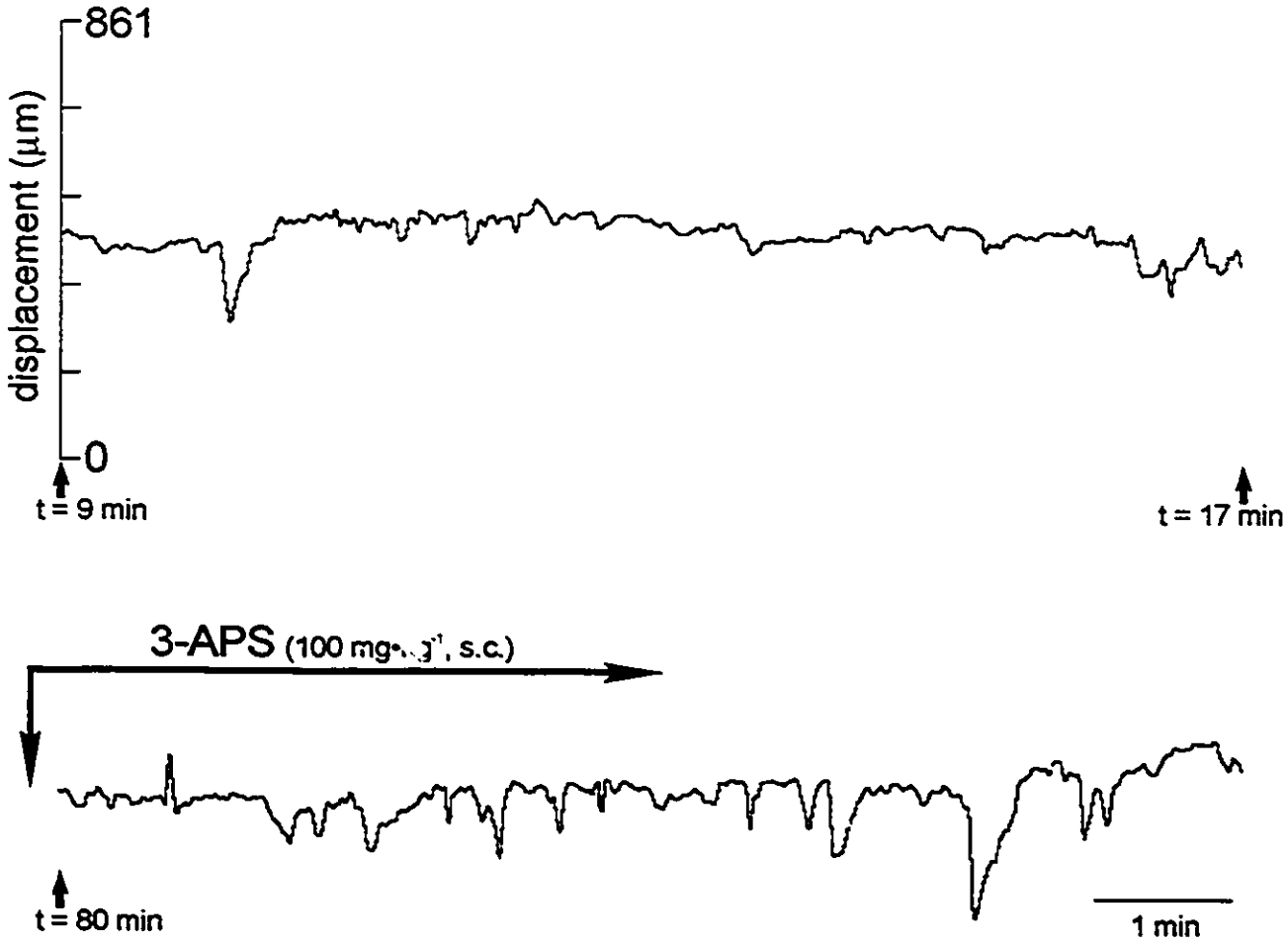


Figure 31 The effects of 3-APS at S_1. The two panels represent 8 min segments pre and post 3-APS (100 mg·kg^{-1}, s.c.) treatment taken from a continuous recording from t = 9 min to t = 88 min in the gastric antrum. 3-APS treatment caused an increase in spontaneous relaxation amplitude. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

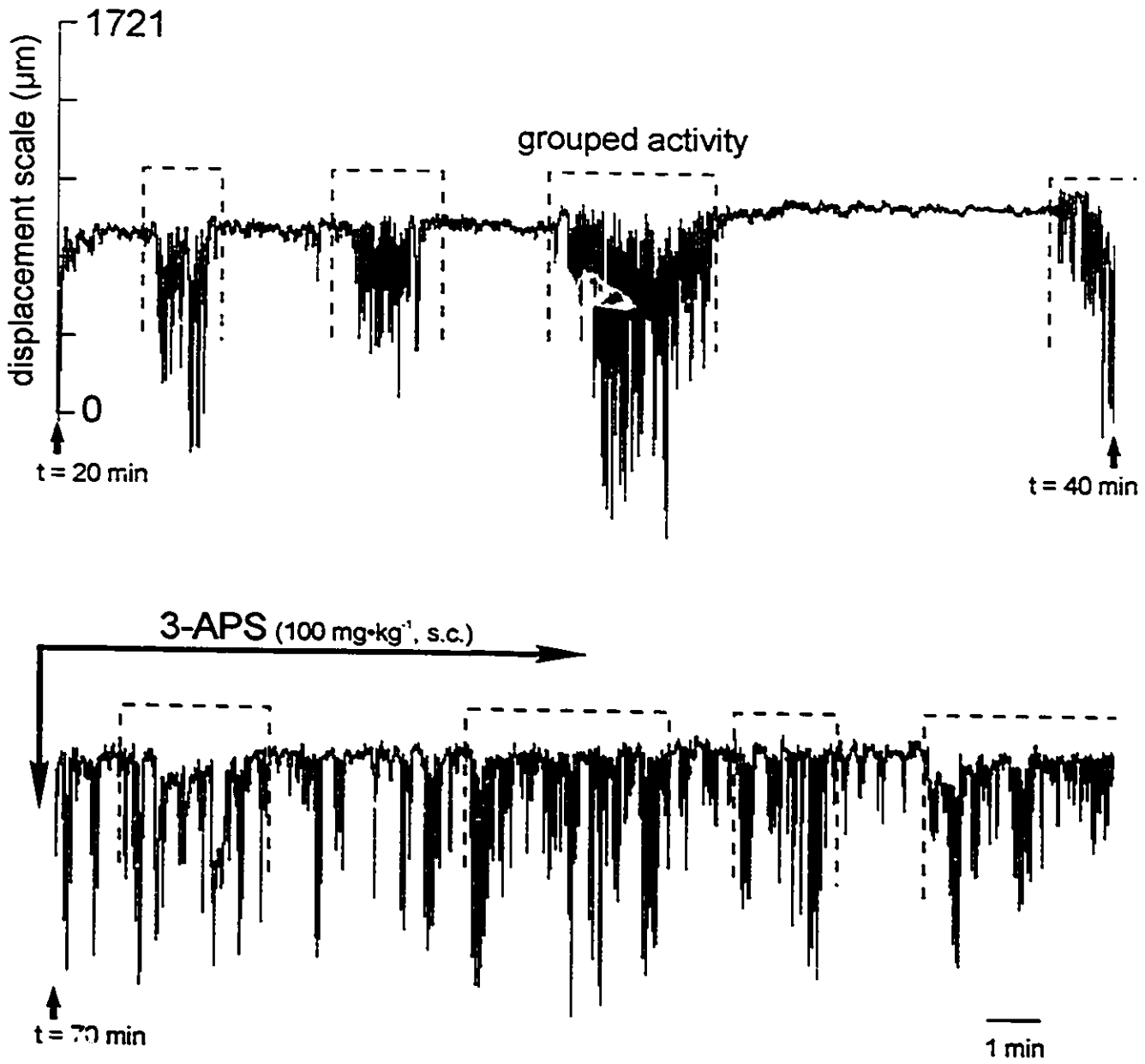


Figure 32 The effects of 3-APS at D_1. Shown is a typical tracing of motor activity pre and post 3-APS (100 mg·kg^{-1}, s.c.) treatment. 3-APS was injected at t = 60 min. Only 'Intergroup' relaxant activity was enhanced by 3-APS. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

TABLE 13

Effects of 3-APS on Amplitude and Frequency of Contractions and Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	3-APS	control	3-APS
S_1 contractions	37±7.4	90±13*	0.58±0.1	0.1±0.04*
S_1 relaxations	39±4.0	92±22*	1.28±0.2	0.65±0.1*
D_1 Grouped contractions	252±35	268±30.2	0.8±0.3	0.5±0.2
D_1 Grouped relaxations	278±13	291±21	9.8±0.9	10.7±0.7
D_1 Intergroup contractions	49±42	15±0	0.1±0	0.03±0.3
D_1 Intergroup relaxations	139±15	200±10*	1.2±0.4	6.7±0.6*

* denotes significance ($p < 0.05$) compared to control; n = 6

3-APS = 3-APS (100 mg·kg^{-1}, s.c.)

amplitude = displacement (μm); frequency = events·min^{-1}

All tabulated data is presented as mean ± SEM

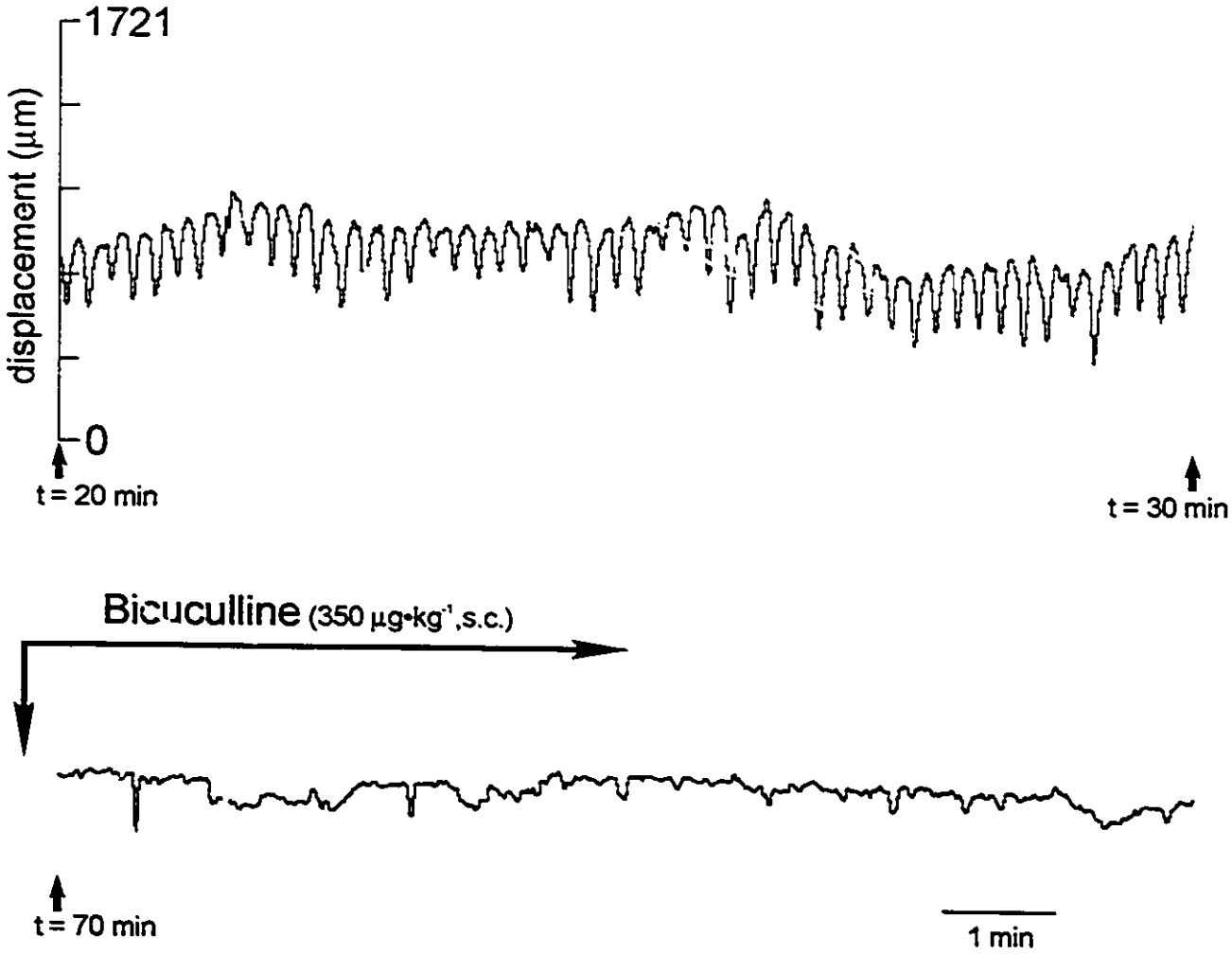


Figure 33 The effects of bicuculline at S_1. Typical antral control activity (10 min) is shown in the top panel. Post bicuculline motor activity is shown in the bottom panel. Bicuculline (350 $\mu g \cdot kg^{-1}$, s.c.) was administered at t = 60 min. Following bicuculline treatment, antral relaxations were reduced. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

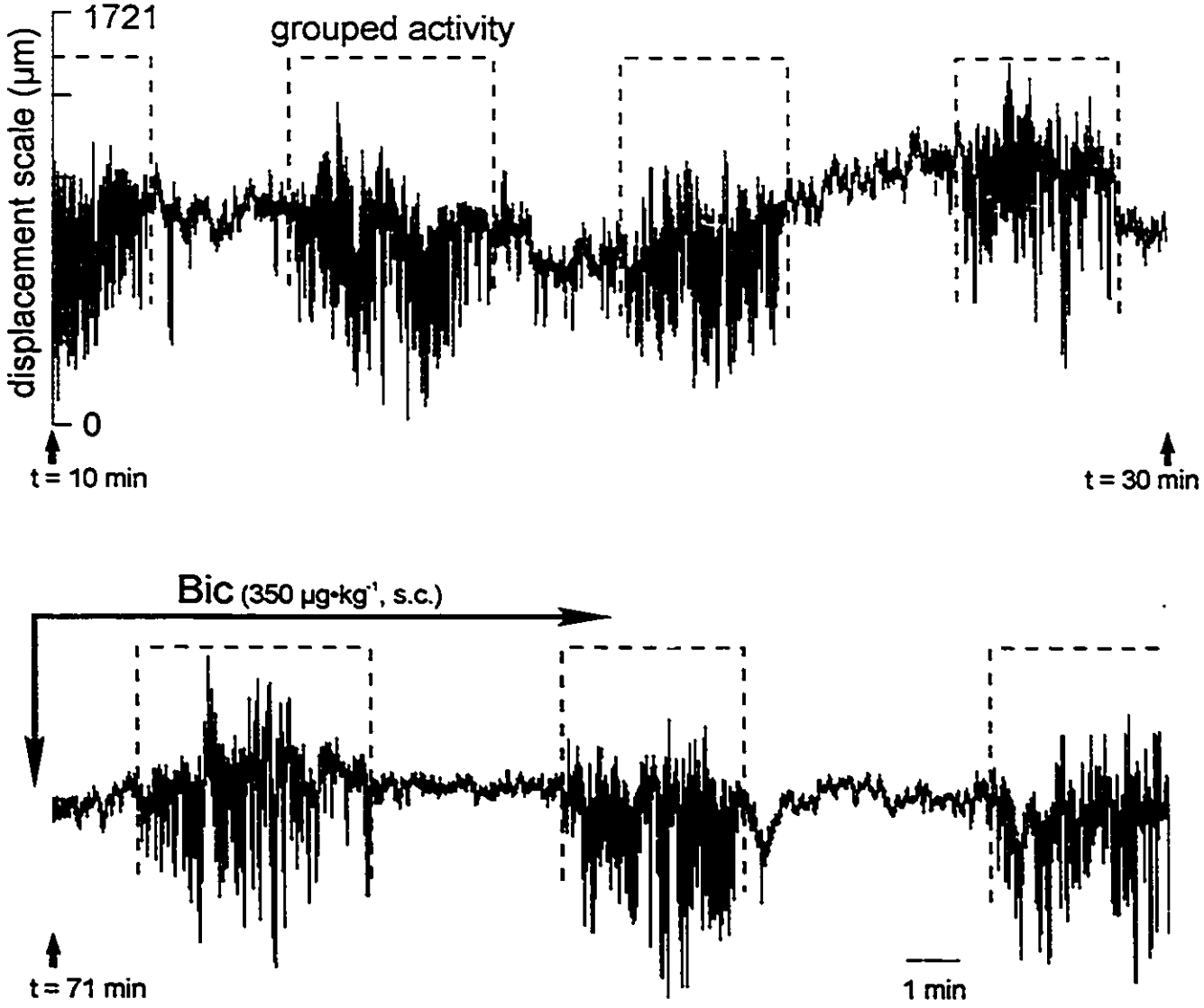


Figure 34 The effects of bicuculline at D_1. The two panels represent 20 min of motor activity taken from a continuous recording from t = 10 to t = 91 min. At t = 60 min. bicuculline (350 $\mu g \cdot kg^{-1}$, s.c.) was injected. Only 'Intergroup' activity was affected by bicuculline treatment. Both relaxations and contractions were reduced. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

TABLE 14

Effects of Bicuculline on Amplitude and Frequency of Contractions and Relaxations at S_1 and D_1

Region + event type	Amplitude		Frequency	
	control	BIC	control	BIC
S_1 contractions	22±3.0	28±1.5*	0.4±0.1	2.5±0.2*
S_1 relaxations	28±2.5	29±3.1	1.0±0.1	0.61±0.1*
D_1 Grouped contractions	125±7.5	107±6.3	4.3±0.7	4.1±0.6
D_1 Grouped relaxations	175±5.8	172±6.9	12.7±0.6	11.8±0.6
D_1 Intergroup contractions	85±11	0.1±0*	1.8±0.4	0±0*
D_1 Intergroup relaxations	91±7.2	53±6.8*	6.5±0.6	2.0±0.4*

* denotes significance ($p < 0.05$) compared to control; n = 4

Bic = Bicuculline (350 $\mu g \cdot kg^{-1}$)

amplitude = displacement (μm); frequency = $events \cdot min^{-1}$

All tabulated data is presented as mean ± SEM

DISCUSSION

As shown in the studies presented in this thesis, spontaneous relaxant activity in the gastroduodenum of the anaesthetized rat occurs within distinct patterns which can be distinguished by their dependence upon either NO or ATP. All relaxant activity in the gastric antrum and in the duodenum within 'Intergroup' periods is dependent upon NO. Further pharmacologic examination of these motor activities, as presented in this chapter, demonstrate that all antral and 'Intergroup' relaxant activity is reduced by treatment with the $GABA_A$ receptor antagonist bicuculline. Furthermore, application of the specific $GABA_A$ receptor agonist 3-APS evoked relaxations in the antrum and in the duodenum (only during the 'Intergroup' period). These results provide clear functional evidence that the 'A-GABAergic' system targets the NO mediated relaxation responses occurring spontaneously in the rat gastroduodenum.

In this laboratory, Krantis *et al*[110] demonstrated the localization of $GABA_A$ receptors on NO synthase positive neurons in the myenteric plexus of the rat colon and in the ileum (personal communication, Krantis *et al*). A subpopulation (30-35%) of myenteric neurons displaying $GABA_A$ receptor immunoreactivity was shown to be NO synthase positive. However, almost 90% of NO synthase positive myenteric neurones displayed $GABA_A$ receptor immunoreactivity. Of the nitrergic cells colocalizing $GABA_A$ immunoreactivity, some were morphologically identified as being Dogiel Type I, which are proposed to be motor neurons [62]. This localization study provided an anatomical basis for the functional findings presented herein. Additional functional evidence that GABA evokes NO mediated responses in the mammalian gut has been provided by Boeckxstaens *et al*[15]. Using *in vitro* preparations

of the canine ileum, they found that GABA induced relaxations are TTX sensitive and inhibited by the NOS inhibitors, L-NMMA and L-NNA. The localization of $GABA_A$ receptors on nitrergic neurons in the rat intestine suggests that GABA evoked NO mediated relaxations are most likely due to direct A-GABAergic innervation of the nitrergic-NANC inhibitory neurons. Irrespective of where the GABAergic neurones occur within this pathway, it is clear that the $GABA_A$ sites represent a primary active component.

In addition to the NO mediated relaxations of the antrum and duodenum, NO can modulate the ATP dependent 'Grouped' relaxations of the duodenum. Enteric NO neurons and nerve fibres represent an extensive and rich innervation of the rat and guinea pig intestine [132,133]. The existence of NO positive neurons in the myenteric plexus with their fibres innervating the gut muscularis supports the role of NO as a NANC inhibitory transmitter. However, the histochemical evidence also supports the notion that NO interneurons are present in both rat and guinea pig intestine since NO positive varicose fibres form a rich neuropil surrounding myenteric ganglionic neurons [2,132,133]. Indeed, functional evidence supporting the existence of nitrergic interneurons has already been shown by Young *et al* [175]. The NO donor, sodium nitroprusside was used to stimulate an increase in intracellular levels of cGMP in gut tissue. Induced cGMP immunoreactivity (compared to control levels) was not only found in smooth muscle cells, but in a subpopulation of myenteric neurons. From the present work, it would appear that NO interneurons are involved in modulating purinergic related 'Grouped' relaxations and these neurones are not targeted by the A-GABAergics. This differential sensitivity of NO related responses to bicuculline treatment indicates that at least two distinct populations of NO neurons may exist in the duodenum: NO NANC neurons with

$GABA_A$ receptors, and NO interneurons without $GABA_A$ receptors. Neurochemical confirmation of the nature of the NO neurones in the gastroduodenum is the focus of ongoing studies in this laboratory by other researchers

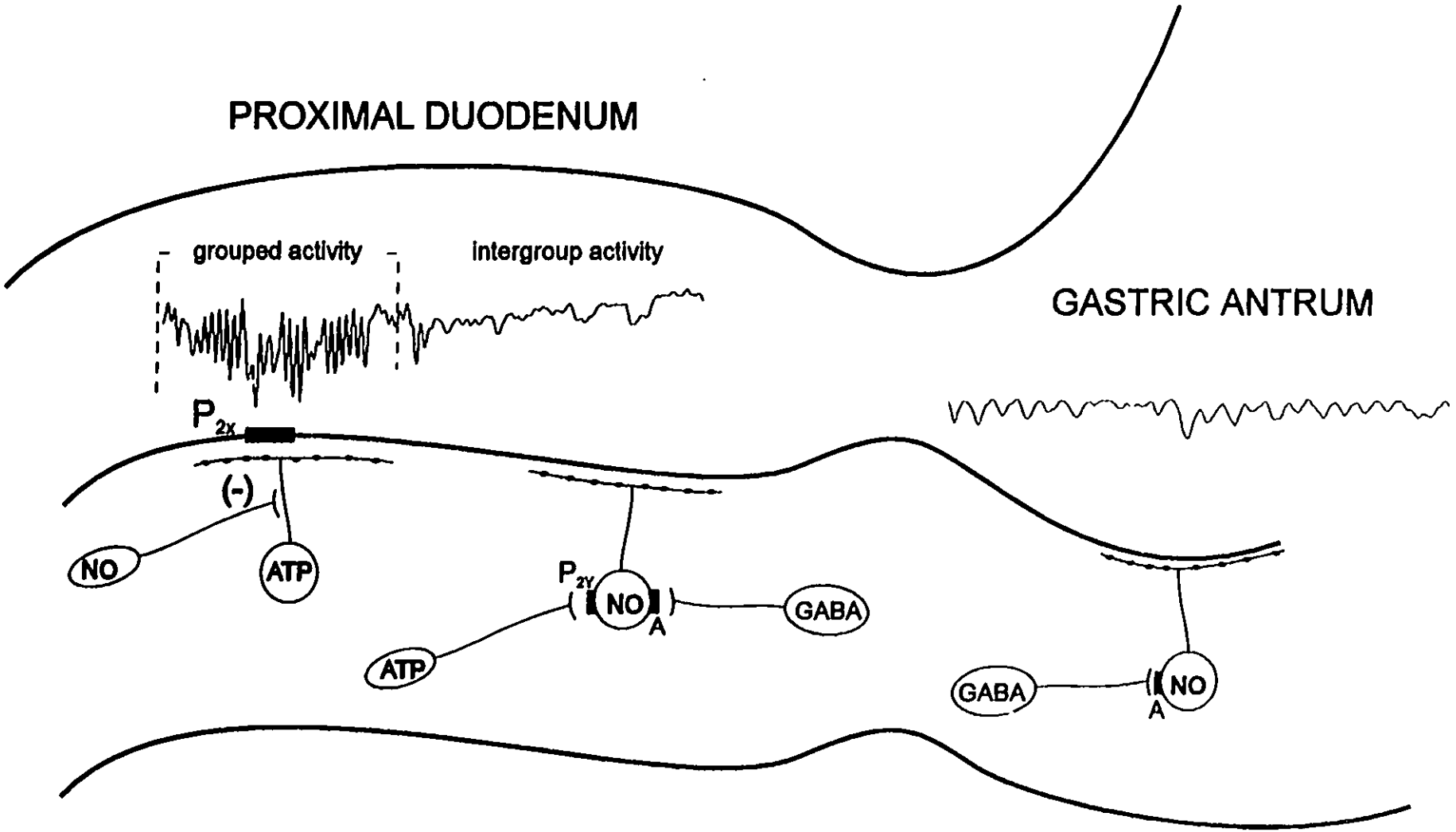
In Chapter 3, it was shown that P_{2y}-purinoceptors are involved in a pathway targeting nitrergic inhibitory motor neurons in the proximal duodenum. However, it was unknown if P_{2y} receptors existed directly on nitrergic inhibitory motor neurons or on a population of interneurons which target nitrergic neurons. The results of the experiments presented in this chapter raise the possibility that P_{2y}-purinoceptors and $GABA_A$ receptors are involved in the same pathway targeting NO motor neurons. Since there is strong anatomical indications that there is direct A-GABAergic innervation of nitrergic neurons it is highly likely that P_{2y} receptors may in fact be targeting this A-GABAergic component. Further studies are needed to determine the nature of purinergic (via P_{2y}-purinoceptors) and A-GABAergic interaction in the control of duodenal relaxant activity.

Studies show that NO colocalizes with other neurotransmitters in the myenteric plexus of various species. In the human colon, studies in this laboratory have shown that NOS positive neurons colocalize with Neuropeptide Y (NPY) [134]. In another study of the human colon, Nichols *et al* presented evidence that a subpopulation of GABAergic myenteric neurons is NOS positive [135]. In the guinea-pig small intestine and rat gut, VIP coexists with NO synthase [2,37]. Thus the nitrergic components identified in the present study may well represent one or the other of these NO neuronal subpopulations. More importantly, it may be possible to distinguish nitrergic interneurons and motor neurons. One could speculate that the NO releasing NANC motor neuron would not colocalize GABA.

To my knowledge, histo-anatomical studies investigating whether purinergic neurons display $GABA_A$ sites have not been carried out. However, I would expect that such studies would show no GABAergic innervation of purinergic neurons, since, as demonstrated herein, ATP related relaxation of the proximal duodenum ('Grouped' activity) is unaffected by A-GABAergic agonist and antagonist drugs. This is not the case *in vitro*, in the rat duodenum, since GABA induced relaxations could be blocked by ATP desensitization [117]. Furthermore, relaxations induced by ATP, GABA and electrical stimulation were all antagonized by apamin[36] which has been shown to block ATP mediated relaxations. Studies using the quinacrine-staining method [9,110] for identifying ATP neurons in combination with $GABA_A$ receptor immunolocalization might provide answers to this discrepancy between the *in vivo* and *in vitro* studies.

In conclusion, the results in this chapter demonstrate that $GABA_A$ receptors occur within pathway(s) controlling spontaneous NO- mediated relaxations of both the gastric antrum and the proximal duodenum. Furthermore, 'Grouped' relaxations, which are ATP mediated, are unaffected by the A-GABA'ergic system. A schematic summary of the inhibitory innervation of the gastroduodenum and the A-GABAergic involvement in this innervation is presented in Figure 35.

Figure 35 The inhibitory innervation of the rat gastroduodenum. The A-GABAergic innervation appears to target only the nitrergic system mediating relaxations of the gastroduodenum. It is possible that P_{2y}-purinoceptors and $GABA_A$ receptors are involved in the same pathway targeting NO inhibitory motor neurons.


PROXIMAL DUODENUM
grouped activity
intergroup activity
GASTRIC ANTRUM
P2X
(-)
NO
ATP
P2Y
NO
A
GABA
ATP
GABA
NO
A

CHAPTER 5

The Role of VIP in Rat Gastroduodenal Motor Activity

Although the studies in this thesis indicate that the mediators responsible for all spontaneous relaxant activity in the rat gastroduodenum *in vivo* have been accounted for, still unknown is the of role VIP in the inhibitory innervation of the rat gastroduodenum (see Fig. 35). As discussed earlier, there is adequate evidence to support the notion that VIP is a NANC inhibitory neurotransmitter in different regions of the gut of a variety of species (see Introduction). Furthermore, it has been proposed that there may be an interplay between the peptidergic and nitrergic systems in the mediation of relaxations. This interplay has been demonstrated in the rat colon by Grider[70] where the NANC relaxation has been characterized to have a dual component, consisting of NO and VIP releasing neurons. In this proposed model of VIP-NO interplay for the control of the descending relaxation phase of peristalsis in the rat colon (see Fig. 7), not only is there neurogenic release of VIP and NO, but VIP is also proposed to induce relaxations by activating NOS localized in the smooth muscle. This raises the possibility that VIP may be operating within the nitrergic pathways of the antrum and proximal duodenum, not as the primary motor transmitter, but as some neuromodulator or else as a transmitter of interneurons within the pathways controlling the nitrergic response. On this basis, if VIP were to have a role in the relaxant activity in the rat gastroduodenum *in vivo*, it is possible this role might involve NO. In Chapter 4 it was shown that a subpopulation of nitrergic NANC inhibitory neurons are targeted by GABA via $GABA_A$ receptors. Therefore, it is of interest to determine if VIP and GABA have any relationship

with regards to nitrergic relaxant activity. **Toward answering this question, the experiments described in this chapter sought to i) establish the nature of VIP actions in the rat gastroduodenum and ii) investigate whether VIP targets NO related responses and/or a $GABA_A$ergic-NO related pathway.**

METHODS

In Vivo experiments

Sprague Dawley rats were prepared for in vivo experiments as presented in Chapter 1. To test VIP involvement with the nitrergic and A-GABAergic systems two antral foil strain gauges were used: S_1 and S_2. The S_1 foil strain gauge was glued in the axis of the longitudinal muscle layer while the S_2 foil strain gauge was attached juxtaposed to the S_1 foil strain gauge but oriented to the circular muscle layer (as presented in Chapter 1).

Intra-arterial Cannulation

In experiments that required close intra-arterial administration of VIP, the animals were surgically prepared and connected to the IBM data acquisition system as described in Chapter 1. However, the jugular vein was not cannulated. Instead, the femoral artery was exposed (after a 2-3 cm incision of the skin in the area of the right hind limb) and a cannulae (PE-10 polyethylene tubing, Intramedic) was introduced and fed retrogradely into the lower part of the thoracic aorta with the tip positioned close to the opening of the superior mesenteric artery. From measurements obtained in animals at necropsy, it was deemed that a approximately 6 cm of the cannulae had to be inserted to approximate a tip position close

the opening of the superior mesenteric artery. To further ensure proper tip position, a small injection of VIP (< 3 $\mu g \cdot kg^{-1}$) was given via the cannulae to validate that the injection not only caused a hemodynamic effect, but also induced a motor response. With the patency of the close intra arterial cannulation, the hind limb incision was closed with wound clips, and the animals placed in a prone position and allowed to stabilize for 60 minutes. At the conclusion of the experiment and after euthanization of the animal, the position of the intra-arterial cannulae was verified. Animals that did not respond to this VIP injection, or did not have a correctly positioned cannulae, were not included for analysis.

Ex Vivo experiments

Animals were surgically prepared for *ex vivo* experiments as described in Chapter 1.

Chemicals

The drugs used were: vasoactive intestinal polypeptide (VIP; Bachem, Babendorf, Switzerland), Bicuculline and N^G-nitro L-arginine methyl ester (L-NAME; Sigma Chemicals, St.Louis, MO, USA).

RESULTS

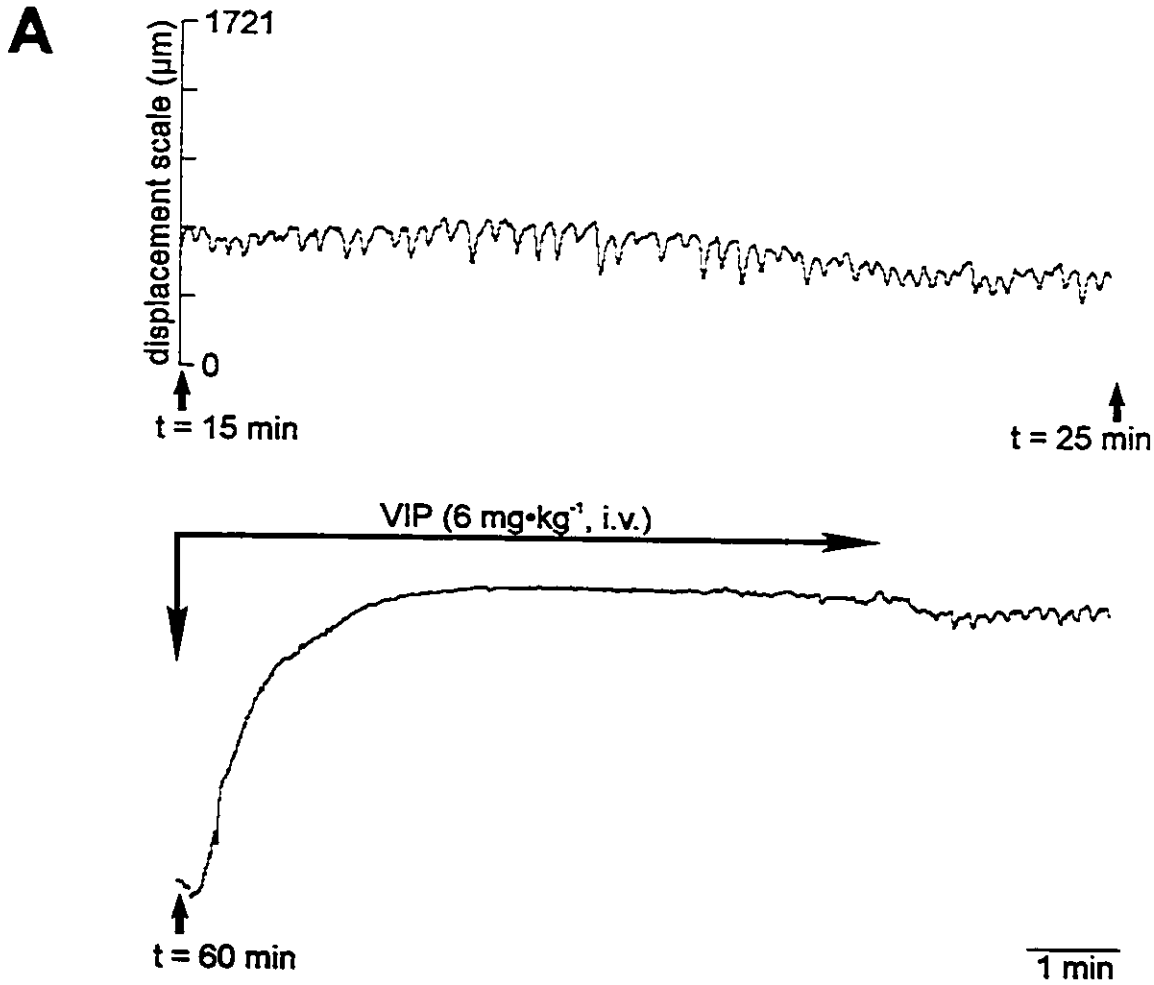
Effects of Intravenously Administered VIP

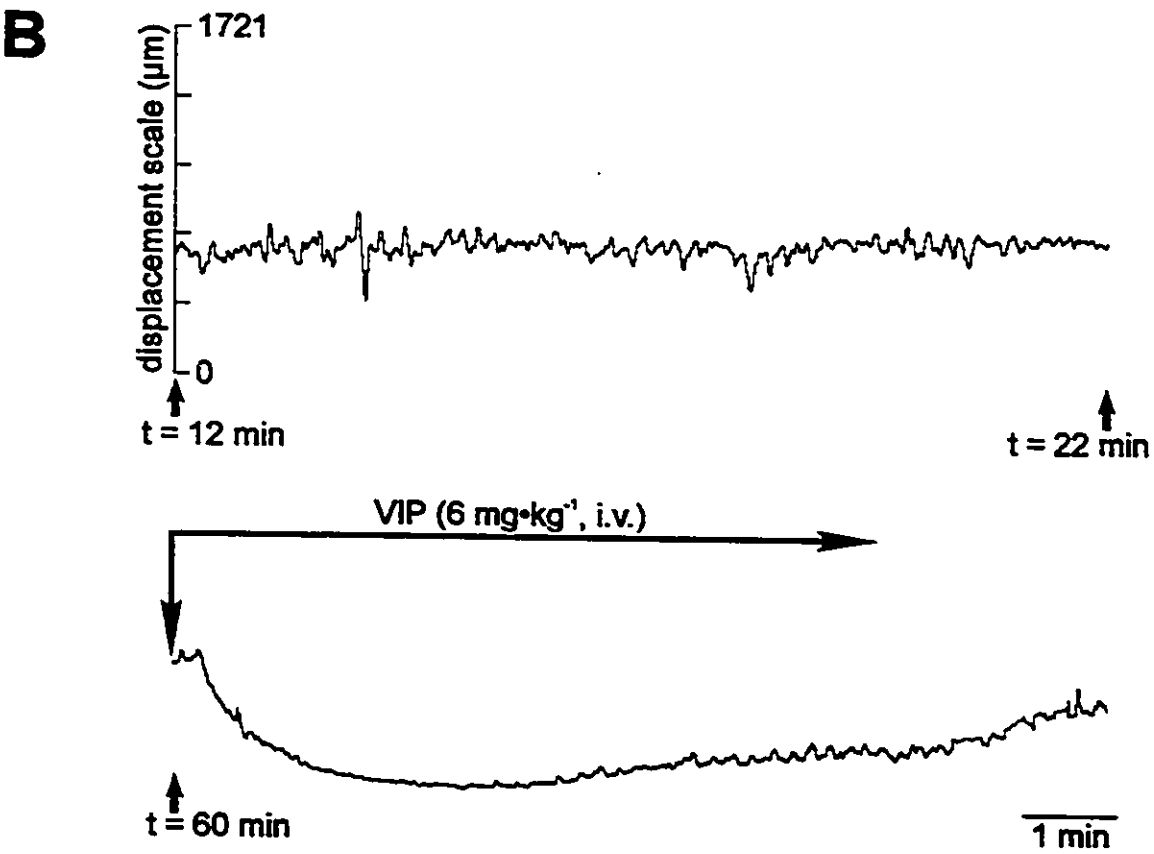
Initial experiments with VIP treatment utilised only one antral recording site (S_1). VIP (6 $\mu g \cdot kg^{-1}$, i.v.) administered after 60 minutes of control recording caused either a single contraction or a single relaxation of the gastric antrum (Fig. 36). The VIP induced response was apparent within 1 minute post injection and was maximal 1 minute after the initiation of the response. This response lasted for up to 60 minutes before a return to control levels. In contrast, in the proximal duodenum VIP always caused an immediate transient relaxation that lasted no longer than 2 min (Fig. 37).

VIP evoked contractions of the gastric antrum were unexpected since VIP in previous studies has not been shown to induce contractions. Therefore, these experiments were repeated but with two antral foil strain gauges, S_1 (oriented to the longitudinal muscle) and (S_2 oriented to the circular muscle). In this way it was possible to test if these VIP effects were specific for the longitudinal muscle layer, and whether the VIP related relaxations involve the A-GABAergic and/or the nitrergic systems.

After 60 minutes of control recording animals were injected with VIP (6 $\mu g \cdot kg^{-1}$, i.v.) to establish a control response at the recording sites. Following 60 minutes of VIP treatment, the NOS inhibitor L-NAME (10 $mg \cdot kg^{-1}$, i.v.) was then administered and, 30 minutes later, VIP was retested. In this way a minimum of 90 minutes elapsed between the two VIP administrations, thereby avoiding any contamination due to a lingering response from the first administration of VIP. As observed in the preliminary experiments on VIP treatment alone, the pre-L-NAME VIP injection induced either single contraction or single relaxation

Figure 36 The effects of VIP at S_1. Shown in **A** and **B** are typical recordings pre and post VIP administration (6 $\mu g \cdot kg^{-1}$, i.v., at t = 60 min) presented in two panels. In the gastric antrum VIP treatment was able to evoke either a single contraction (**A**) or a single relaxation (**B**). These responses lasted up to 60 minutes before a return to control levels. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.


A
1721
displacement scale (µm)
0
t = 15 min
t = 25 min
VIP (6 mg•kg⁻¹, i.v.)
t = 60 min
1 min


B
1721
displacement scale (µm)
0
t = 12 min
t = 22 min
VIP (6 mg•kg⁻¹, i.v.)
t = 60 min
1 min

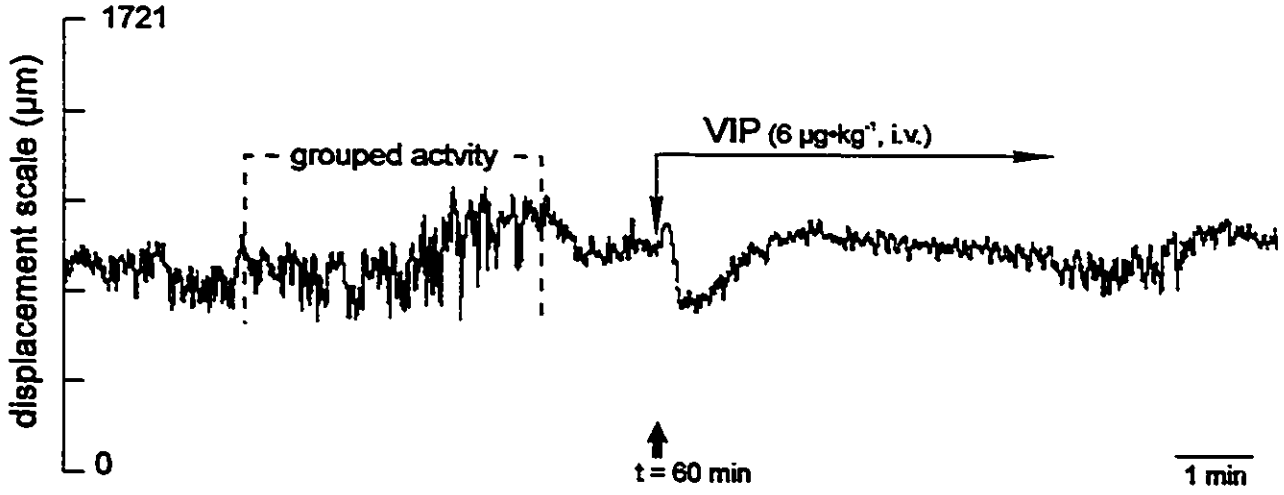


Figure 37 The effects of VIP at D_1. VIP (6 µg·kg^{-1}, i.v.) was injected as a single bolus at t= 60 min. In the proximal duodenum, an immediate relaxation was always evoked. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

at both antral recording sites. Interestingly, VIP induced contractions were not significantly affected by pretreatment with L-NAME. However, all VIP induced antral relaxations were reduced by L-NAME treatment (at S_1: to 55% of the control response and at S_2 : to 49% of the control response), Table 15.

Effects of Bicuculline on VIP Actions

The preceding experimental protocol was repeated using the $GABA_A$ antagonist, bicuculline (350 $\mu g \cdot kg^{-1}$, i.v.). VIP administered alone induced responses comparable to those observed previously. However, in contrast to the preceding experiment, only VIP induced relaxations at S_1 were affected by bicuculline administration, Table 16.

The Effects of Intra-arterial Injection of VIP

Experiments with two antral foil strain gauges (described above) demonstrated that the contraction responses to applied VIP occurred in both the longitudinal and circular muscle layers. To further confirm these observations, VIP (6 $\mu g \cdot kg^{-1}$) was injected intra arterially (after the method of Bojo *et al*[18]) via a cannulae in the thoracic artery positioned to allow VIP to be administered close to the opening of the superior mesenteric artery and therefore, directly into the blood supply of the antrum. The contraction inducing actions of VIP (i.a.) were comparable to those observed with intravenous administration of VIP (Fig. 38).

The Effects of VIP and 'Coupled' Activity

In Chapter 1 it was reported that during control recordings 'coupled' events were

TABLE 15

Effects of VIP, pre/post L-NAME, on Contraction and Relaxation Amplitude at S_1

antral strain gauge + response	VIP (control)	VIP (post L-NAME)
S_1 relaxation	168±20	92±11*
S_2 relaxation	93±16	46±6*
S_1 contraction	485±34	346±109
S_2 contraction	80±17	58±28

* denotes significance ($p < 0.05$) compared to control; n = 4

VIP = VIP (6 $\mu g.kg^{-1}$, i.v.)

amplitude = displacement (μm)

All tabulated data is presented as mean ± SEM (μm)

TABLE 16

Effects of VIP, pre/post Bicuculline, on Contraction and Relaxation Amplitude at S_1

antral strain gauge + response	VIP (control)	VIP (post BIC)
S_1 relaxation	585±37	150±29*
S_2 relaxation	162±13	184±15
S_1 contraction	300±110	219±40
S_2 contraction	238±107	153±69

* denotes significance ($p < 0.05$) compared to control; n = 4

VIP = VIP (6 $\mu g.kg^{-1}$, i.v.)

amplitude = displacement (μm)

All tabulated data is presented as mean ± SEM (μm)

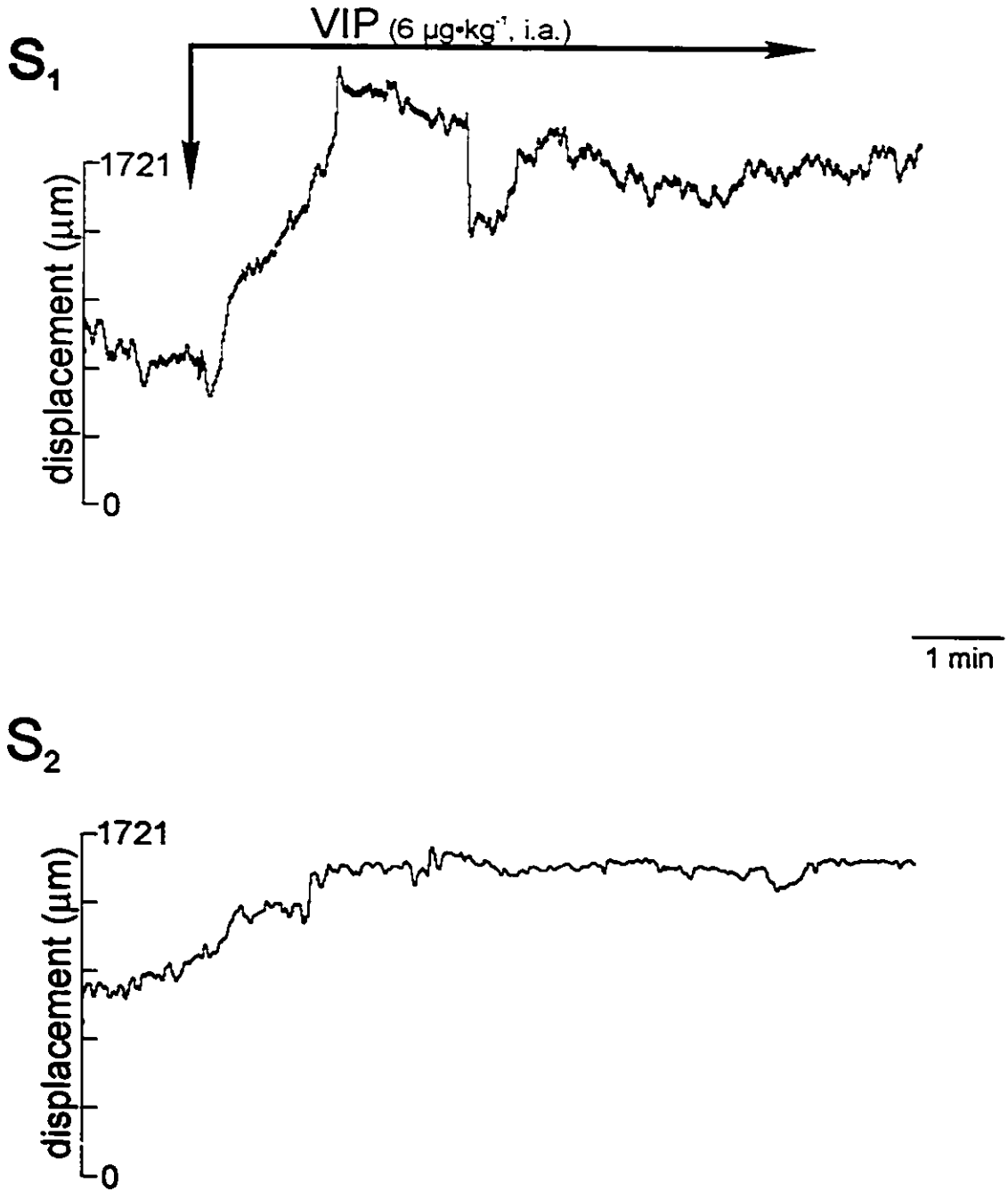


Figure 38 The effects of VIP (i.a.) at S_1 and S_2. VIP (6 µg·kg^{-1}) was injected at t = 60 min. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

occasionally seen to occur. These 'coupled' responses consisted of a random large amplitude event occurring in the gastric antrum that was temporally coupled with a simultaneous large amplitude response in the proximal duodenum. Intravenous injection of VIP (6 $\mu g \cdot kg^{-1}$) evoked either an antral contraction or relaxation and a transient duodenal relaxation. In addition to these responses, always within approximately 10 minutes after VIP injection, a 'coupled' response was observed (Fig 39). However, unlike 'coupled' activity observed during control motor activity, the amplitude of VIP induced 'coupled' activity was smaller.

Ex vivo experiments were carried out to further examine whether VIP could in fact induce 'coupled' activity. VIP (6 $\mu g \cdot kg^{-1}$, 100 μl) directly applied to the serosa near the antral recording site resulted in only a transient antral relaxation (Fig. 40). Coupled activity never occurred. However, VIP directly applied to the serosa near the duodenal foil strain gauge site induced not only a response in the duodenum but a response in the gastric antrum that was temporally coupled to the duodenal response (Fig. 41). These responses displayed identical onset and offset with an average duration of approximately 3.75 minutes. However, the profiles of the evoked responses were always opposite: a contraction (amplitude 481±42 μm) occurred in the gastric antrum while in the duodenum there was a relaxation (amplitude: 903±12 μm).

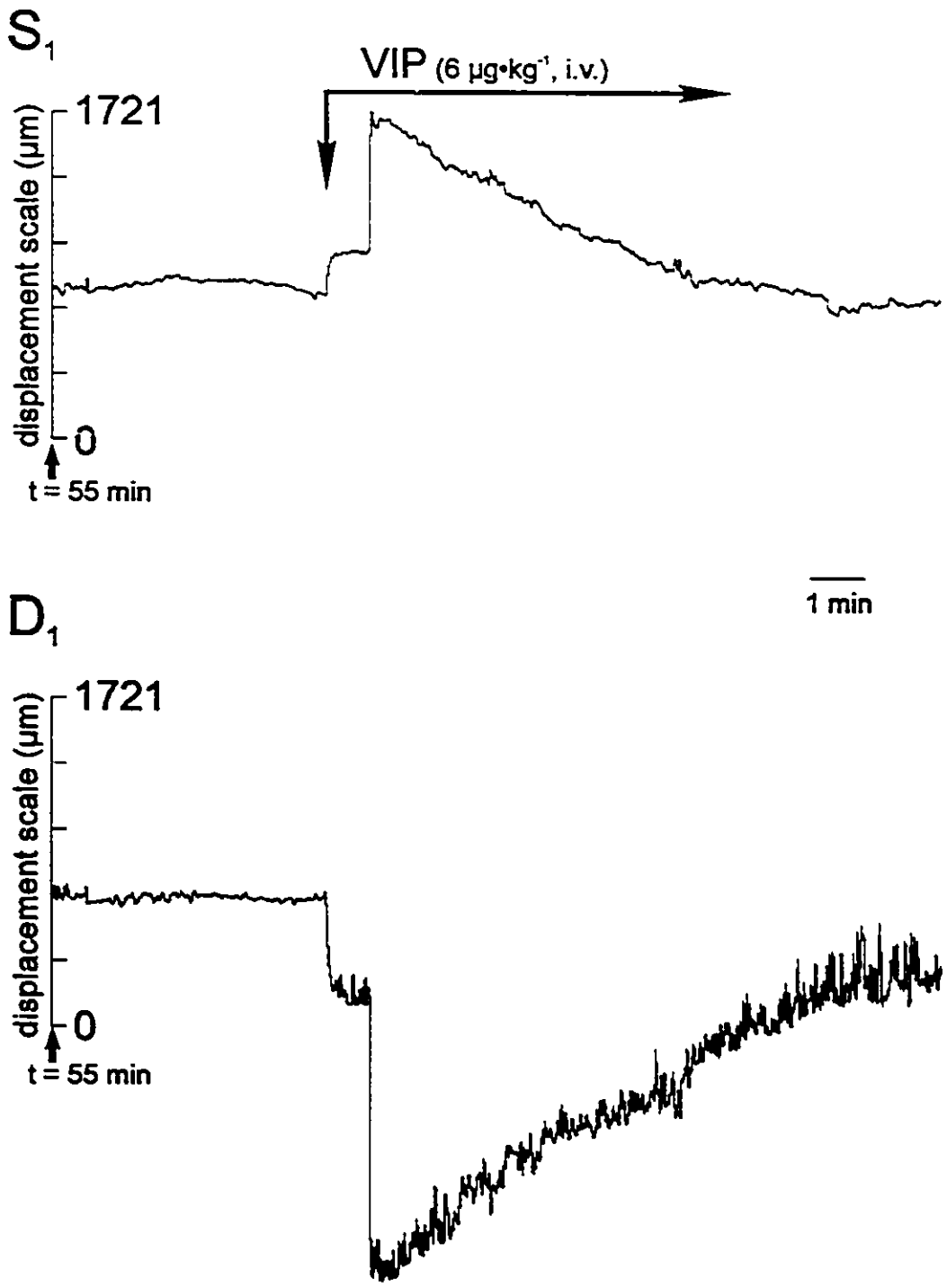


Figure 39 The effects of VIP at S_1 and D_1 showing coupled responses. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

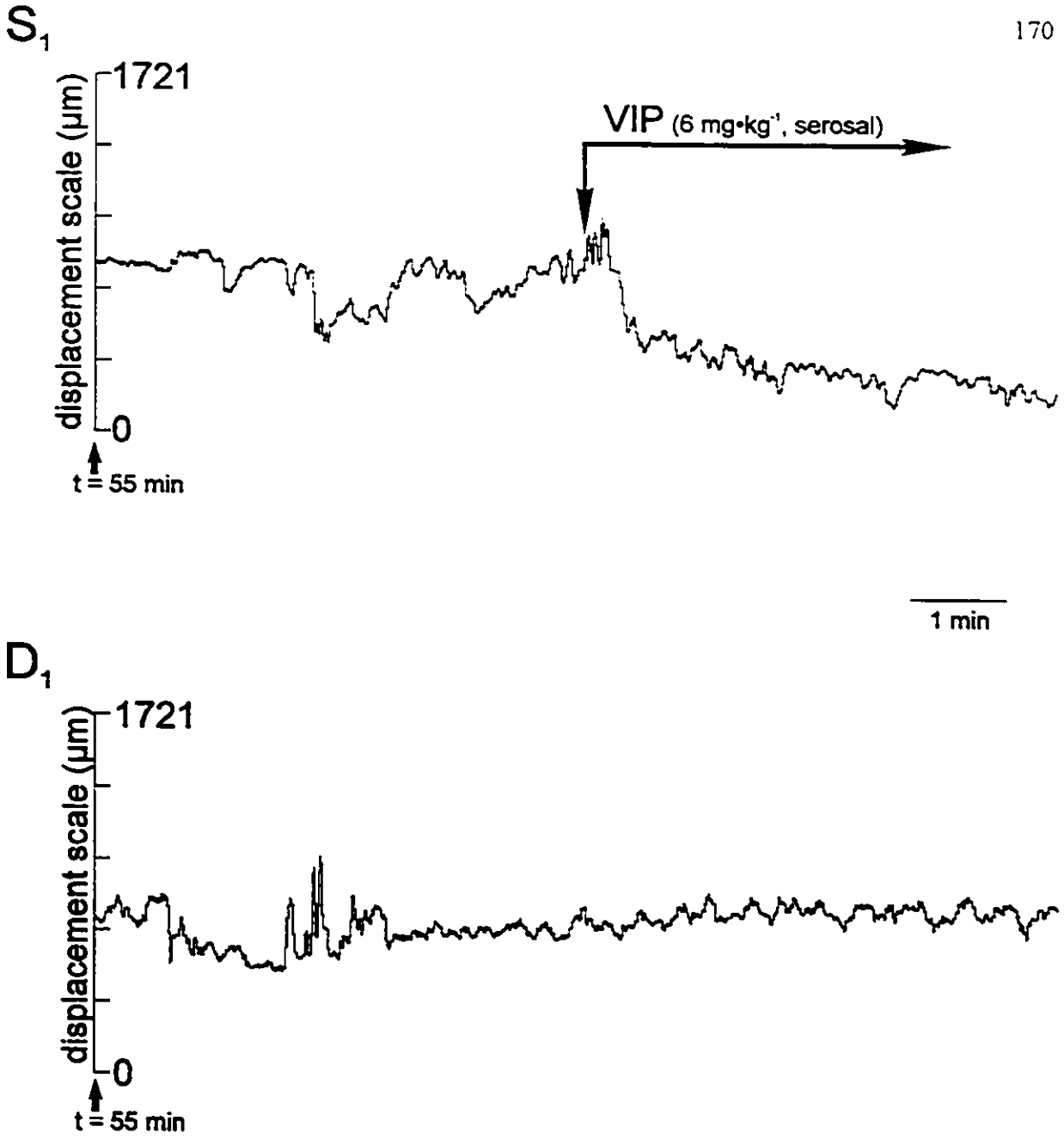


Figure 40 The effects of VIP at S_1 and D_1, *ex vivo*. The two panels present 10 minute segments demonstrating the responses of the gastric antrum (panel 1) and the proximal duodenum (panel 2) to the direct application of VIP (6 $\mu g \cdot kg^{-1}$, 100 μl) to the serosa of the gastric antrum around the S_1 foil strain gauge. VIP evoked only a transient antral relaxation. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

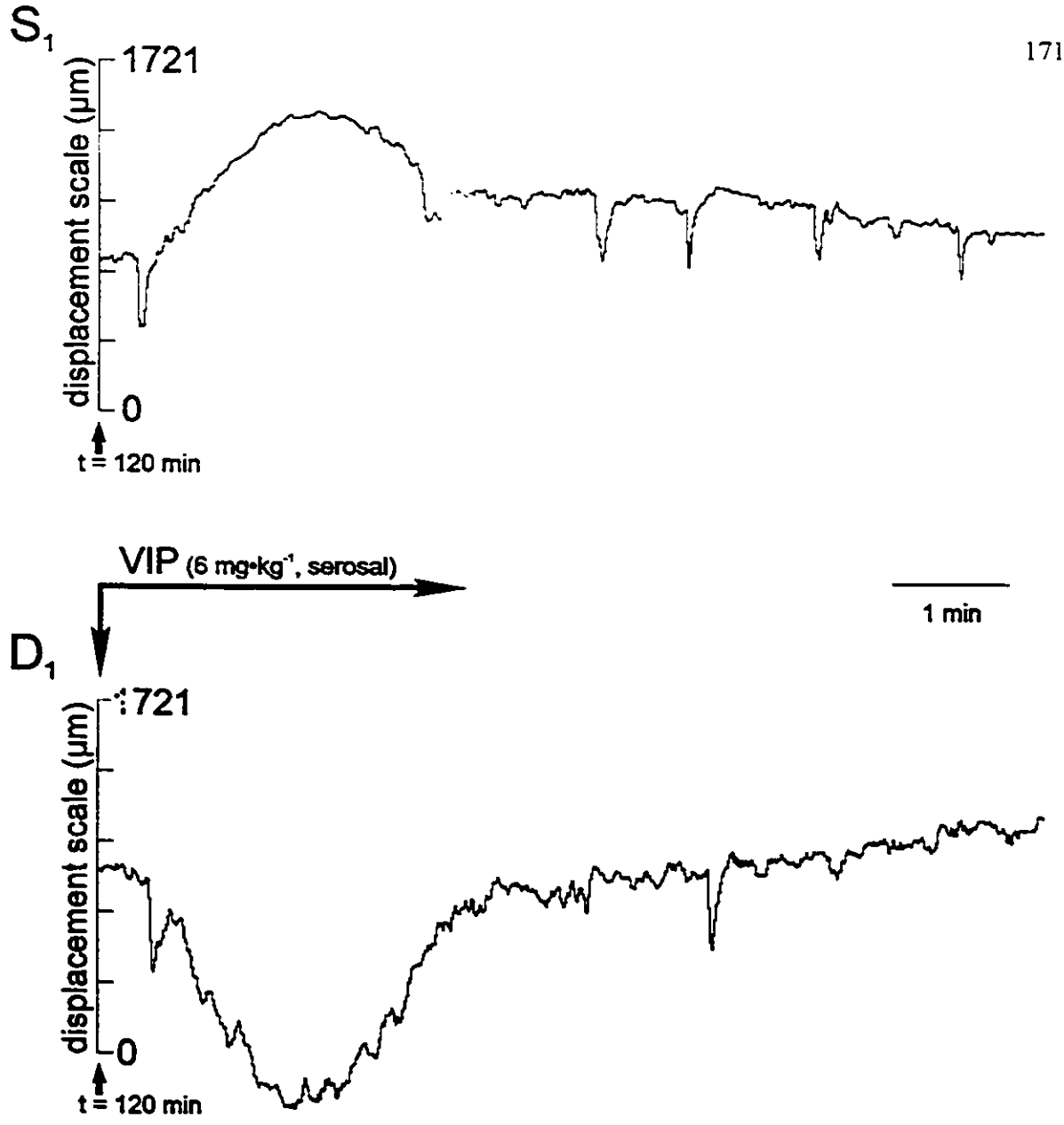


Figure 41 The effects of VIP at S_1 and D_1, *ex vivo*. The two panels present 10 minute segments demonstrating the responses of the gastric antrum (panel 1) and the proximal duodenum (panel 2) to the direct application of VIP (6 $\mu g \cdot kg^{-1}$, 100 μl) to the serosa of the proximal duodenum around the D_1 foil strain gauge. VIP induced a relaxation response in duodenum as well as an antral contraction that was temporally coupled to the duodenal response. The displacement scale represented can be used to compare the alteration in tone and amplitude of the responses. The time bar represents 1 minute.

DISCUSSION

The results of this study show that VIP applied either systemically, close intra-arterially or directly onto the serosa of the rat gastroduodenum, can evoke relaxations of the antrum and duodenum, of these, only the antral relaxant responses were blocked by pretreatment with the NOS inhibitor, L-NAME. It would appear therefore, that all VIP induced antral relaxant actions in the rat gastroduodenum are mediated by NO. In the rabbit stomach, it has been shown that VIP receptors exist on smooth muscle via which VIP can evoke relaxations directly [131]. Moreover, VIP appeared to be targeting NO myogenic components. This phenomenon also occurs in the rat large intestine [70]. In the present study, we could not determine whether NO in smooth muscle cells was involved in VIP actions. However, VIP actions were characterized based on the sensitivity of VIP evoked relaxations to the $GABA_A$ receptor antagonist bicuculline. Only relaxations to VIP recorded at the longitudinal muscle foil strain gauge (S_1) were blocked by bicuculline. Therefore, in the rat gastric antrum, VIP must be inducing relaxation of the circular and longitudinal muscle layers via different NO related pathways. The nitrergic neurons innervating the longitudinal muscle layer are stimulated via a GABAergic mechanism and this pathway is targeted by VIP interneurons. In addition, VIP is targeting a separate NO innervation of the antral circular muscle that does not have a GABAergic component. Alternatively, NO might be synthesized by circular smooth muscle cells which are stimulated directly by VIP. Figure 42 summarizes the possible VIP interactions with NO releasing cells, and the A-GABAergic- NO pathway.

In the proximal duodenum, VIP evoked a transient relaxation that was insensitive to both L-NAME and bicuculline. Therefore, in the duodenum, VIP actions do not involve the nitrergic

system. Since all spontaneous activity has been accounted for in the rat proximal duodenum it is unlikely that peptidergic (ie. VIP) NANC inhibitory motor neurons are involved in this activity. However, it remains to be determined whether VIP targets the purinergic system. Future experiments could resolve this issue by testing whether desensitizing P_{2x}-purinoceptors with α,β Me-ATP or else an antagonist of these sites, can affect VIP actions.

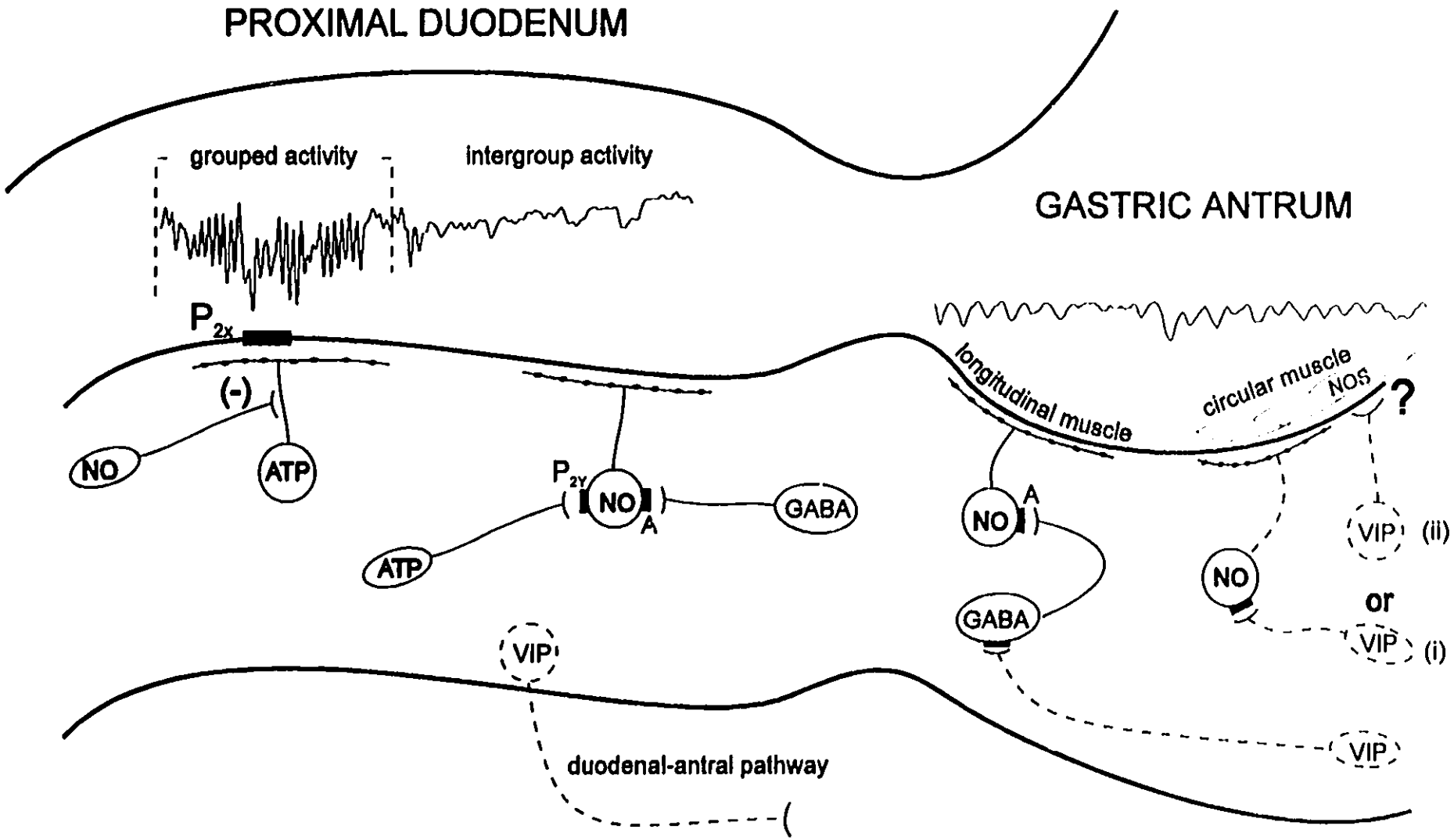
The ability of VIP to induce contractions of both the longitudinal and circular muscle layers of the rat gastric antrum, regardless of the route of administration (i.v. or close i.a.), was a particularly interesting and unexpected finding. There is a great deal of evidence supporting VIP as a candidate NANC inhibitory neurotransmitter, but scant evidence for VIP to be an excitatory agent. In the guinea-pig intestine, VIP can evoke a TTX sensitive contraction [21,61,93]. Moreover, electrophysiological studies show that VIP can excite about 45% of the nerve cells in the myenteric plexus of the ileum (for review see [61]). Therefore, the findings of the present study may represent functional evidence for VIP to induce antral contractions via cholinergic and/or non-cholinergic excitatory motor neurons. Further studies would be needed to more fully define the nature of these VIP induced antral contractions, in particular whether they involve VIP targeting of excitatory motor neurons.

In addition to the VIP (i.v.) evoked responses that were confined to each recording site, VIP was also able to induce a 'coupled' response in the rat gastroduodenum. These responses were further investigated using *ex vivo* experiments. Only direct application of VIP onto the proximal duodenum resulted in a 'coupled' response, which suggests that some reflex (or feedback) pathway involving VIP exists between the proximal duodenum and the gastric antrum. In this regard, functional and morphological evidence strongly supports the existence

of a duodeno-gastric reflex pathway(s). The stomach, unlike the intestine, is believed to be under a greater extrinsic neural influence, either through central input or intestinal input via prevertebral ganglion[62]. In addition, it has been shown that a duodeno-gastric inhibitory reflex pathway (to delay gastric emptying), involving a projection through the coeliac prevertebral ganglia, occurs when the duodenal mucosa is exposed to hyperacidity or hyperosmality [62,127]. Morphological studies in the guinea pig small intestine demonstrate that VIP positive neurons in the myenteric plexus project to coeliac ganglia [35]. Efferent pathways from the coeliac ganglia include innervation of the stomach [35,62,127].

In conclusion, it appears that VIP interacts with the antral nitrergic system in two ways: **i)** by stimulating, the A-GABAergic-nitrergic system and **ii)** the antral circular muscle is innervated either directly by VIP motor neurons (exerting effects via a myogenic NO system) or a non-GABAergic pathway involving VIP. Furthermore, VIP acting at the duodenum can evoke coupled activity of the antrum and duodenum which supports the notion of a duodeno-antral reflex pathway involving this peptide.

Figure 42 Inhibitory innervation of the rat gastroduodenum. The results in this chapter suggest two possibilities of peptidergic innervation: i) The antral circular muscle is innervated directly by VIP or a pathway involving VIP. This possibility involves targeting of NOS by VIP in antral circular smooth muscle cells to induce relaxation (proposed by Grider; 1993) ii) In the gastric antrum, the A-GABAergic-nitrergic system is targeted by VIP. VIP is involved in a duodeno-antral reflex pathway.


PROXIMAL DUODENUM
grouped activity
intergroup activity
GASTRIC ANTRUM
P2x
(-)
NO
ATP
P2Y
NO
A
GABA
ATP
longitudinal muscle
circular muscle
NOS
?
NO
A
GABA
VIP
(ii)
NO
or
VIP
(i)
VIP
VIP
duodenal-antral pathway

CONCLUSION

A consistent characteristic of normal spontaneous motor activity recorded from the gastroduodenum of anaesthetized Sprague-Dawley rats, was the existence of distinct motility patterns. Antral motor activity comprised both contractions and relaxations; however, relaxations were more predominant and often occurred in an oscillatory pattern. Contractions and relaxations were also present in the proximal duodenum, but within two distinct patterns of motor activity. Intense periods of motor activity ('Grouped') were interposed with more quiescent periods of motor activity ('Intergroup'). This characterization of normal gastroduodenal motor activity provided the basis for the investigation of putative NANC transmitter involvement in relaxant activity of these regions of the rat gut.

The motor activity recording technique used here was based upon a method developed for use with the conscious unrestrained rat. The results of these studies show that the same recording technique can be adapted for use in the anaesthetized rat and provides valuable information regarding the enteric neural control of gut motility. In addition, the anaesthetized rat could potentially be used to investigate migrating motor complex (MMC's). Observations of control motor activity in Chapter 1 showed that 'Grouped' activity occurred in the proximal duodenum with a frequency and profile that was reminiscent of MMC's recorded in other studies. Further studies will be needed to confirm this. If the patterned activity in the proximal duodenum is indeed true migrating motor complex activity, the anaesthetized rat could represent an alternate to the current animal of choice, the conscious, unrestrained canine, for the *in vivo* study of MMC. The use of the anaesthetized rat would represent

several advantages over the use of the canine model. First, the rat requires only a 60 minute equilibration (recovery) period (post surgery) as opposed for the need of 8-10 days recovery that occurs with the use of conscious dogs [84]. Secondly, the rat model can be utilised in combination with a recording technique that allows for simultaneous evaluation of contractions and relaxations. Studies with the canine and other species have paid scant attention to the assessment of relaxations. Within the studies presented in this thesis, the identity of the inhibitory innervation mediating/modulating the different relaxations within the spontaneous motor patterned activity of the rat gastroduodenum was revealed. The precise model of control for these two systems remains obscure. This provides a basis for the study of control mechanism(s) underlying gastroduodenum motor function. Lastly, the anaesthetized rat model represents a major cost saving, not only in relation to the cost of a rat compared to a dog, but also due to the reduction in experimental cost associated with the use of an acute versus a chronic model. Furthermore, the recording technique utilises inexpensive and reusable foil strain gauges glued to the serosa, which represents the active component of a full wheatstone bridge, the remaining elements of which are outside the animal. In this way, we have avoided the need to implant costly or cumbersome transducer assemblies.

Results from the *in vivo* studies presented in this thesis provides evidence that all three putative NANC inhibitory neurotransmitters are involved in relaxant motor activity. However, only ATP and NO are involved in the control of spontaneous inhibitory motor activity in the rat gastroduodenum. Each neurotransmitter was capable of evoking relaxations of the gastric antrum and the proximal duodenum. However, in the gastric antrum, all spontaneous relaxant activity was NO mediated. In contrast, in the duodenum, relaxations

within the distinct patterns of spontaneous motor activity involved different transmitters: 'Intergroup' relaxations were mediated by NO while 'Grouped' relaxations were shown to mediated by ATP via P_{2x}-purinoceptors.

In the present study, A-GABAergic innervation was found to specifically target nitrergic motor neurons in the gastroduodenum. These findings supports anatomical evidence in the rat large intestine that 90% of nitrergic myenteric neurons contain $GABA_A$ receptors[110]. Furthermore, the observation that $GABA_A$ receptor blockade inhibits spontaneous nitrergic relaxations demonstrates the requirement for functionally active GABAergic neurons within the pathways controlling NO mediated relaxations of the rat antrum and duodenum. Although there was no evidence that VIP is functionally important within spontaneous relaxant motor activity mediated by ATP or NO, the administration of VIP could, nevertheless, stimulate relaxations of the gastroduodenum. In the antrum, VIP relaxed longitudinal muscle via an A-GABAergic-NO mechanism. VIP induced relaxations of antral circular muscle were also NO mediated but insensitive to A-GABAergic blockade. Hence it appears that only GABAergic neurones within the inhibitory innervation of the antral longitudinal muscle are sensitive to VIP. Since these sites are not requisite for the spontaneous relaxation of the antrum, this VIP action may represent a purely pharmacological circumstance or else and more likely, this represents a site for integration of this A-GABAergic-NO pathway with other reflex control mechanisms. In antral circular smooth muscle, the results presented here supports the scheme proposed by Grider (see Introduction); VIP stimulates NOS present in the smooth muscle and/or belongs in a pathway that can stimulate nitrergic motor neurons and may well represent a distension evoked reflex pathway. The results from experiments involving VIP

provide the foundation for further studies to investigate whether VIP directly targets the A-GABAergic-nitrergic innervation of longitudinal smooth muscle. Peptidergic (VIP) innervation of the antral circular muscle also requires further investigation. At this point it is unknown if VIP causes relaxation of antral circular muscle via activation of NOS within neurons inhibitory motor neurons or within smooth muscle cells.

Intravenously administered VIP was also able to induce 'coupled' responses of the antrum and duodenum. Further *ex vivo* experimentation showed that only direct application of VIP onto the serosa of the proximal duodenum evoked a response that was temporally coupled to a response in the antrum. Conversely, VIP applied directly onto the antrum did not evoke 'coupled' responses. This strongly suggests that VIP neurons originating in the duodenum may be involved in a duodeno-antral reflex pathway. This provides functional evidence to corroborate morphological studies from other laboratories which show a VIP neural projection from the duodenum to prevertebral ganglia with projections to the stomach [35].

In addition to determining that all spontaneous relaxant activity within the rat gastroduodenum is mediated by either purinergic or nitrergic inhibitory motor neurons, these studies also revealed that the ATP mediated 'Grouped' relaxations are under tonic inhibitory modulation by NO. This purinergic nitrergic interaction may be even more extensive since it was found that NO mediated relaxations of the duodenum can be stimulated by the P_{2y}-purinoceptors agonist, 2-Me-S-ATP. P_{2y}-purinoceptors exist either directly on duodenal nitrergic inhibitory motor neurons or some other unknown interneuron that is part of an excitatory innervation of nitrergic inhibitory motor neurons. The fact that duodenal nitrergic

motor neurons are targeted by A-GABAergics, suggest that the GABAergic interneurons may well be stimulated by ATP via P_{2y} receptors. Whether ATP and GABA are involved in the same or separate excitatory pathways controlling NO inhibitory motor neurons in the proximal duodenum requires urgent attention. Current dogma states that ATP mediates relaxations of the gut via P_{2y}-purinoceptors on smooth muscle [27,98]. However, evidence presented herein strongly suggests the existence of P_{2x}-purinoceptors on duodenal smooth muscle whereby ATP mediates 'Grouped' relaxant activity. This notion is supported by other studies reporting P_{2x} receptors to be responsible for mediating ATP evoked relaxations of the gut (see Chapter 3 and Introduction). Hence these various studies taken together suggest ATP may well mediate relaxations via both P_2-purinoceptor subtypes but the receptor of choice may be species and region specific.

Nitrergic modulation of ATP mediated 'Grouped' relaxations may represent a population of nitrergic neurons that are not targeted by A-GABAergic neurons. This raises the possibility of the existence of two populations of nitrergic neurons in the proximal duodenum and a basis for distinguishing NO motor neurons from NO interneurons using the criteria of whether $GABA_A$ receptors exist on the cell soma or not.

The logical treatment of most gastrointestinal disorders depends on an understanding of the underlying physiology. GI diseases involving the disruption of motor activity requires that the enteric neuronal control of gut motor activity be first characterised. Such characterization reveals possible target sites for drug interaction to rectify the disruption of motor activity. In this laboratory, there is considerable interest in the human duodenal disease, duodenal ulceration (DU). This is a multifactorial disease in which the disruption of

gastroduodenal motor activity is an important contributing factor. From studies of experimental duodenal ulceration using the cysteamine-HCl treated Sprague Dawley rat model, excitatory cholinergic and intestinal NANC inhibitory motor neurons appears to be disrupted. Moreover, this disruption of motor activity in the gastroduodenum occurs very early in DU development. The observations from Chapter 1 show that cysteamine-HCl does indeed disrupt duodenal patterning. Hence the studies presented in this thesis have important implications beyond just the understanding of how NO, ATP and VIP are involved in the control of inhibitory motor activity in the rat. This provides a basis for further investigation as to which, if any, of these neurons and pathways are affected in the development of DU.

REFERENCES

1. Abrahamsson, H. Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch. int. Pharmacodyn.* 1986. 280,Suppl.: 50-61.
2. Aimi, Y., Kimura, H., Kinoshita, T., Minami, Y., Fujimura, M. and Vincent, S.R. Histochemical localization of nitric oxide synthase in rat enteric nervous sytem. *Neuroscience* 1993. 53: 553-560.
3. Allescher, H.D., Tougas, G., Vergara, P., Lu, S. and Daniel, E.E. Nitric oxide as a putative nonadrenergic noncholinergic inhibitory transmitter in the canine pylorus *in vivo*. *Am. J. Physiol.* 1992. 262: G695-G702.
4. Baccari, M.C., Calamai, F. and Staderini, G. Effects of arterial infusions of adenosine 5'-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP) on vagal excitatory motor responses in the rabbit stomach '*in vivo*'. *J. Auton. Nerv. Syst.* 1990. 30: S15-S18.
5. Baer, H.P. and Frew, R. Relaxation of guinea-pig fundic strip by adenosine, adenosine triphosphate and electrical stimulation: lack of antagonism by theophylline or ATP treatment. *Br. J. Pharmacol.* 1979. 67: 293-299.
6. Barbier, A.J. and Lefebvre, R.A. Involvement of the L-arginine: nitric oxide pathway in nonadrenergic noncholinergic relaxation of the cat gastric fundus. *J. Pharmacol. Exp. Ther.* 1993. 266: 172-178.
7. Bauer, V., Matusak, O. and Kuriyama, H. Non-Cholinergic, Non-Adrenergic Responses to Nerve Stimulation of Different Regions of the Guinea-Pig Small Intestine. *Arch. Pharmacol.* 1982. 319: 108-114.
8. Beck, K., Calamai, F., Staderini, G. and Susini, T. Gastric motor responses elicited by vagal stimulation and purine compouds in the atropine-treated rabbit. *Br. J. Pharmacol.* 1988. 94: 1157-1166.
9. Belai, A. and Burnstock, G. Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and anococcygeus muscle. *Cell Tissue Res.* 1994. 278: 197-200.
10. Belai, A., Schmidt, H.H.H.W., Hoyle, C.H.V., Hassall, C.J.S., Saffrey, M.J., Moss, J.,

Forstermann, U., Murad, F. and Burnstock, G. Colocalization of nitric oxide synthase and NADPH-diaphorase in the myenteric plexus of the rat gut. *Neurosci. Lett.* 1992. 143: 60-64.

11. Berezin, I., Snyder, S.H., Bredt, D.S. and Daniel, E.E. Ultrastructural localization of nitric oxide synthase in canine small intestine and colon. *Am. J. Physiol.* 1994. 266: C981-C989.
12. Biancani, P., Walsh, J. and Behar, J. Vasoactive Intestinal peptide: A neurotransmitter for relaxation of the rabbit internal anal sphincter. *Gastroenterol.* 1985. 89: 867-874.
13. Bitar, K.N. and Makhlouf, G.M. Relaxation of isloated gastric smooth muscle cells by vasoactive intestinal peptide. *Sci.* 1982. 216: 531-533.
14. Boeckxstaens, G.E., Pelckmans, P.A., Bogers, J.J., Bult, H., De Man, J.G., Oosterbosch, L., Herman, A.G. and Van Maercke, Y.M. Release of nitric oxide upon stimulation of nonadrenergic noncholinergic nerves in the rat fundus. *J. Pharmacol. Exp. Ther.* 1991. 256: 441-447.
15. Boeckxstaens, G.E., Pelckmans, P.A., Bult, H., De Man, J.G., Herman, A.G. and Van Maercke, Y.M. Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br. J. Pharmacol.* 1991. 102: 434-438.
16. Boeckxstaens, G.E., Pelckmans, P.A., Herman, A.G. and Van Maercke, Y.M. Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. *Gastroenterol.* 1993. 104: 690-697.
17. Boeckxstaens, G.E., Pelckmans, P.A., Rampart, M., Ruytjens, I.F., Verbeuren, T.J., Herman, A.G. and Van Maercke, Y.M. $GABA_A$ receptor-mediated stimulation of non-adrenergic non-cholinergic neurones in the dog ileocolonic junction. *Br. J. Pharmacol.* 1990. 101: 460-464.
18. Bojo, L., Lefebvre, R.A., Nellgard, P. and Cassuto, J. Involvement of vasoactive intestinal polypeptide in gastric reflex relaxation. *Eur. J. Pharmacol.* 1993. 236: 443-448.
19. Bornstein, J.C. Local neural control of intestinal motility: Nerve circuits deduced for the guinea-pig small intestine. *Clinical and Experimental Pharmacology and Physiology*. 1994. 21: 441-452.
20. Bredkjaer, H.E., Wulff, B.S., Emson, P.C. and Fahrenkrug, J. Location of PHM\VIP mRNA

in human gastrointestinal tract detected by *in situ* hybridization. *Cell Tissue Res.* 1994. 276: 229-238.

21. Briejer, M.R., Akkermans, L.M.A., Meulemans, A.L., Lefebvre, R.A. and Schuurkes, J.A.J. 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn Schmiedebergs Arch. Pharmacol.* 1995. 351: 126-135.

22. Brookes, F.P. The pathophysiology of peptic ulcer disease. *Digestive Diseases and Sciences* 1985. 30: 15S-29S.

23. Bryant, M.G., Polak, J.M., Modlin, I., Bloom, S.R., Albuquerque, R.H. and Pearse, A.G.E. Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. *Lancet* 1976. 1976:1: 991-993.

24. Bueno, L., Ferre, J. and Ruckebusch, Y. Effects of anesthesia and surgical procedures on intestinal myoelectric activity in rats. *Dig. Dis.* 1978. 23: 690-695.

25. Bult, H., Boeckxstaens, G.E., Pelckmans, P.A., Jordaens, F.H., Van Maercke, Y.M. and Herman, A.G. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* 1990. 345: 346-347.

26. Burleigh, D.E. N^G-Nitro-L-Arginine reduces Nonadrenergic, Noncholinergic Relaxations of Human Gut. *Gastroenterol.* 1992. 102: 679-683.

27. Burnstock, G. Purinergic Mechanisms. *Ann NY Acad Sci.* 1-17.

28. Burnstock, G., Campbell, G., Satcheel, D. and Smythe, A. Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.* 1970. 40: 668-688.

29. Burnstock, G., Satchell, D.G. and Smythe, A. A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *Br. J. Pharmacol.* 1972. 46: 234-242.

30. Caenepeel, J.J., Vantrappen, G., Eyssen, H. and Coremans, G. Interdigestive myoelectric complex in germ-free rats. *Digestive Diseases and Sciences* 1989. 34: 1180-1184.

31. Calignano, A., Whittle, B.J.R., Di Rosa, M. and Moncada, S. Involvement of endogenous nitric oxide in the regulation of rat intestinal motility *in vivo*. *Eur. J. Pharmacol.* 1992. 229:

273-276.

32. Chayvialle, J., Miyata, M. and Thompson, J.C. Release of vasoactive intestinal peptide by distension of the proximal stomach in dogs. *Gut* 1980. 21: 745-749.

33. Costa, M. and Brookes, S.J.H. The Enteric Nervous System. *The American Journal of Gastroenterology* 1994. 89: s129-s137.

34. Costa, M. and Furness, J.B. The origins, pathways and termination of neurons with VIP-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* 1983. 8: 665-676.

35. Costa, M., Furness, J.B., Buffa, R. and Sai, S.I. Distribution of enteric nerve cell bodies and axons showing immunoreactivity for vasoactive intestinal poypeptide in the guinea-pig intestine. *Neuroscience* 1980. 5: 587-596.

36. Costa, M., Furness, J.B. and Humphreys, C.M.S. Apamin distinguishes two types of relaxation mediated by enteric nerves in the guinea-pig gastrointestinal tract. *Naunyn Schmiedebergs Arch. Pharmacol.* 1986. 332: 79-88.

37. Costa, M., Furness, J.B., Pompolo, S., Brookes, S.J.H., Bornstein, J.C., Bredt, D.S. and Synder, S.H. Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. *Neurosci. Lett.* 1992. 148: 121-125.

38. Crist, J.R., He, X.D. and Goyal, R.K. Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J. Physiol.* 1992. 447: 119-131.

39. Crowe, R. and Burnstock, G. Comparitive studies of quinacrine-positive neurones in the myenteric plexus of stomach and intestine of guinea-pig, rabbit and rat. *Cell Tissue Res* 1981. 221: 93-107.

40. Cuffari, C., Rubin, S.Z., Nichols, K., Staines, W. and Krantis, A. Evidence for nitric oxide (NO) synthesizing neurons and blood vessels in normal human and Hirschsprung's intestine (abstr). *Gastroenterol.* 1992. 102: A922

41. D'Amato, M., Curro, D. and Montuschi, P. Evidence for dual components in the non-adrenergic non-cholinergic relaxation in the rat gastric fundus: role of endogeneous nitric oxide and vasoactive intestinal polypeptide. *J. Auton. Nerv. Syst.* 1992. 37: 175-186.

42. D'Amato, M., DE Beurme, F.A. and Lefebvre, R.A. Comparison of the effect of vasoactive intestinal polypeptide and non-adrenergic non-cholinergic neurone stimulation in the cat gastric fundus. *Eur. J. Pharmacol.* 1988. 152: 71-82.

43. D'Amato, M., Montuschi, P., Ciabattoni, G., Ragazzoni, E. and Lefebvre, R.A. Release of vasoactive intestinal peptide from rat gastric fundus. *Br. J. Pharmacol.* 1992. 105: 691-695.

44. DE Beurme, F.A. and Lefebvre, R.A. Influence of α-chymotrypsin and trypsin on the non-adrenergic non-cholinergic relaxation in the rat gastric fundus. *Br. J. Pharmacol.* 1987. 91: 171-177.

45. DE Beurme, F.A. and Lefebvre, R.A. Vasoactive Intestinal Polypeptide as possible mediator of relaxation in the rat gastric fundus. *J. Pharm. Pharmacol.* 1988. 40: 711-715.

46. De Giorgio, R., Parodi, J.E., Brecha, N.C., Brunicardi, F.C., Becker, J.M., Go, V.W. and Sternini, C. Nitric oxide producing neurons in the monkey and human digestive system. *J. Comp. Neurol.* 1994. 342: 619-627.

47. Delbro, D. and Fandriks, L.ATP induces non-cholinergic, non-adrenergic gastric relaxation *in vivo*. *Acta Physiol. Scand.* 1982. Suppl. 508: 67

48. Delbro, D. and Fandriks, L.Inhibition of vagally induced non-adrenergic, non-cholinergic gastric relaxation by P_2-purinoceptor desensitization. *Acta Physiol. Scand.* 1984. 120: 12A

49. Desai, K.M., Zembowicz, A., Sessa, W.C. and Vane, J.R. Nitroxergic nerves mediate vagally induceed relaxation in the isolated stomach of the guinea pig. *Proc. Natl. Acad. Sci. USA* 1991. 88: 11490-11494.

50. Dudeck, O., Bultmann, R. and Starke, K. Two relaxation-mediating P_2-purinoceptors in guinea-pig taenia caeci. *Naunyn Schmiedebergs Arch. Pharmacol.* 1995. 351: 107-110.

51. Dunn, P.M. and Blakeley, A.G.H. Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.* 1988. 93: 243-245.

52. Edin, R., Lundberg, J.M., Ahlman, H., Dahlstrom, A., Fahrenkrug, J., Hokfelt, T. and Kewenter, J. On the VIP-ergic innervation of the feline pylorus. *Acta Physiol. Scand.* 1979. 107: 185-187.

53. Ekbald, E., Ekelund, M., Graffner, H., Hakanson, R. and Sundler, F. Peptide-containing nerve fibers in the stomach wall of rat and mouse. *Gastroenterol.* 1985. 89: 73-85.

54. Ekblad, E., Mulder, H., Uddman, R. and Sundler, F. NOS-containing neurons in the rat gut and coeliac ganglia. *Neuropharmacol.* 1994. 33: 1323-1331.

55. Eklund, S., Jodal, M., Lundgren, O. and Sjoqvist, A. Effects of vasoactive intestinal peptide on blood flow, motolity and fluid transport in the gastrointestinal tract of the cat. *Acta*

Physiol. Scand. 1979. 105: 461-468.

56. Erdo, S.L. Peripheral GABAergic mechanisms. *TiPS* 1985. 205-208.

57. Erdo, S.L. and Bowery, N.G. *GABAergic Mechanisms in the Mammalian Periphery*. 1st ed. New York: Raven Press, 1986.

58. Foreter, E.R., Green, T. and Dockray, G.J. Efferent pathways in the reflex control of gastric emptying in rats. *Am. J. Physiol.* 1991. 260: G499-G504.

59. Forster, E.R. and Southam, E. The intrinsic and vagal extrinsic innervation of the rat stomach contains nitric oxide synthase. *Neurochem.* 1993. 4: 275-278.

60. Frew, R. and Lundy, P.M. Evidence against ATP being the nonadrenergic, noncholinergic inhibitory transmitter in guinea pig stomach. *Eur. J. Pharmacol.* 1982. 81: 333-336.

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

62. Furness, J.B. and Costa, M. *The Enteric Nervous Sytem*. 1st ed. New York: Churchill Livingstone, 1987.

63. Furness, J.B., Li, Z.S., Young, H.M. and Forstermann, U. Nitric oxide synthase in the enteric nervous system of the guinea-pig: a quantitative description. *Cell Tissue Res.* 1994. 277: 139-149.

64. Furness, J.B., Trusell, D.C., Pompolo, S., Bornstein, J.C., Maley, B.E. and Storm-Mathisen, J. Shapes and projections of neurons with immunoreactivity for gamma-aminobutyric in the guinea-pig small intestine. *Cell Tissue Res.* 1989. 256: 293-304.

65. Gally, J.A., Mantague, P.R., Reeke, G.N. and Edelman, G.M. The NO hypothesis: Possible effects of a short-lived, rapidly diffusable signal in the development and function of the nervous system. *Proc.Natl.Acad.Sci.* 1990. 87: 3547-3551.

66. Giotti, A., Luzzi, S., Maggi, C.A., Spagnesi, S. and Zilletti, L. Modulatory role of $GABA_B$ receptors on cholinergic tone in guinea-pig distal colon. *Br. J. Pharmacol.* 1985. 84: 883-895.

67. Giotti, A., Luzzi, S., Spagnesi, S. and Zilletti, L. $GABA_A$ and $GABA_B$ receptor mediated effects in guinea-pig ileum. *Br. J. Pharmacol.* 1983. 78: 469-478.

68. Granger, D.N., Barrowman, J.A. and Kvietys, P.R. *Clinical Gastrointestinal Physiology*. Philadelphia: W.B. Saunders, 1985.

69. Grider, J.R. Identification of neurotransmitters by selective protection of postjunctional receptors. *Am. J. Physiol.* 1990. 258: G103-G106.

70. Grider, J.R. Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. *Am. J. Physiol.* 1993. 264: G334-G340.

71. Grider, J.R., Cable, M.B., Bitar, K.N., Said, S.I. and Makhlouf, G.M. Vasoactive intestinal peptide. Relaxant neurotransmitter in tenia coli of the guinea pig. *Gastroenterol.* 1985. 89: 36-42.

72. Grider, J.R., Cable, M.B., Said, S.I. and Makhlouf, G.M. Vasoactive intestinal peptide as a neural mediator of gastric relaxation. *Am. J. Physiol.* 1985. 248: G73-G78.

73. Grider, J.R. and Makhlouf, G.M. Colonic peristaltic reflex: identification of vasoactive intestinal peptide as mediator of descending relaxation. *Am. J. Physiol.* 1986. 251: G40-G45.

74. Grider, J.R. and Makhlouf, G.M. Prejunctional inhibition of vasoactive intestinal peptide release. *Am. J. Physiol.* 1987. 253: G7-G12.

75. Grider, J.R. and Makhlouf, G.M Regulation of peristaltic reflex by peptides of the myenteric plexus. *Arch. int. Pharmacodyn.* 1990. 303: 232-251.

76. Grider, J.R. and Makhlouf, G.M. Transmitter of Inhibitory Motor Neurons of the Gut. *Ann NY Acad Sci* 1990. 369-377.

77. Grider, J.R. and Makhlouf, G.M. Enteric GABA: mode of action and role in the regulation of peristaltic reflex. *Am. J. Physiol.* 1993. 262: G690-G694.

78. Grider, J.R., Murthy, K.S., Jin, J.-G. and Makhlouf, M. Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancment of VIP release. *Am. J. Physiol.* 1992. 262: G774-G778.

79. Grider, J.R. and Rivier, J.R. Vasoactive Intestinal Peptide (VIP) as transmitter of inhibitory motor neurons of the gut: evidence from the use of selective VIP antagonists and VIP antiserum. *J. Pharmacol. Exp. Ther.* 1990. 253: 738-742.

80. Grozdanovic, Z., Bruning, G. and Baumgarten, H.G. Nitric oxide- a novel autonomic neurotransmitter. *Acta Anat.* 1994. 150: 16-24.

81. Grundy, D., Gharib-Naseri, M.K. and Hutson, D. Role of nitric oxide and vasoactive intestinal polypeptide in vagally mediated relaxation of the gastric corpus in the

anaesthetized ferret. *J. Auton. Nerv. Syst.* 1993. 43: 241-246.

82. Gustafsson, B.I. and Delbro, D.S. Tonic inhibition of small intestinal motility by nitric oxide. *J. Auton. Nerv. Syst.* 1993. 44: 179-187.

83. Gustafsson, B.I. and Delbro, D.S. Neurogenic inhibition of duodenal and jejunal motility in the anaesthetized rat. *Eur. J. Pharmacol.* 1994. 257: 227-233.

84. Haba, T. and Sarna, S.K. Regulation of gastroduodenal emptying of solids by gastropyloroduodenal contractions. *Am. J. Physiol.* 1993. 264: G261-G271.

85. Hawking, F. Suramin: with special reference to onchoceriasis. *Advances in Pharmacology and Chemotheraphy* 1978. 15: 289-322.

86. Hoyle, C.H.V., Knight, G.E. and Burnstock, G. Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.* 1990. 99: 617-621.

87. Huizinga, J.D. and Hertog, D.A. Inhibition of fundic strips from guinea-pig: the effect of theophylline on responses to adennosine, ATP and intramural nerve stimulation. *Eur. J. Pharmacol.* 1980. 63: 259-265.

88. Ito, S., Ohga, A. and Ohta, T. Gastric vasodilation and vasoactive intestinal peptide output in response to vagal stimulation in the dog. *J. Physiol.* 1988. 404: 669-682.

89. Ito, S., Ohga, A. and Ohta, T. Gastric relaxation and vasoactive intestinal peptide output in response to reflex vagal stimulation in the dog. *J. Physiol.* 1988. 404: 683-693.

90. Jessen, K.R. and Hills, J.M. GABA as an autonomic neurotransmitter: studies on intrinsic GABAergic neurons in the myenteric plexus of the gut. *TINS* 1987. 10: 255-262.

91. Jessen, K.R., Hills, J.M., Dennison, M.E. and Mirsky, R. γ-aminobutyrate as an autonomic neurotransmitter: release and uptake of [^{3}H]γ-aminobutyrate in guinea pig large intestine and cultered enteric neurons using physiological methods and electron microscope autoradiography. *Neuroscience* 1983. 10: 1427-1442.

92. Jessens, K.R., Hills, J.M. and Saffrey, M.J. Immunohistochemical demonstration of GABAergic neurons in the enteric nervous system. *J. Neurosci.* 1986. 6: 1628-1634.

93. Kachelhoffer, J., Mendel, C., Dauchel, J., Hohmatter, D. and Grenier, J.F. The effects of VIP on intestinal motility. *Dig. Dis.* 1976. 21: 957-962.

94. Kamata, K., Sakamoto, A. and Kasuya, Y. Similarities between the relaxations induced by

vasoactive intestinal peptide and stimulation of the non-adrenergic non-cholinergic neurons inthe rat stomach. *Naunyn Schmiedebergs Arch. Pharmacol.* 1988. 338: 401-406.

95. Kanada, A., Hata, F., Suthamnatpong, N., Maehara, T., Ishii, T., Takeuchi, T. and Yagasaki, O. Key roles of nitric oxide and cyclic GMP in nonadrenergic and noncholinergic inhibition in rat ileum. *Eur. J. Pharmacol.* 1992. 216: 287-292.

96. Kanada, A., Hosokawa, M., Suthamnatpong, N., Maehara, T., Takeuchi, T. and Hata, F. Neuronal pathways involved in nitric oxide-mediating descending relaxation in rat ileum. *Eur. J. Pharmacol.* 1993. 250: 59-66.

97. Keef, K.D., Du, C., Ward, S.M., McGregor, B. and Sanders, K.M. Enteric inhibitory neural regulation of human colonic circular muscle: Role of nitric oxide. *Gastroenterol.* 1993. 105: 1009-1016.

98. Kennedy, C. P_1- and P_2-Purinoceptor subtypes - an update. *Arch. int. Pharmacodyn.* 1990. 303: 30-50.

99. Kerr, D.I.B. and Krantis, A. Uptake and stimulaus-evoked release of [^{3}H]-γ-aminobutyric acid by myenteric nerves of guinnea-pig intestine. *Br. J. Pharmacol.* 1983. 78: 271-276.

100. Kerr, D.I.B., Ong, J., Prager, R.H., Gynther, B.D. and Curtis, D.R. Phaclofen: a peripheral and central baclofen antagonist. *Brain Res.* 1987. 405: 150-154.

101. Krantis, A. The effects of cysteamine on neurogenic responses in the stomach and small intestine of the rat. *Eur. J. Pharmacol.* 1987. 144: 257-266.

102. Krantis, A. Selective uptake of gamma-[^{3}H]aminobutyric acid by neural elements and vascular nerves of the rat intestinal submucosa. *Neurosci. Lett.* 1990. 109: 1-6.

103. Krantis, A. and Clark, D. Localization of [^{3}H]GABA-labelled nerve fibre networks in the rat intestinal mucosa. *J. Auton. Nerv. Syst.* 1991. 34: 195-200.

104. Krantis, A. and Clark, D. High affinity uptake of [^{3}H]GABA by submucous ganglion cells, nerve fibres and peri-and para-vascular fibres in guinea-pig and rat intestine. *J. Auton. Nerv. Syst.* 1991. 31: 251-258.

105. Krantis, A., Costa, M., Furness, J.B. and Orbach, J. γ-aminobutyric acid stimulates intrinsic inhibitory and excitatory nerves in the guinea-pig intestine. *Eur. J. Pharmacol.* 1980. 67: 461-468.

106. Krantis, A. and Harding, R.K. The distribution of GABA-transaminase-dehydrogenase

activity in the myenteric plexus of rat small intestine: a histochemical analysis. *Neurosci. Lett.* 1986. 64: 85-90.

107. Krantis, A. and Harding, R.K. GABA-related actions in isolated *in vitro* preparations of the rat small intestine. *Eur. J. Pharmacol.* 1987. 141: 291-298.

108. Krantis, A. and Kerr, D.I.B. The effect of GABA antagonism on propulsive activity of the guinea-pig large intestine. *Eur. J. Pharmacol.* 1981. 76: 111-114.

109. Krantis, A. and Krause, C. Interactions of the ulcerogen cysteamine with enteric inhibitory nerves and putative transmitters in the rat small intestine. *J. Auton. Pharmacol.* 1989. 9: 219-230.

110. Krantis, A., Shabnavard, L., Nichols, K., de Blas, A.L. and Staines, W. Localization of $GABA_A$ receptor immunoreactivity in NO synthase postive myenteric neurones. *J. Auton. Nerv. Syst.* 1995. 53: 157-165.

111. Krantis, A. and Webb, T. Autoradiographic localization of [^{3}H] γ-aminobutyric acid in neuronal elements of the rat gastric antrum and intestine. *J. Auton. Nerv. Syst.* 1989. 29: 41-48.

112. Lancaster, J.R., Jr. Nitric oxide in cells. *American Scientist* 1992. 80: 248-259.

113. Lefebvre, R.A., Baert, E. and Barbier, A.J. Influence of N^G-nitro-L-arginine on non-adrenergic non-cholinergic relaxation in the guinea-pig gastric fundus. *Br. J. Pharmacol.* 1992. 108: 173-179.

114. Lefebvre, R.A., Hasrat, J. and Gobert, A. Influence of N^G-nitro-L-arginine methyl ester on vagally induced gastric relaxtion in the anaesthetized rat. *Br. J. Pharmacol.* 1992. 105: 315-320.

115. Leff, P., Wood, B.E. and O'Connor, S.E. Suramin is a slowly-equilibrating but competitive antagonist at P_{2x}-receptors in the rabbit isolating ear artery. *Br. J. Pharmacol.* 1990. 101: 645-649.

116. Li, C.G. and Rand, M.J. Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.* 1990. 191: 303-309.

117. Maggi, C.A., Manzini, S. and Meli, A. Evidence that $GABA_A$ receptors mediate relaxation of the rat duodenum by activating intramural nonadrenergic-noncholinergic neurones. *J.*

Auton. Pharmacol. 1984. 4: 77-85.

118. Manzini, S., Maggi, C.A. and Meli, A. Pharmacological evidence that at least two different non-adrenergic non-cholinergic inhibitory systems are present in the rat small intestine. *Eur. J. Pharmacol.* 1986. 123: 229-236.

119. Matharu, M.S. and Hollingsworth, M. Purinoceptors mediating relaxation and spasm in the rat gastric fundus. *Br. J. Pharmacol.* 1992. 106: 395-403.

120. Matusak, A. and Bauer, V. Effect of desensitization induced by adenosine 5'-triphosphate, substance P, bradykinin, serotonin, GABA and endogenous noncholinergic-nonadrnergic transmitter in the guinea-pig ileum. *Eur. J. Pharmacol.* 1986. 126: 199-209.

121. McConalogue, K. and Furness, J.B. Projection of nitric oxide synthesizing neurons in the guinea-pig colon. *Cell Tissue Res.* 1993. 271: 545-553.

122. McKay, A.E. and Krantis, A. *In vivo* recording of gastroduodenal motlity during experimental duodenal ulceration. *Can. J. Physiol. Pharm.* 1991. 69: AXXI

123. McKay, A.E., Krantis, A. and Johnson, F. *In vivo* gastroduodenal motility recordings in conscious, unrestrained rats: characterization during experimental duodenal ulcer. *Gastroenterol.* 1992. 102: A934

124. McKirdy, H.C., Marshall, R.W. and Taylor, B.A. Control of the human ileocaecal junction: an *in vitro* analysis of adrenergic and non-adrenergic non-cholinergic mechanisms. *Digestion* 1993. 54: 200-206.

125. McKirdy, H.C., McKirdy, M.L., Lewis, M.J. and Marshall, R.W. Evidence for Involvement of Nitric Oxide in the Non-Adrenergic Non-Cholinergic (NANC) Relaxation of Human Lower Oesophageal Sphinter Muscle Strips. *Exp. Physiol.* 1992. 77: 509-511.

126. Mercier-Parot, L. and Tuchmann-Duplessis, H. Abortifacient and teratogenic effect of suramin, a trypanocide. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales* 1973. 167: 1518-1522.

127. Messenger, J.P. and Furness, J.B. Distribution of enteric nerve cells that project to the coelic ganglia of the guinea-pig. *Cell Tissue Res.* 1992. 269: 119-132.

128. Meulemans, A.L., Helsen, L.F. and Schuurkes, A.J. Role of NO in vagally-mediated relaxations of guinea-pig stomach. *Naunyn Schmiedebergs Arch. Pharmacol.* 1993. 347: 225-230.

129. Middleton, S.J., Cuthbert, A.W., Shorthouse, M. and Hunter, J.O. Nitric oxide affects mammalian distal colonic smooth musccle by tonic inhibition. *Br. J. Pharmacol.* 1993. 108: 974-979.

130. Murray, J.A. and Clark, E.D. Characterization of nitric oxide synthase in the opossum esophagus. *Gastroenterol.* 1994. 106: 1444-1450.

131. Murthy, K.S., Zhang, K.-M., Jin, J.-G., Grider, J.R. and Makhlouf, G.M. VIP-mediated G protein-coupled Ca^{2+} influx activates a constitutive NOS in dispersed gastric muscle cells. *Am. J. Physiol.* 1993. 265: G660-G671.

132. Nichols, K., Krantis, A. and Staines, W. Histochemical Localization of Nitric Oxide-Synthesizing Neurons and Vascular Sites in the Guinea-pig Intestine. *Neuroscience* 1992. 51: 791-799.

133. Nichols, K., Staines, W. and Krantis, A. Nitric oxide sythase distribution in the rat intestine: A histochemical analysis. *Gastroenterol.* 1993. 105: 1651-1661.

134. Nichols, K., Staines, W. and Krantis, A. Neural sites of the human colon colocalize nitric oxide synthase-related NADPH diaphorase activity and neuropeptide Y. *Gastroenterol.* 1994. 107: 968-975.

135. Nichols, K., Staines, W., Wu, J.-Y. and Krantis, A. Immunopositive GABAergic neural sites display nitric oxidde synthase-related NADPH diaphorase activity in the human colon. *J. Auton. Nerv. Syst.* 1995. 50: 253-262.

136. Ny, L., Alm, P., Ekstrom, P., Hannibal, J., Larsson, B. and Andersson, K. Nitric oxide syntahse-containing, peptide-containing, and acetylcholinesterase-positive nerves in the cat lower oesophagus. *Histochemical Journal* 1994. 26: 721-733.

137. Ohno, N., Ito, K.M., Yamamoto, Y. and Suzuki, H. Suramin selectively inhibits the non-adrenergic non-cholinergic inhibitory junction potential in the guinea pig stomach. *Eur. J. Pharmacol.* 1993. 249: 121-123.

138. Olson, L., Alund, M. and Norberg, K.-A. Fluorescence-microscopical demonstration of a population of gastro-intestinal nerve fibres with a selective affinity for quinacrine. *Cell Tissue Res.* 1976. 171: 407-423.

139. Ong, J. and Kerr, D.I.B. $GABA_A$- and $GABA_B$-receptor-medeiated modification of intestinal motility. *Eur. J. Pharmacol.* 1983. 86: 9-17.

140. Osthaus, L.E. and Galligan, J.J. Antagonists of Nitric Oxide Synthesis Inhibit Nerve-mediated Relaxations of Longitudinal Muscle in Guinea Pig Ileum. *J. Pharmacol. Exp. Ther.* 1991. 260: 140-145.

141. Pascaud, X.B., Genton, M.J.H. and Bass, P. A minature transducer for recording intestinal motility in unrestrained chronic rats. *Am. J. Physiol.* 1993. 235: E532-E538.

142. Pearson, G.T. Structural organization and neuropeptide distributions in the equine enteric nervous system: an immunohistochemical study using whole-mount preparations from the small intestine. *Cell Tissue Res.* 1994. 276: 523-534.

143. Said, S.I. and Mutt, V. Polypeptide with broad biological activity: Isolation from small intestine. *Sci.* 1970. 169: 1217-1218.

144. Sanders, K.M. and Ward, S.M. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.* 1992. 262: G379-G392.

145. Satchell, D.G., Burnstock, G. and Dann, P. Antagonism of the effects of purinergic nerve stimulation and exogenously applied ATP on the guinea-pig taenia coli by 2-substituted imidazolines and related compounds. *Eur. J. Pharmacol.* 1973. 23: 264-269.

146. Satchell, D.G., Lynch, A., Bourke, P.M. and Burnstock, G. Potentiation of the effects of exogenously applied ATP and purinergic nerve stimulation on the guinea-pig taenia coli by dipyridamole and hexobendine. *Eur. J. Pharmacol.* 1972. 19: 343-350.

147. Schlicker, E., Urbanek, E. and Gothert, M. ATP, α,β-methylene ATP and suramin as tools for characterization of vascular P_{2x} receptors in the pithed rat. *J. Auton. Pharmacol.* 1989. 9: 357-366.

148. Schmidt, H.H.H.W., Lohmann, S.M. and Walter, U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochemica et Biophysica Acta* 1993. 1178: 153-175.

149. Schultzberg, M., Hokfelt, T., Nilsson, G., Terenius, L., Rehfeld, J.F., Brown, M., Elde, R., Goldstein, M. and Said, S. Distribution of peptide and catecholamine-containing neurons in the gastrointestinal tract of rat and gunea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamin β-hydroxylase. *Neuroscience* 1980. 5: 689-744.

150. Serio, R., Mule, F. and Postorino, A. Noadrenergic, noncholinergic inhibitory junction potentials in rat proximal colon: role of nitric oxide. *Can. J. Physiol. Pharm.* 1995. 73: 79-84.

151. Shuttleworth, C.W.R., Murphy, R. and Furness, J.B. Evidence that nitric oxide participates in non-adrenergic inhibitory transmission to intestinal muscle in the guinea-pig. *Neurosci. Lett.* 1991. 130: 77-80.

152. Shuttleworth, C.W., Xue, C., Ward, S.M., De Vente, J. and Sanders, K.M. Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. *Neuroscience* 1993. 56: 513-522.

153. Slater, M., Knowles, R.G. and Moncada, S. Widespread tissue distribution, species distribution and changes in activity of Ca^{++} dependent and Ca^{++} independent nitric oxide synthases. *FEBS* 1991. 291: 145-149.

154. Small, R.C. and Weston, A.H. Theophylline antagonizes some effects of purines in the intestine but not those of intramural inhibitory nerve stimulation. *Br. J. Pharmacol.* 1979. 67: 301-308.

155. Sneddon, P. and Graham, A. Role of nitric oxide in the autonomic innervation of smooth muscle *J. Auton. Pharmacol.* 1992. 12: 445-456.

156. Stark, M.E., Bauer, A.J., Sarr, M.G. and Szyrszewski, J.H. NItric Oxide Mediates Inhibitory Nerve Input in Human and Canine Jejunum. *Gastroenterol.* 1993. 104: 398-409.

157. Stark, M.E. and Szurszewski, J.H. Role of Nitric Oxide in Gastrointestinal and Hepatic Function and Disease. *Gastroenterol.* 1992. 103: 1928-1949.

158. Synder, S.H. Nitric oxide: First in a new class of neurotransmitters. *Sci.* 1992. 257: 494-496.

159. Szabo, S., Pihan, G., Gallagher, G.T. and Brown, A. Role of local secretory and motility changes in the pathogenesis of experimental duodenal ulcer. *Scand. J Gastroenterol.* 1984. 19: 106-111.

160. Szabo, S., Reynolds, E.S. and Lichtenberger, L.M. Pathogenesis of duodenal ulcer. Gastric hyperacidity caused by propionitrile and cysteamine in rats. *Res. Commun. Chem. Pathol. Pharmacol.* 1977. 16: 311-322.

161. Takeuchi, K., Nishiwaki, H. and Okabe, S. Role of local motility changes in the pathogenesis of duodenal ulcers induced by cysteamine in rats. *Digestive Diseases and Sciences* 1987. 32: 295-304.

162 Taniyama, K., Kusunoki, M., Saito, N. and Tanaka, C. Release of γ-aminobutyric acid from cat colon. *Sci.* 1982. 217: 1038-1040.

163. Taniyama, K., Saito, N., Miki, Y. and Tanaka, C. Enteric γ-aminobutyric acid-containing neurons and the relevance to motility of the cat colon. *Gastroenterol.* 1987. 93: 519-525.

164. Timmermans, J., Barbiers, M., Scheuermann, D.W., Stach, W., Adriaensen, D., Mayer, B. and De Groodt-Lasseel, M.H.A. Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. *Ann. Anat.* 1994. 176: 515-525.

165. Tonini, M., Crema, A., Frigo, G.M., Rizzi, C.A., Manzo, L., Candura, S.M. and Onori, L. An *in vitro* study of the relationship between GABA receptor function and propulsive motility in the distal colon of the rabbit. *Br. J. Pharmacol.* 1989. 98: 1109-1118.

166. Tottrup, A., Svane, D. and Forman, A. Nitric oxide mediating NANC inhibition on opossum lower esophageal sphincter. *Am. J. Physiol.* 1991. 260: G385-G389.

167. Trezise, D.J., Kennedy, I. and Humphrey, P.P.A. Characterization of purinoceptors mediating depolarization of rat isolated vagus nerve. *Br. J. Pharmacol.* 1993. 110: 1055-1060.

168. Ward, S.M., Xue, C. and Sanders, K.M. Localization of nitric oxide synthase in canine ileocolonic and pyloric sphincters. *Cell Tissue Res.* 1994. 275: 513-527.

169. Waterman, S.A. and Costa, M. The role of ascending excitaory and descending inhibitory pathways in peristalsis in the isolated guinea-pig small intestine. *J. Physiol.* 1994. 481: 223-232.

170. Waterman, S.A. and Costa, M. The role of enteric inhibitory motoneurons in peristalsis in the isolated guinea-pig small intestine. *J. Physiol.* 1994. 477: 459-468.

171. Wattchow, D.A., Furness, J.B. and Costa, M. Distribution and coexistence of peptides in nerve fibers of the external muscle of the humen gastrointestinal tract. *Gastroenterol.* 1988. 95: 32-41.

172. Wechsung, E. and Houvenaghel, A. Effect of vasoactive intestinal polypeptide on porcine gastrointestinal electrical activity. *Peptides* 1994. 15: 1373-1376.

173. Wiklund, N.P., Leone, A.M., Gustafsson, L.E. and Moncada, S. Release on nitric oxide evoked by nerve stimulation in guinea-pig intestine. *Neuroscience* 1993. 53: 607-611.

174. Young, H.M., Furness, J.B., Shuttleworth, C.W.R., Bredt, D.S. and Synder, S.H. Co-localization of nitric oxide synthase immunoreactivity and NADPH diaporase staining in neurons of the guinea-pg intestine. *Histochem*. 1992. 97: 375-378.

175. Young, H.M., McConalogue, K., Furness, J.B. and De Vente, J. Nitric oxide targets in the guinea-pig intestine identified by induction of cyclic GMP immunoreactivity. *Neuroscience* 1993. 55: 583-596.

176. Ziganshin, A.U., Hoyle, C.H.V., Bo, X., Lambrecht, G., Mutschler, E., Baumert, H.G. and Burnstock, G. PPADS selectively antagonizes P_{2x}-purinoceptors-mediated responses in the rabbit urinary bladder. *Br. J. Pharmacol.* 1993. 110: 1491-1495.

177. Zimmerman, R.P., Gates, T.S., Mantyh, C.R., Vigna, S.R., Boehmer, C.G. and Mantyh, P.W. Vasoactive Intestinal Peptide (VIP) receptors in the Canine gastrintestinal tract. *Peptides* 1989. 9: 1241-1253.

178. Zimmermann, H. Signalling via ATP in the nervous system. *TINS* 1994. 17: 420-426.