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AN INVESTIGATION OF THE MECHANISMS BY WHICH OPIATES AFFECT THE MOTILITY OF THE GUT

A thesis presented for the degree of

Doctor of Philosophy

in the University of Glasgow

by

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DEDICATION

This thesis is dedicated to those scientists in the developing nations, who, despite all the odds are making meaningful contributions to the advancement of science.

SUMMARY

(1) The object of this study was to investigate the effect and mechanisms by which morphine and the opioid peptides affect gut motility. Emphasis was placed on the examination of the neuronal basis of these effects, particularly the involvement of a tonic non-adrenergic, non-cholinergic inhibitory mechanism postulated to be responsible for the suppression of myogenic activity and the release of 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) by these drugs. Alternative explanations of these effects were also sought.

(2) The preparation chosen for this study was the rat isolated colon, which permits demonstration of the responses to opioids and other drugs _in vitro_.

(3) The isolated colon of the rat contracts rhythmically to morphine and other opioid peptides. These rhythmic contractions could be divided into the initial contraction and the subsequent waves of contractions. The 5-HT antagonist, methysergide, non-competitively antagonised the initial response but had no effect on the waves of rhythmic contractions. In contrast, the specific opioid antagonist, naloxone, competitively antagonised the initial contraction and abolished the rhythmic contractile activity.

(4) The rhythmic waves of contractions were unaffected by pretreatment with parachlorophenylalanine (PCPA) which depleted the intestinal 5-HT as measured spectrofluorometrically. Contractions were still produced in tissues made subsensitive to 5-HT by a process of autodesensitisation and were not abolished by atropine, casting doubt on the 5-HT/ACh hypothesis. The ineffectiveness of reserpine in depleting the 5-HT content of the colon was also confirmed in the study.

(5) Several other drugs having in common the ability to block conductance in neural pathways or neuro-effector transmission, i.e. tetrodotoxin (TTX), apamin, tolazoline, phentolamine, oxprenolol and clonidine, produced similar patterns of rhythmic contractile activity in the rat colon. This suggested that the inherent myogenic activity of the colonic muscle might normally be suppressed by nervous influence.

(6) Electrical field stimulation of the colon provided evidence about the innervation of this tissue. It was demonstrated that there is a motor cholinergic response to nerve stimulation which was reduced or abolished by atropine or morphine and potentiated by 6-hydroxydopamine pretreatment or apamin. Indirect evidence for the presence of an inhibitory adrenergic influence was provided. The inability of adrenergic and cholinergic antagonists to block inhibitory responses of the colon to nerve stimulation provided evidence for the existence of non-adrenergic, non-cholinergic (NANC) inhibitory nerves in the colon. In addition, the optimum frequency of stimulation of the inhibitory response was less than that characteristic of either an adrenergic or cholinergic mechanism.

(7) The observation that this NANC inhibitory, nerve-mediated response to electrical field stimulation could still be elicited in the presence of drugs producing rhythmic waves of contractions, made it unlikely that the removal of a non-adrenergic, non-cholinergic inhibitory mechanism was responsible for producing the rhythmic contractile activity in the colon.

(8) The similarity between the effects of the opioids, the adrenergic neurone blocker and adrenoceptor antagonists, clonidine and apamin, raised the possibility that the actions of these drugs might be mediated through adrenergic neurones. This possibility was examined using the techniques of High Performance Liquid Chromatography (HPLC) with electrochemical detection and also in tritium efflux studies.

(9) Preliminary experiments with the HPLC were concerned with the optimisation of the conditions necessary for chromatographic separation. It was demonstrated that changes in the electrode potential voltage, mobile phase composition and flow rate affected the detection and separation of catecholamines. The catecholamine content of the rat colon, mouse, guinea-pig and rat vasa deferentia were also measured. Transmitter overflow from the mouse and guinea-pig vasa deferentia occurring spontaneously and in response to electrical field stimulation were measured. No spontaneous release of noradrenaline or its metabolites was demonstrated in the rat colon.

(10) Morphine, clonidine and TTX did not affect tritium efflux at concentrations at which they produce rhythmic waves of contractions in the colon.

(11) The implications of these results for the hypothesis previously postulated and the one suggested in this study to explain the rhythmic contractions are discussed.

INTRODUCTION

The importance of the gastro-intestinal tract to the body's homeostatic control mechanisms cannot be over-emphasised. For contraction of intestinal muscle, integrated and co-ordinated by the nervous system, promotes the transport of ingested foodstuff along the alimentary tract and in the process ensures adequate mixing and exposure to absorptive surfaces (Hirst, 1979). The mammalian gastro-intestinal tract handles ingested materials in different ways, adapting its response to the different characteristics of food consumed, thereby ensuring efficient utilisation of nutrient for the body's homeostatic mechanisms.

It is no exaggeration to say that higher animals are the differences between what their small intestines absorb and their kidneys excrete. With the exception of oxygen, every substance gains entrance across a digestive - absorptive surface. The movement of material within this lumen is complex, and unlike the cardiovascular system, the intestine acts both as the conduit and the pump, transporting its content in an aboral direction (Weisbrodt, 1981).

The small intestine is the most-studied part of the gastro-intestinal tract (Weisbrodt, 1981). The reason for this may perhaps be the easier accessibility of the small intestine compared to the colon or as bluntly put by Spior (1975):-

> "The small intestine after all,
> provides the main reason for
> the existence of the gut, all
> the rest is a prologue or
> epilogue, for man can live
> without his stomach or
> oesophagus and may thrive
> without his colon."

However, this judgement may be too harsh. Although the mammalian colon is not actively involved in absorbing the principal products of our foodstuffs such as sugar, amino acids, small peptides (Phillips, 1969). The human colon is nevertheless responsible for the final modification of the 500-1,000 ml of fluid that enters it daily and in so doing appears to be responsive to body's requirements (Phillips, 1969; Cummings, 1975; Schultz, 1981).

It is difficult to determine when the first studies of intestinal motility were conducted. According to previous reviews (Texter, 1964; Christensen, 1971; Bortoff, 1972; Daniel & Sarna, 1978), detailed investigation began about the middle of the last century. It is, however, apparent that the study of motor activity of the gastro-intestinal tract has had a long and distinguished history. Despite the long interest in the study of gastro-intestinal motility, the interaction of the factors controlling motility are in many ways still poorly understood. The statement by Bayliss and Starling (1899) that:

> "On no subject of physiology do we meet so many discrepancies of facts and opinion as in the physiology of the intestinal movement"

is even true today.

The movements of the intestinal muscle depend on three primary factors:-

(a) The intrinsic properties of the musculature itself (myogenic factors)

(b) Intrinsic and extrinsic nervous influences

(c) Local and systemic chemical control

Myogenic factors are properties intrinsic to the smooth muscle that are involved in control of contraction. They include the electrical

activities of smooth muscle cells, the communication between muscle layers, the metabolism and transduction of energy in smooth muscle and the way in which the myo-electric events are superimposed on the energy metabolism of muscle to produce contraction (Weisbrodt, 1981). Intestinal muscle, like cardiac muscle, is capable of generating electrical signals that are responsible for both the initiation and integration of contraction.

Integration of contraction is achieved by a slow electrical transient (a slow change in the electrical properties of the membrane) called the electrical slow wave, while the initiation of contraction is accomplished by a burst of much more rapid electrical activity called the spike burst or action potential (Tomita, 1981). Smooth muscle may also generate brief potentials known as prepotentials (Gillespie, 1968). These slow waves defined as spontaneous, slow, periodic fluctuations in transmembrane potential of smooth muscle cells (Papsova, Nagai & Prosser, 1968; Prosser & Bortoff, 1968) have been given various names such as basic electrical rhythm (Bass, Code & Lambert, 1961), pace-setter or synchronizer potentials (Code & Carlson, 1968), basic organ specific rhythm (Golenhofen & Lammel, 1972), control potentials (El-Sharkawy & Daniel, 1975a). The generation and propagation of slow waves occur in the presence of tetrodotoxin or atropine and thus these processes are likely to be myogenic (Tomita, 1981). They are responsible both for the rhythmicity and polarity of intestinal contraction (Bortoff, 1976).

There appear to be considerable regional and species differences in the frequency, amplitude and the rate of propagation of slow waves (Prosser & Bortoff, 1968; Ruckebusch & Fioramonti, 1975; Stoddart & Duthie, 1968;

McCoy & Baker, 1979). The site of origin of slow waves in the small intestine differs from that in the colon.

In the small intestine, slow waves are generated by the longitudinal muscle and spread electrotonically into the circular layer (Bortoff, 1961; Kobayashi, Nagai & Prosser, 1966), while in the colon, they are generated by the circular muscle and spread into the longitudinal muscle (Christensen, Caprilli & Lund, 1969; Caprilli & Onori, 1972). The muscle cells of the two layers seem to be electrically coupled by means of low resistance pathways provided by nexus or gap junctions (Bortoff, 1976). Slow waves function not only to control the pattern of contractions at any one locus but also influence the pattern of adjacent loci. They give rise to both segmenting (localised) and peristalsis (propagated) contractions (Bortoff, 1976).

The second type of electrical activity which can be recorded from intestinal muscle is the spike potential. Spike potentials are rapid membrane depolarizations, which occur primarily during the depolarising phase of the slow waves. Each spike is followed by a small increment of tension and the frequency of spike discharge determines the degree of tension development (Bulbring, 1955). Bozler (1945) was first to suggest that spontaneous fluctuations in membrane potential, associated with mechanical changes, could result from fluctuations in cellular metabolism. Although the process involved remains to be clarified, it appears that a smooth muscle may have more than one mechanism for generating oscillations in membrane potential (Prosser, 1973; Tomita & Watanabe, 1973a).

The interaction of electrogenic transport systems and/or slow voltage-dependent changes in ionic conductance appear to be primarily reponsible

(Connors, Prosser & Weems, 1974). Bolton (1971, 1972), however, showed that in the guinea-pig, the intermittent release of acetylcholine may be involved in the initiation of rhythmic activity.

An increment in intracellular calcium (Ca^{++}) concentration is related to the appearance of spikes and is considered to be due both to its release by intracellular stores and to a rapid calcium influx from outside the cell (Job, 1969; Syson & Huddart, 1973). Smooth muscles vary in their requirement for extracellular Ca^{++}; for example, the taenia coli is almost totally dependent on extracellular Ca^{++} and rapidly ceases contracting in a Ca^{++}-free media. In contrast, some vascular smooth muscles, e.g. rabbit main pulmonary artery which utilise principally intracellular stores of Ca^{++}, remain functional for a considerably longer period of time in Ca^{++}-free media (Devine, Somlyo & Somlyo, 1972).

The gut receives its extrinsic innervation from both branches of the autonomic nervous system. Sympathetic nerves are inhibitory except for those to the sphincters whereas parasympathetic nerves contain two distinct nerve fibre populations, one excitatory and the other inhibitory (Youmans, 1968). The sympathetic post ganglionic fibres to the gut end mainly at the intramural nerve plexuses, mostly the myenteric plexus with few if any adrenergic fibres visible within the layer of smooth muscle (Norberg, 1964; Jacobowitz, 1965; Costa, Furness & Gabella, 1971; Furness & Costa, 1974).

This observation may, however, be species-dependent. In the guinea-pig caecum both the longitudinal (taenia coli) and circular muscle are profusely innervated. Fluorescent adrenergic fibres are also quite common among the muscle cells of the small and large intestine of rabbits and rats (Holland & Vanov, 1965) although Gillespie (1968) could not confirm the

innervation of the colonic musculature reported by Holland and Vanov in rabbit or guinea-pig.

The concentration of noradrenergic axons within the enteric plexuses has led to a radical change of the view of how these axons actually affect gastro-intestinal motility. The concept of reciprocal control of motility by inhibitory adrenergic and excitatory cholinergic fibres may not be correct. Norberg and Sjoqvist (1966) proposed that the action of the sympathetic transmitter is an indirect one, to cause the inhibition of excitatory ganglionic transmission rather than a direct relaxant action on smooth muscle. The relaxation thus produced was due to removal of an excitatory cholinergic tone (Paton & Visi, 1969; Wikberg, 1977; Gershon & Erde, 1981). It would appear that adrenergic fibres terminating at the intramural ganglion cells are primarily concerned with modulation of local reflex activity (Furness & Costa, 1974).

That the intestinal muscle can function independently of its extrinsic innervation is evident from continued activity of intestine after both vagotomy and sympathectomy (Kosterlitz, 1968).

This relatively benign result of severing the extrinsic intestinal innervation cannot contrast more with the intestinal obstruction and the inability of the bowel to propel luminal content that occur when a segment of the gut is aganglionic as a result of congenital defects in Hirschsprung's disease (Bodian, Stephens & Ward, 1949) and in certain piebald and spotted strains of mice (Bolande, 1975). The aganglionic segments are not denervated as they contain both cholinergic (Kamijo, Halt & Koelle, 1953) and adrenergic axons (Bennett, Garrett & Howard, 1968; Gannon, Noblett & Burnstock, 1969; Garrett, Howard & Nixon, 1969). The

absence of ganglion cells and the processes of at least some of the intrinsic enteric neurones reduce the activity of the local nervous system or enteric nervous system (E.N.S.). The E.N.S. it would seem, is apparently more involved in regulating the motility of the bowel than are the brain and spinal cord. Ultrastructural and electrophysiological studies of enteric neurones indicate synaptic mechanisms similar to those of the central nervous system. Both acetylcholine (ACh) (Dale, 1973) and noradrenaline (NA) (Finkleman, 1930; Gillespie & Mackenna, 1961) are enteric neuro-transmitters but they are not the only ones. Electrophysiological, histochemical and immunocytochemical studies have demonstrated the presence of nerves utilising transmitters other than NA and ACh (Burnstock, 1972; Gershon & Erde, 1981). Transmission in such nerves has been given the negative, cumbersome but descriptive name non-adrenergic, non-cholinergic (NANC).

In retrospect, pharmacological evidence for control of intestinal motility by transmitters other than NA and ACh abound in the literature. As early as 1898, Bayliss and Starling reported that in the dog small intestine, vagal stimulation caused relaxation followed by a powerful contraction and neither component was abolished by atropine.

The inhibitory response to transmural nerve stimulation in the guinea-pig, kitten or mouse stomach were also unaffected by atropine and adrenergic neurone blocking agents (Paton & Vane, 1963). Such responses have also been reported in the stomach, duodenum, ileum, caecum and colon, in the lower oesophageal, pyloric, ileo-caecal and internal anal sphincters. These reports have been extensively documented (Burnstock, 1972; Burnstock, 1979; Gillespie, 1982).

The study of NANC transmission has revealed new putative neurotransmitters in the peripheral nervous system, especially the E.N.S. The idea of there being a bipartite division of the ANS with only two opposing transmitters ACh and NA seems misguided today as an oversimplification (Gershon & Erde, 1981). However, although the literature abounds with putative neuro-transmitters, established neurotransmitters are far less common. A formidable case has been made for adenosine triphosphate (ATP) or related nucleotides by Burnstock (Burnstock, 1972; Burnstock, 1975; Burnstock, 1979) but the evidence is not impregnable (Gillespie, 1982).

For example, rabbit distal colon and rat stomach are supplied by non-adrenergic inhibitory nerves but in these tissues ATP produces a contractile response (Burnstock, Campbell, Satchell & Smythe, 1970; Mackay & McKirdy, 1972). Weston (1973) also showed that in the guinea-pig ileum, stimulation of the non-adrenergic inhibitory nerves in the longitudinal muscle either directly or as part of the peristaltic reflex produced an inhibitory response, unaffected by the presence of large desensitising doses of ATP. Moreover, the concentration of ATP sometimes required to produce an effect was occasionally very high (Ambache & Zar, 1970; Ambache, Killick & Woodley, 1977). Rapid breakdown of ATP to adenosine may not be a sufficient reason to explain this discrepancy (Ambache et al, 1977). Other putative neurotransmitters include 5-hydroxytryptamine (5-HT), vaso-active intestinal peptide (VIP), substance P, somatostatin, enkephalin, neurotensin and gama aminobutyric acid (Furness & Costa, 1982). It seems likely that more than one neurotransmitter may be involved in transmission of the wide array of responses gathered under the umbrella of NANC transmission.

I would like to end this section on putative neurotransmitters with the cautionary note voiced by Charles F. Code (1982):-

> "Finally the perspective of years should provide some warnings, of past mistakes to be avoided, things along the road of progress to be wary of. In the area of chemotransmitter substances, I see a prospective complication, a cloud in the distant horizon. Experience has led me to the conclusion that in the development of a species, the forces at work tend to keep all options open.
>
> A mechanism, a chemical compound useful at one stage in the evolutionary process but superseded by another may exist in vestigial form. It becomes a reminder of the past, a footprint on the way, an option available but little used. Do some of the chemotransmitters being identified these days in the gut represent such vestiges of the past? It is a disturbing thought. It could be true."

The importance of another control mechanism in the regulation of intestinal muscle motility, namely the role of locally-released and systemic hormones, has been recognised. In addition to their effect on secretion (Burks, 1976), gastrin, cholecystokinin, caerulein and secretin all have diverse effects on gastrointestinal motility (Walsh, 1981). Gastrin can increase the tone of the lower oesophageal sphincter (Cohen & Lipshutz, 1971), increase both the antral slow waves and force of contraction (Cooke, Chvasta & Weisbrodt, 1972) and in relatively high doses increase spike bursts and contractile activity of intestine (Waterfall, Duthie & Brown,

1973). Secretin reduces gastric motility, delays gastric emptying, decreases intestinal motility and causes relaxation of the lower oesophageal sphincter (Waterfall _et al_, 1973). Cholecystokinin and caerulein also exhibit diverse effects on gastro-intestinal motility (Burks, 1976). They are potent stimulants of gall bladder contraction and relaxation of the sphincter of Oddi (Lin, 1975). Cholecystokinin causes relaxation of the human lower esophageal sphincter and has been shown to antagonise the contracting action of gastrin. It is also a strong stimulant of pancreatic enzyme secretion _in vivo_ (Walsh, 1981).

Other hormones may also have profound effects on intestinal musculature. Clinicians have for a long time been aware of a link between gastro-intestinal symptoms and thyroid disorders. Hyperthydroidism is characterised by increased intestinal motility while hypothyroid states can induce atony and intestinal obstruction (Middleton, 1971). It both Addison's disease and severe diabetes mellitus, gastro-intestinal symptoms are common and may include diarrhoea (Truelove, 1966). Thus it would appear that the systemic hormones may have a general role in regulating the responsiveness of smooth muscle to neural and possibly local chemical control (Gibson, 1981).

In summary, it thus becomes clear that there are many potential mechanisms for control of intestinal motility. It seems likely that all these mechanisms operate simultaneously and their operations are integrated with one another to produce the various patterns of contraction seen in the intact animals. It is only by studying these mechanisms separately and in the presence of other related mechanisms that we can ever hope to gain complete understanding of what controls intestinal motility.

<u>Opiates and the gastro-intestinal tract</u>

The discovery in both brain and gut of specific opioid receptors that mediate opiate activity has inspired intense research interest (Pert & Synder, 1973; Ambinder & Schuster, 1979; Konturek, 1980). The reasons for the interest are probably three-fold. First, better understanding of the pharmacology of the opiates in the intestine may improve our knowledge of the physiological control of motility. Secondly, such studies may provide a better understanding of the relationship between neurotransmitter functions and the possible endocrinological role of the opioids. Thirdly, such studies may elucidate the mechanisms involved in opiate withdrawal. With the discovery of opioid receptors, it was apparent that some endogenous substances, different from any known neurotransmitter, could exist and that these might bind to those receptors. That such substances exist was first demonstrated in pig brain extract by Kosterlitz and his colleagues (Hughes, Smith & Kosterlitz, 1975). Two penta-peptides were identified. These two enkephalins differed only by the presence of a methionine or leucine residue at their C-terminus. Pituitary extracts were also found to have opioid activity (Goldstein, 1976). This activity resides in three long-chain polypeptides known as alpha, beta and gamma endorphins, all of which were derived from beta-lipotropin discovered in the mid-1960s (Li, Barnafi, Chretien & Chung, 1965).

Radio-immunoassay and immunocytochemistry techniques have also demonstrated the presence in humans, of enkephalins in nerve fibres of myenteric plexuses of the stomach, intestine, gall bladder and cystic duct as well as in special endocrine cells (Amine Precursor Uptake and Decarboxylase (APUD)) in the gastric, antral and duodenal mucosa as well as the pancreas (Polak, Sullvan, Bloom, Facer & Pearse, 1977). Similar techniques have

been employed in various species to demonstrate enkephalinergic nerves in the myenteric plexus and circular muscle (Elde, Hokfelt, Johansson, Terenius, 1976; Schultzberg, Dreyfus, Gershon, Hokfelt, Elde, Nilsson, Said & Goldstein, 1978; Furness & Costa, 1980). In contrast to most of the other brain - gut peptides such as bombesin, neurotensin, and substance P, many of the central and peripheral actions of the opioids are indirectly familiar because of the remarkable similarity between their biological activities and those of the opium alkaloids and their congeners, which have been subjected to physiological and pharmacological scrutiny since the 1800s.

Opium and later morphine have been used through the ages for relief of diarrhoea and dysentery, and investigation of the mechanism by which morphine and its surrogates exert their constipating action dates from the 19th century. These studies have been reviewed several times (see Krueger, 1937; Vaughan Williams, 1954; Kosterlitz & Lees, 1964; Weinstock, 1971; Daniel, 1982; Furness & Costa, 1982). A number of factors complicate analysis of the action of opiate agonists. First, the effect of morphine on the motility of the intestinal tract is dependent on the species, the dose, the region of intestine under investigation, and the choice of experimental method (Reynolds & Randall, 1957). Secondly, propulsion is modified both by central actions of morphine and by its direct effect on the intestine. Thirdly, the ileum of the guinea-pig which is the organ most extensively examined (e.g. Kosterlitz & Lees, 1964; North & Tonini, 1977; North, Katayama & Williams, 1979) is clearly not a universal model for investigating the effect of opiates on the gut motility. In the guinea-pig ileum, opiates inhibit acetylcholine release but in most species, including humans, there is no evidence that morphine or other opiates act by inhibiting acetylcholine release (Daniel, 1982).

Morphine and related drugs relax the smooth muscle of the distal portion of the oesophagus both in normal individuals and in patients with diffuse oesophageal spasm (Schmidt, 1939). They also exert profound effects on gastric motility, tone, peristalsis and emptying, increasing the amplitude but decreasing the rate of gastric emptying. Electrical activity in terms of slow waves and spike activity are also increased by morphine and the enkephalins (Silbiger & Donner, 1968). Early investigators attributed this delay to pylorospasm (Krueger, 1937) but duodenal spasm is now generally accepted as the mechanism of delay (Konturek, 1980). Plant and Miller (1926) originally demonstrated that the primary effect of morphine on the intestine of man and dog was an increase in muscular tone, frequency and amplitude of peristaltic waves and an increase in the amplitude but a decrease or no change in the frequency of the rhythmic contraction. After a time, the frequency of the peristaltic waves decreased while the tone remained high. These observations have been repeatedly confirmed using a variety of experimental techniques (Kreuger, 1937; Silbiger & Donner, 1968). Early radiological studies showed delayed passage of radio-opaque contrast materials, demonstrating the non-propulsive nature of the small intestine motility induced by morphine (Pancoast & Hopkins, 1915). Studies in patients with ileostomies in which morphine produces a marked reduction in collected ileal effluent, also provide further evidence (Adler, Atkinson & Ivy, 1942).

In contrast to other mammalian species, gastro-intestinal tone and contractile activity are consistently diminished in the guinea-pig (Schultz, 1978) thus emphasizing species differences in the nature of response to morphine.

Plant and Miller (1928) observed that the most pronounced and lasting effect of morphine in the human colon was an increase in tone. Morphine causes an increase in basal luminal pressure and stimulates segmenting motility. This segmenting motility has been invoked as the principal explanation for morphine's constipating action. Painter and Truelove (1964) and Garrett, Sauer and Moertel (1967) reported increased sensitivity to morphine in patients with diverticulosis or ulcerative colitis. The high pressure caused by morphine in such colons may be a factor contributing to perforation in ulcerative colitis.

In dogs and cats, intravenous administration of morphine produces marked and sustained contractions of internal and external anal sphincters. These contractions were not affected by high thoracic transection of the cord or vagotomy, suggesting that the effect may be peripheral (Koppanyi & Murphy, 1933).

Many studies have sought to explain the mechanism of the gastro-intestinal effects of morphine. The constipating effect of morphine has been attributed variously to its effect on gastric emptying, small intestinal and colonic motility. Morphine and the enkephalins may also act on the CNS to reduce the urge to defaecate in spite of accumulation of faeces in the large bowel (Jaffe & Martin, 1980). The intestinal mucosa may be an additional peripheral site at which opiates can produce their anti-diarrohea effect, unrelated to changes in intestinal motility (Powell, 1981). _In vitro_ studies have shown that opiate agonists can stereo-specifically enhance electrolyte absorption by the intestinal mucosa and that this effect is naloxone-sensitive (Racusen, Binder & Dobbins, 1978; McKay, Linaker & Turnberg, 1981). Electrolyte absorption would also promote water reabsorption by the mucosa, decreasing the volume of intraluminal content and faecal output.

Controversy still exists as to whether the primary site of action for the effects of morphine in the intestine is central or peripheral. For example, the spasmogenic response of the small intestine has been attributed to an action of morphine directly on the smooth muscle or indirectly on Auerbach's plexus (Plant & Miller, 1926, 1928; Burks & Long, 1967a,b; Burks, 1973, 1976).

The former explanation seems likely since this excitatory effect persisted after administration of ganglionic blockers (Vaughan Williams & Streeten, 1950), decapitation (Burks, 1976) and vagal sectioning (Stahl, Van Bever & Janssen, 1977). A peripheral mechanism has also been suggested by studies in which low doses of morphine given intraperitoneally inhibited movement along the intestine of an orally administered charcoal meal. The same doses of morphine administered intravenously had no effect on gastro-intestinal transit (Tavani, Bianchi, Geretti & Manara, 1980).

A peripheral mechanism is also suggested by the antagonistic effect of a quaternary analogue of nalorphine, diallyl-morphine, on morphine-induced slowing of intestinal transit without affecting morphine-induced analgesia (Tavani et al, 1979). The effectiveness of loperamide, which exerts peripheral opiate activity without any central effect, in the control of diarrhoea is also a strong argument in favour of a peripheral site of action (Stahl et al, 1977).

Morphine-induced changes in gastro-intestinal motility resulting from an effect within the CNS have been reported in a number of species including rat (Margolin, 1963; Parolaro, Sala & Gori, 1977; Stewart, Weisbrodt &

Burks, 1977; Schultz, Wuster & Hertz, 1979; Galligan & Burks, 1983), cat (Stewart et al, 1977) and dog (Bueno & Fioramonti, 1982). Evidence for a central site of morphine action in the rat is based on the ability of low doses of morphine given intracerebrally to inhibit intestinal transit while much larger systemic doses are required to produce a comparable anti-transit effect.

Burleigh, Galligan and Burks (1981) used a quaternary opiate receptor antagonist, diallyl-morphine, to block central opioid receptors. The intestinal effects of subcutaneously administered morphine were inhibited by this pretreatment, whereas diallyl-morphine did not alter the intestinal effect of centrally-administered morphine. The spinal cord has recently been identified as an additional site, where opioids act to influence gastro-intestinal motor activity (Porreca & Burks, 1983). Thus, the relative contributions of centrally- and peripherally-mediated constipating effects remain unresolved.

Mechanism of action of morphine on gastro-intestinal tract

With the possible exception of the guinea-pig ileum, where opiate agonists inhibit peristalsis and reduce the spontaneous and stimulated release of acetylcholine, morphine and the enkephalins have spasmogenic effect on isolated pieces of gut *in vitro* (Weinstock, 1971). The most thoroughly studied species are the dog and rat. In the dog, Burks and Long (1967a) and Burks (1973, 1976) have demonstrated that the direct excitatory action of morphine and related agents is due to the release of 5-HT (from an unknown source) which, in turn, stimulates cholinergic nerves that act upon the muscle. The involvement of 5-HT was deduced from the observations that 5-HT antagonists, cyproheptadine and cinnaserin, inhibit the motor response to 5-HT and morphine. Also pretreatment with reserpine, which supposedly depletes the tissue level of 5-HT, decreases the motor response to morphine but not exogenous 5-HT. Daniel, Gonda, Donnoto, Oki and Yanaihara (1981), however, found that doses of 5-HT sufficient to produce tachyphylaxis did not affect the response to enkephalin. Thus the role of 5-HT in the action of opiates in the dog intestine may require further studies.

In the isolated colon of rat intestine, morphine and opioid peptides were spasmogenic (Kaymakcalan & Temelli, 1964; Weinstock, 1971; Gillan & Pollock, 1980; Nijkamp & Van Ree, 1980; Scheurer, Drack, Varga & Halter, 1981; Huidobro-Toro & Way, 1981; Moritoki, Takei, Kotani, Kiso, Ishida & Endoh, 1984). In addition to this excitatory effect, the opiates also produce rhythmic waves of contractile activity *in situ*, in the pithed rat and in isolated segments of the colon.

The mechanisms underlying this motor action is unknown but two hypotheses have been postulated to explain it. The first, similar to the one proposed for the dog intestine, suggests that morphine has an excitatory effect because it releases ACh and 5-HT, which then act on intestinal smooth muscle to cause it to contract (Burks, 1976). The second hypothesis suggests that opiates inhibit a tonically-active neural inhibitory mechanism that normally suppresses myogenic activity. Thus opiates acting at a pre-synaptic site to inhibit the tonic release of an inhibitory transmitter could reveal myogenic activity that is normally restrained by this tonic inhibitory influence (Gillan & Pollock, 1980). The nature of this inhibitory influence is still equivocal. Gillan and Pollock (1980) suggested that it is a non-adrenergic, non-cholinergic influence whereas Nijkamp and Van Ree (1980) suggested the adrenergic mechanism as the inhibitory influence. In addition, Gillan and Pollock (1980) proposed as an alternative hypothesis a direct excitatory effect of opiates on intestinal smooth muscle.

Weaknesses exist in these hypotheses, e.g. the ACh/5-HT hypothesis is contradicted by evidence that morphine causes the colon to contract in the presence of an ACh antagonist and in tissue rendered unresponsive to 5-HT by repeated exposure to 5-HT (Gillan & Pollock, 1980). The 5-HT hypothesis also does not explain why morphine is constipating and 5-HT generally is not. The myogenic hypothesis was based on the proposition by Wood (1972, 1975) and Tonini, Secchinini, Frigo and Crema (1974) that intrinsic neurones are tonically active in intestine, that this activity is predominantly inhibiting and that the natural state of intestinal muscle after withdrawal of this inhibition is electrical activity with contraction. The data cited to support this hypothesis, which include

increased muscle motility after administration of tetrodotoxin (TTX), atropine and local anaesthetic, have been interpreted on the basis of two assumptions (a) that the excitatory effects of these agents result from withdrawal of nervous inputs and (b) that these drugs inhibit release of nerve transmitters or the effects of these transmitters. So far no-one has found such strips to have continous release of mediators or to have junctional potentials affecting most circular smooth muscle cells as this hypothesis requires (Daniel & Sarna, 1978).

It becomes obvious that in spite of the many extensive studies that have been published, there are many gaps yet to be filled before a clear and complete account can be given of the effect of morphine on the gastro-intestinal tract.

This study, therefore, examined these two hypotheses and sought to answer the following questions:-

Are the excitatory effects of opioids in the colon affected when the intestinal content of 5-HT is reduced by pretreatment of rats with parachlorphenylalanine (PCPA) to inhibit the synthesis of 5-HT?

Are the excitatory effect of opioids in the colon inhibited by 5-HT- and ACh-antagonists?

How similar are the effects of opioids and TTX which blocks sodium conductance and consequently abolishes the nerve action potential, and apamin which inhibits inhibitory mechanisms that depend on increased potassium permeability in smooth muscle?

Could clonidine, reported to possess anti-diarrhoea activity (Shearman, Lal & Ursillo, 1980; Lal, Shearman & Ursillo, 1981; Lal & Shearman, 1981) through α_2-receptor stimulation, produce rhythmic waves of activity similar to morphine?

What is the nature of the hypothetical neural inhibitory mechanism that is inhibited by morphine to reveal myogenic activity in the colon?

MATERIALS and METHODS

Recording Responses of Rat Isolated Colon

Male Wistar rats (230-300 g) were stunned and killed by bleeding. From each rat, one 3-4 cm length of terminal colon was excised, emptied of contents and suspended in an organ bath containing 20 ml of Krebs' bicarbonate solution (mM NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 2.5 and glucose 11.1), maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. The lower end of the colon was anchored to the hook of a ring electrode, and the upper end attached by a thread to a Grass FT03 force-displacement transducer mounted vertically above the organ bath. The initial resting force of 2 g applied to each tissue gradually fell to 1 g during the 30 minute equilibration period, throughout which the Krebs' solution was changed at 15 minute intervals but no drugs were added to the organ bath. Responses were recorded isometrically and displayed on a Grass Polygraph.

Field Stimulation of the Isolated Colon

The responses of the colon to electrical field stimulation of the intramural nerves and the effects of various drugs on these responses were investigated. Segments of colon, suspended in organ bath containing Krebs' solution (37°C) were stimulated electrically through silver ring electrode with square wave pulses of supramaximal voltage, 0.5 ms duration and variable frequency (1-50 Hz) supplied either by a Palmer stimulator or a Grass S88 stimulator.

Assay of 5-Hydroxytryptamine (5-HT)

The 5-HT content of the colon was assayed flurometrically using o-phthalaldehyde (OPT), which forms a fluorescent complex with 5-HT (Curzon, Kantamaneni and Tricklebank, 1981).

Control rats and rats pretreated with either parachlorophenylalanine (PCPA) or reserpine, were stunned and killed by bleeding. From each rat, a segment (0.5 g) of terminal colon was removed, dissected free of mesentery and blood vessels and transferred to cold Krebs' solution. Each segment of colon was homogenised in 5 ml of acidified butanol (850 µl HCl in 1 litre butanol, $0^{o}C$) in a glass-tube by a motor-driven teflon pestle. The volume of each homogenate was adjusted to 25 ml and the homogenate centrifuged (3,000 g, 10 min, $4^{o}C$).

The 5-HT content of each supernatant was determined by a slightly modified version of the method of Curzon and Green (1970). The modification was necessary for the preparation of tissue blank. A tissue blank was prepared by adding 10 µl of potassium ferricyanide (0.2% W/V) to an aliquot of the supernatant. This procedure oxidises all of the 5-HT present (Anden & Magnuson, 1967).

A 2 ml aliquot of each supernatant was transferred to a tube containing 5 ml of n-heptane and 600 µl of an acid solution of cysteine (1% W/V in HCl 0.1N). The contents of each tube were mixed for 2 minutes and centrifuged (3,000 g, 5 min, $4^{o}C$).

From each tube the upper organic phase together with the disc at the organic/aqueous interphase were removed by suction and discarded. A 200 µl aliquot of the lower acidic aqueous phase was incubated for 15 mins at $77^{o}C$ with 20 µl 1% cysteine and 800 µl conc HCl containing 0.004% OPT. The fluorescence that developed was measured, when the tubes had cooled to room temperature, in an Aminco-Bowman spectrophotofluorimeter at an activation wavelength of 370 nm and an emission wavelength of 480 nm.

Standards were prepared by dissolving 5-HT in distilled water so that 200 μl volume containing between 50 and 200 ng 5-HT could be added to tissue extracts to serve as internal standards, which were carried through the entire assay procedure. The relationship between concentration and fluorescence that developed was linear (Figure 1).

Radioactive (^{3}H)-L-Noradrenaline Experiments

(1) Incubation procedure and ^{3}H efflux

The rat terminal colon was dissected out, free of adhering blood vessels. Each tissue (2-3 cm) was then incubated at 37°C in Krebs' solution containing 444 KBq/ml of (^{3}H)-NA and incubated for 20 minutes at 37°C. Uptake of (^{3}H)-NA was stopped by transferring tissues from the amine-containing solution into tubes containing normal ice-cold Krebs' solution.

Having washed off the extracellular and loosely bound radioactivity in the cold Krebs' solution, the colon was then set up for in vitro recording of mechanical activity as previously described. Krebs' solution bathing the tissue was removed and replaced at ten-minute intervals and was dissolved in 10 mls of toluene-Triton X scintillant. The radioactivity present in these samples was counted in a liquid scintillation spectrometer. The effects of drugs on the efflux from and mechanical activity of the colon were determined by adding drugs from the 80th minute of incubation onwards.

The ^{3}H remaining in the tissue at the end of each experiment was determined by digesting the tissue in 1 ml of 4M KOH at 60°C for 1 hour before adding 10 ml of toluene Triton X scintillant and counting.

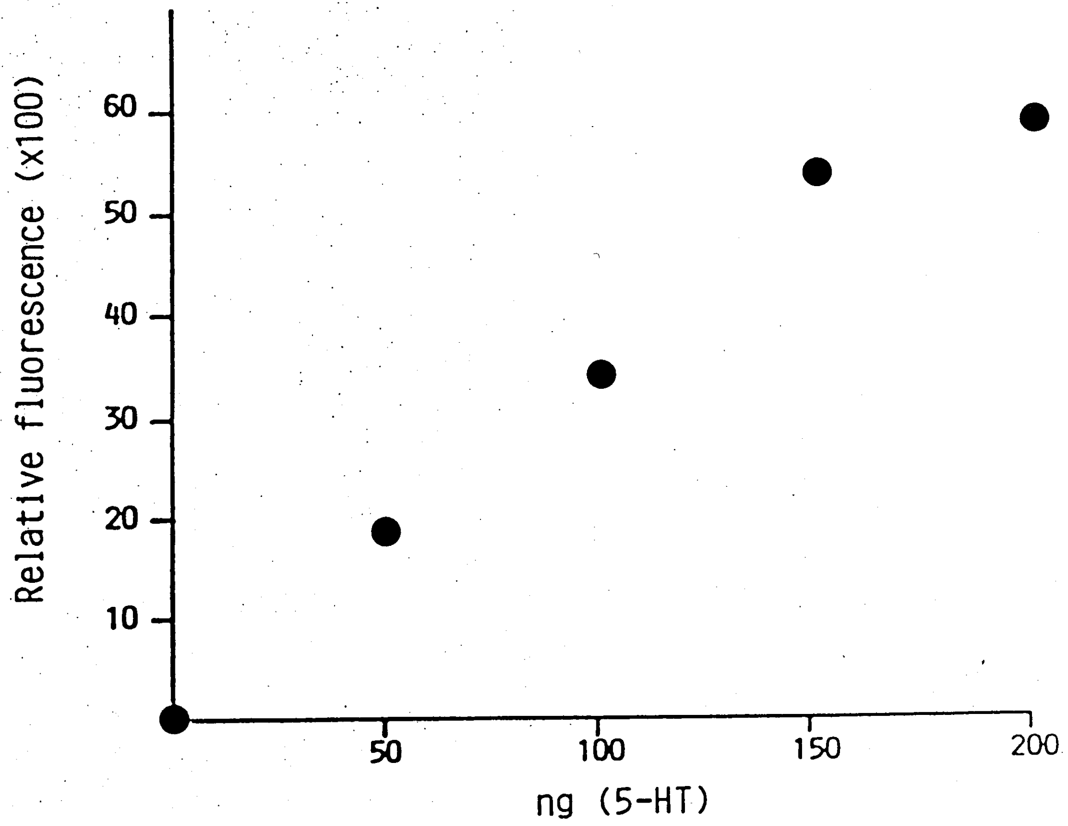


Fig. 1: Standard graph for 5-hydroxytryptamine (5-HT)

Efflux was expressed as fractional tritium release of the total tritium content of the tissue. No correction for tritium in the extracellular space was made.

(2) (^{3}H) Uptake by the rat colon

The uptake of (^{3}H)-NA by the rat colon was carried out essentially as described by Hermann and Graefe (1977) for isolated rat tissues. The colon, dissected free of adhering blood vessels was blotted dry, cut into pieces and weighed. Each tissue sample was mounted on the tip of a stainless steel rod and incubated at 37°C in tubes containing Krebs' solution.

After 30 minutes, tissues were transferred into media containing 444 KBq/ml of (^{3}H)-NA for various time intervals (1-60 minutes). Uptake of (^{3}H) was stopped by transferring the tissues from the amine-containing solution into tubes containing ice-cold Krebs' solution and by chilling (0°C) them for 1 minute. Thereafter the tissues were rapidly removed from the stainless steel rod and blotted dry. Each tissue was digested in 1 ml of 4M KOH at 60°C for 1 hour. Toluene scintillant was added to each vial and the radioactivity counted by a liquid scintillation counter. Uptake was expressed as activity per mg tissue. Correction for quenching was carried out by plotting external standard ratio (E.S.R.) against counting efficiency and sample counts were converted from CPM to DPM.

High Performance Liquid Chromatography Experiments

(1) Chromatographic System

The technique of High Performance Liquid Chromatography with electrochemical detection was used to measure the catecholamine contents

of the rat colon and the vasa deferentia of the guinea-pig, rat and mouse. Both spontaneous and electrical-stimulation-induced release of noradrenaline from the vasa deferentia of the guinea-pig and mouse were similarly measured.

The apparatus consisted of a pressure pump (Laboratory Data Control Model 709) with a pulse dampener to deliver solvent to the column at precise flow rates with a relatively pulse-free output at pressures up to 1,200 p.s.i., an injection valve Rheodyne 7125 (Berkeley, CA USA) with a 100 µl loop, a stainless steel reverse-phase Hypersil column (150 x 5 mm 1D) prepacked with octadecyl bonded silica (HPLC Technology) and an electrochemical detector combined with a battery or mains operated potentiostat amplifier. The detector was operated at +0.55 or +0.7 V with a glassy carbon working electrode, a platinum wire auxillary electrode and a silver/silver chloride reference electrode, all housed in a Faraday cage to avoid electrical disturbance (Figure 2). A mixture of solutes introduced into the system through the injection port is separated into components on travelling down the column. The individual solutes are measured as they pass through the detector, the resultant voltage signals, once amplified, are recorded as peaks on a chart recorder. Peak heights are proportional to quantity.

(2) Chromatography Mobile Phase

The composition of the mobile phase provided the most powerful factor affecting chromatographic separation. As the column aged, the retention capacity and, hence, good separation decreased.

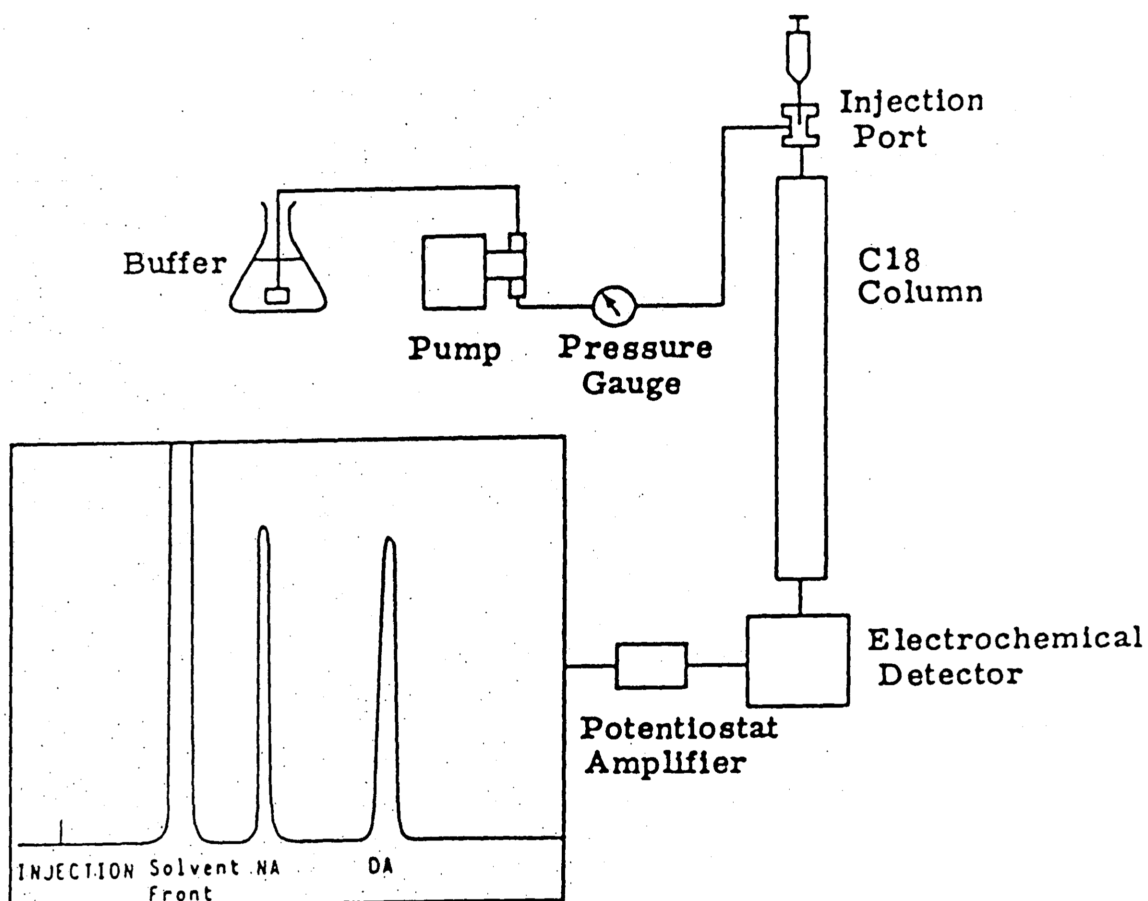


Fig. 2: Diagramatic representation of a High Performance Liquid Chromatography system.

By altering one of the constituents of the buffer, the separation could be improved. Phosphate buffer was used and a typical composition used towards the end of the project is shown below:

Potassium dihydrogen orthophosphate	13.6 g
Sodium octylsulphonate	0.0864 g
EDTA	0.0336 g
Methanol	40 ml
Deionised Water to	1 litre

The pH was adjusted to 3.2 with concentrated orthophosphoric acid. The water used for the mobile phase was deionised and filtered through a Millipore Q reagent grade water system. Prior to use, the mobile phase was filtered and degassed using an Edwards single stage high vacuum pump (Model ISC 50) and a Millipore solvent clarification kit with 0.45 μm aqueous filters.

Extraction of Catecholamines from Tissues

The NA and DA contents of the guinea-pig, rat and mouse vasa deferentia and rat colon were extracted and measured essentially as described for the guinea-pig vas deferens by Macrae (1983).

Appropriate tissues were removed, stripped of adhering blood vessels and connective tissues and individually weighed. They were then finely chopped with scissors and transferred to a 20 ml glass homogeniser with 2 ml of ice-cold 0.1N perchloric acid containing sodium metabisulphite (4×10^{-4} M).

Homogenisation was carried out for 2 periods of 30 seconds with a motor-driven (TRI-R-STIRRER) teflon-glass tissue grinder used at full

power. During homogenisation the temperature of the homogenate rose from 0°C to 4°C. The homogenates were decanted into centrifuge tubes and a final 3 ml of perchloric acid (0.1N) added to transfer the last traces of the homogenates to the centrifuge tubes. The samples were centrifuged (2,000 g, 15 minutes, 4°C) in a Christ centrifuge and the supernatants decanted. Aliquots of 0.5 ml were added together with 50 mg of acid-washed alumina and 1.0 ml of Tris buffer 1 M (pH 8.6) to the polystyrene extraction tubes. The tubes were stoppered, vortexed for 30 seconds and then put onto a Luckham (Model R100) horizontal shaking machine for 10 minutes. Once the alumina had settled, the supernatant was aspirated off and the alumina washed 3 times with 3 ml of a dilute 5 mM Tris buffer. Between washes the alumina was again allowed to settle and wash fluid aspirated off. Catecholamines were then eluted from the alumina with 300 µl of 0.1 N perchloric acid containing 4×10^{-4} M sodium metabisulphite. After adding the acid, the alumina-acid mixture was vortexed for 30 seconds to ensure maximum elution, the alumina was allowed to settle and the supernatant acid aspirated and stored on ice prior to analysis.

In Vitro Experiments

Isolated pairs of either guinea-pig or mouse vasa deferentia were dissected free of adhering blood vessels and connective tissues. Tissues were then set up in a 2 ml or 5 ml plastic syringe bath inserted in a conventional 10 ml organ bath to permit the maintenance of constant temperature. Pairs of vasa were connected to the hook of a ring electrode at the prostatic end and to a Grass FTO 3 strain gauge at the epididymal end to measure longitudinal tension. One gram tension was initially applied to each tissue. Responses were displayed on a Grass polygraph. Krebs' solution was delivered to each organ bath by a

Watson-Marlow constant flow pump. The system allowed Krebs' solution to be aspirated from the top of the inverted syringe or to be washed out from the bottom of organ bath.

Types of Samples

(1) Krebs' solution blank

At the beginning of the experiment, Krebs' solution was passed through the heating coil and into the plastic bath, from which it was withdrawn, extracted and analysed. From this sample it was possible to detect any contaminant in the system.

(2) Spontaneous release sample

This provided a measure of the spontaneous release of catecholamines from the tissue. After the initial equilibration period, the Krebs' solution in contact with the tissue was washed off and replaced with fresh Krebs' solution.

After a period of 6 minutes, equivalent to the duration subsequently used for electrical stimulation and collection, the Krebs' solution was removed and its catecholamine content measured.

(3) Stimulation sample

The vasa were stimulated at a frequency of 20 Hz, with pulses of 1 msec duration, and supramaximal voltage for a period of 4 minutes. The released catecholamines were allowed a further period of 2 minutes for diffusion to be completed. The Krebs' solution was then collected.

(4) Tissue recovery sample

A mixture containing 500 pg NA, DA and DOPEG was added to the tissue and was allowed to remain in contact with the tissue for the same duration as was used for electrical stimulation. The recovery of this sample was, therefore, influenced not only by the extraction losses but also by the tissue inactivation processes.

(5) Krebs' solution recovery sample

A known amount of both NA and DA was added to the Tris buffer and alumina and then extracted. This provided a measure of the extraction efficiency and the values obtained were used to correct experimental results for losses incurred during extraction.

Problems encountered with electrochemical detection

(1) Artefacts occurring after injections of large concentrations of catecholamines

Initially, after one or two injections of a high concentration of the catecholamines into the HPLC, subsequent injections of perchloric acid, distilled water or the mobile phase through the injection port often produced two peaks, occurring at the same positions as the injected catecholamines. These peaks became smaller with repeated injections of perchloric acid until they disappeared completely. These artefacts were due to contamination of either the injection ports or the syringe used for injections. Subsequently, extra care was taken to wash both the injection port and the plastic syringe used for injection and these artefacts no longer occurred.

(2) Artefact associated with extracted Krebs' solution

At the working potential of + 0.7 V, Krebs' solution, which had passed through the reservoir, tubing and heating system and into the plastic syringe organ bath or had passed only through the reservoir and tubing, or Krebs' solution taken directly from the reservoir and then extracted, all produced an artefact at a position between DOPEG and NA. This artefact was initially thought to be either dihydroxymandelic acid (DOMA) or 3-methoxyphenylglycol but neither of these eluted at this position on the chromatogram.

When the working electrode potential was reduced from + 0.7 to + 0.55 V, this artefact disappeared. This observation explains why this peak was not noticed either earlier in this study or by Macrae (1983) since the working potential previously used was + 0.55 V. This peak probably represents an unidentified oxidisable product of Krebs' solution. Macrae (1983) warned against the periodic changing of the tubing system but the arrangement of the apparatus in the present study made this inevitable. However, as long as the separation was good, this unidentified peak could be distinguished from either DOPEG or NA and, therefore, posed no problem. Such unidentified peaks have frequently been reported in HPLC systems (Honma, 1982).

(3) Problems associated with the chromatography system

Various problems were associated with the different components of the system. For example, decreased sensitivity and drifting base lines were usually associated with the working electrode becoming "poisoned" by oxidation products or with the presence of contaminants and trapped air bubbles. In this case the glassy-carbon electrode could be

cleaned in a few minutes by scouring the surface of the electrode with a slurry of fine alumina. Once the alumina has been washed off the electrode, the complete cell was re-assembled and was ready for use.

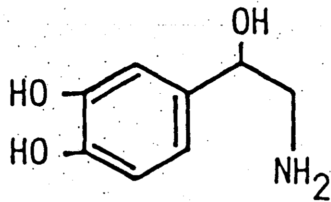
On one occasion, when no signal was obtained to injections of increasing concentrations of the catecholamines, it was found to be caused by a "dead" reference electrode.

Air bubbles in the mobile phase appeared as spikes on the baseline. Spikes were also caused by faulty electrical grounding of the equipment.

Histochemistry - Falck technique

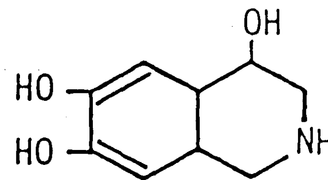
The method was similar to that described by Gillespie and Kirpekar (1966) and is based on the principle that catecholamines can be transformed into fluorescent isoquinoline derivatives by condensation with formaldehyde (Figure 3). Small sections of tissues were removed and immediately frozen in isopentane which had been cooled in liquid nitrogen. The tissues were then freeze-dried in a Pearce Speedivac freeze-drier at -40°C, 0.01 torr overnight. Next day, the temperature was raised to +35°C to prevent condensation and reduce water absorption before breaking the vacuum. The tissues were then removed, wrapped in gauze and pinned to the underside of the lid of a jar containing paraformaldehyde which had been heated to 80°C in a Griffin 1/200 oven. The jar and the tissues were returned to the oven and exposed to formaldehyde vapour for one hour. From the oven the tissues were returned to the freeze drier and dried for a further hour in small vessels containing de-gassed wax.

NORADRENALINE

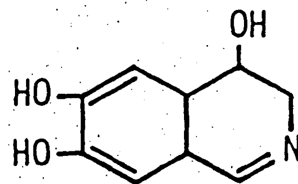


Formaldehyde

4,6,7 - Trihydroxy-
1,2,3,4 - Tetrahydro-
Isoquinoline



Dry protein



4,6,7 -TRIHYDROXY-
3,4 - DIHYDRO-
ISOQUINOLINE

Fig. 3: The Falck Hillarp reaction.

After drying, the temperature was slowly raised to melt the wax, then tissues were left *in vacuo* for 10 minutes to allow them to embed in the wax. Tissues were then removed and aligned in a wax embedding pan and allowed to cool, blocked and placed in the refrigerator for several hours to maintain wax at lower temperature and obtain optimal conditions for tissue sectioning. The blocks were sectioned to give 6 μm sections in a Leitz microtome. These were mounted dry on heated slides with hot liquid paraffin to dissolve the wax and act as mounting medium.

The fluorescing specimens were viewed and photographed on a Carl Zeiss ACM photomicroscope, equipped with a 1 V Fl epi-fluorescene system. The light source was an Osram HB50 mercury lamp, the filters used were: exciter-interference BP 405/8, barrier - LP 418 and dichromatic beam splitter FT 420. Photomicrographs were taken on Ektachrome ASA 400 film using a MC 63 photomicrographic camera.

Animal pretreatment schedules

Reserpine (2 mg/kg, i.p. daily for 4 days) was dissolved in glacial acetic acid (0.2 ml) and diluted with water to 20 ml. Rats received 0.2 ml of this solution/100 g body weight. Control rats received an equivalent volume of an appropriate dilution of acetic acid.

6-Hydroxydopamine (6-OHDA, 2 x 50 mg/kg, i.p. on day 1; then 2 x 100 mg/kg, i.p. on day 5). The concentration of the 6-OHDA solution was adjusted so that rats received 0.2 ml of solution/100 g body weight. Control rats received saline at appropriate intervals and tissues were examined on day 6.

P-Chlorophenylalanine (PCPA, 200 mg/kg, i.p. daily for 4 days). Control rats received saline for a similar period and tissues were examined on day 5.

Preparation of haemolysate

Male rats (250-350 g) were anaesthetised (Nembutal) (65 mg/kg, i.p.) and blood was collected from a cannulated carotid artery. The blood was collected in heparinised tubes, centrifuged (1,000 x g, 20 min, $4^{o}C$) and the plasma and buffy coat removed by aspiration. The erythrocytes was washed twice and resuspended in phosphate buffered isotonic saline to restore the volume to 3 ml; this constituted the washed erythrocyte suspension from which the haemolysate was prepared. 1 ml of the suspension was pipetted into centrifuge tubes containing 19 ml of hypotonic phosphate buffer (20 mM, pH 7.4). This suspension was then centrifuged (20,000 g, 40 min, $4^{o}C$) and the supernatant from this procedure constituted the crude haemolysate. Crude haemolysate (15 ml) was dialysed overnight ($4^{o}C$) against distilled water or hypotonic phosphate buffer (pH 7.4) to remove low molecular weight components. The effect of a 1:100 dilution of the dialysed haemolysate on the inhibitory response of the isolated colon to electric field stimulation was determined.

Analysis of results

All the results on the graphs show the mean $\pm$ standard error of the mean (S.E.M.).

The Student's t-test was used for statistical analysis of results.

A level of probability of $P \leq 0.05$ is taken to indicate statistical significance.

Drugs

Acetylcholine chloride (Koch-Light); Adrenaline bitartrate (Sigma); Apamin (Sigma); Ascorbic acid (B.D.H.); Atropine sulphate (B.D.H.); Carbamoylcholine chloride (Sigma); Clonidine (Boehringer Ingelheim); D-alaglymepheglyol (DAGO) (Sigma); Dihydroxyphenylglycol (Sigma); Diltiazem (Sigma); Dopamine (Sigma); Ethylenediaminetetracetic acid (E.D.T.A.) (Sigma); Flurbiprofen (Boots); Guanethidine monosulphate (Ciba); 6-Hydroxydopamine hydrobromide (Sigma); 5-Hydroxytryptamine creatinine sulphate (Sigma); Leucine enkephalin (Sigma); Methionine enkephalin (Sigma); Methysergide bimaleate (Sandoz); Morphine hydrochloride (Macarthys); Naloxone hydrochloride (Winthrop); L-noradrenaline bitartrate (Koch-Light); Normetanephrine hydrochloride (Sigma); Oxprenolol hydrochloride (Ciba); P-Chlorophenylalamine methylester (Sigma); Phentolamine mesylate (Ciba); Prazosin (Sigma); Propranolol hydrochloride (I.C.I.); Quinidine (B.D.H.); Reserpine (Koch-Light); S.K.F. 525A (Smith, Kline & French); Substance P (Sigma); Tetrodotoxin (TTX) (Sankyo); Tyramine hydrochloride (Sigma); Verapamil (Sigma); Yohimbine hydrochloride (Koch-Light).

RESULTS

1. Effects of morphine and of opioid peptides on the motility of the isolated colon

1.1 Morphine

The isolated colon of the rat generally shows a low level of spontaneous motor activity. The addition of morphine (10^{-6}-10^{-5} M) to the colon caused it to contract, usually immediately but sometimes only after a delay of several minutes. The motor response to morphine was not a sustained contraction and was followed by a relaxation to the baseline, with morphine still in the bath. This initial contraction was followed by waves of contraction and relaxation which continued at intervals thereafter (Fig. 4). The frequency of these rhythmic waves of contraction was variable both between animals and in tissues from the same animal during the course of an experiment. These waves of contraction and relaxation were abolished by the specific opiate antagonist naloxone (10^{-6} M) (Table 1a).

Above the threshold required to produce the rhythmic contractions, no dose-response relationship was observed. Increasing the dose merely increased the probability that the rhythmic waves of contraction would occur. In most studies of the excitatory effects of morphine in the gut, the drug is usually allowed to remain in contact with the tissue briefly and then is washed from the bath. In this study the initial contraction was, therefore, examined separately from the rhythmic contractions subsequently produced by prolonged exposure to morphine. A very steep concentration-response relationship was obtained. This contraction was competitively antagonised by the specific opiate antagonist naloxone. In contrast, the 5-HT antagonist methysergide

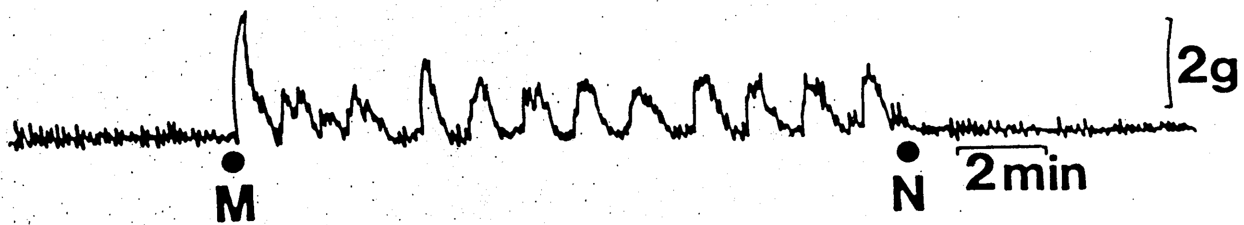


FIGURE 4 Excitatory effect of morphine on the isolated colon of the rat. Morphine (M, 5 x 10^{-6} M) produced an immediate contraction followed by rhythmic waves of contractions. Naloxone (N, 10^{-6} M) abolished the contractions.

shifted the dose response curve for morphine non-competitively (Fig. 5). Other agonists, which produce dose-dependent contractile responses in the rat colon, included carbachol, 5-HT and substance P. The mean $E.C._{50}$ values for these agonists and the mean maximum force developed by the colon in response to these drugs are shown in Table 1.

1.2 Enkephalins

The ability of morphine to produce rhythmic waves of contraction and relaxation was shared by the synthetic opioid agonists DAGO, leucine enkephalin and methionine enkephalin. When compared with morphine, the enkephalins were more potent and, therefore, lower concentrations of these agonists were required to cause contractions. DAGO (2×10^{-8} M), leucine enkephalin (10^{-8} M) and methionine enkephalin (2×10^{-7} M) caused the colon to contract rhythmically (Fig. 6).

The waves of contraction produced by the synthetic agonists sometimes gradually diminished in amplitude probably due to destruction by tissue enkephalinase and were abolished by low concentrations of the opiate antagonist naloxone (10^{-7}-2×10^{-7} M).

1.3 Comparison of the response of the colon to morphine, 5-HT, ACh and KCl

The pattern of rhythmic waves of contraction produced by morphine was unlike the responses of the rat colon to other agonists. KCl produced a rapidly-developed contraction which was well-maintained until the KCl was washed out of the organ bath (Fig. 7). ACh also caused the colon to contract and remain contracted for several minutes. Morphine-induced rhythmic contractions were also unlike those produced by 5-HT, which caused the colon to contract and relax irregularly and

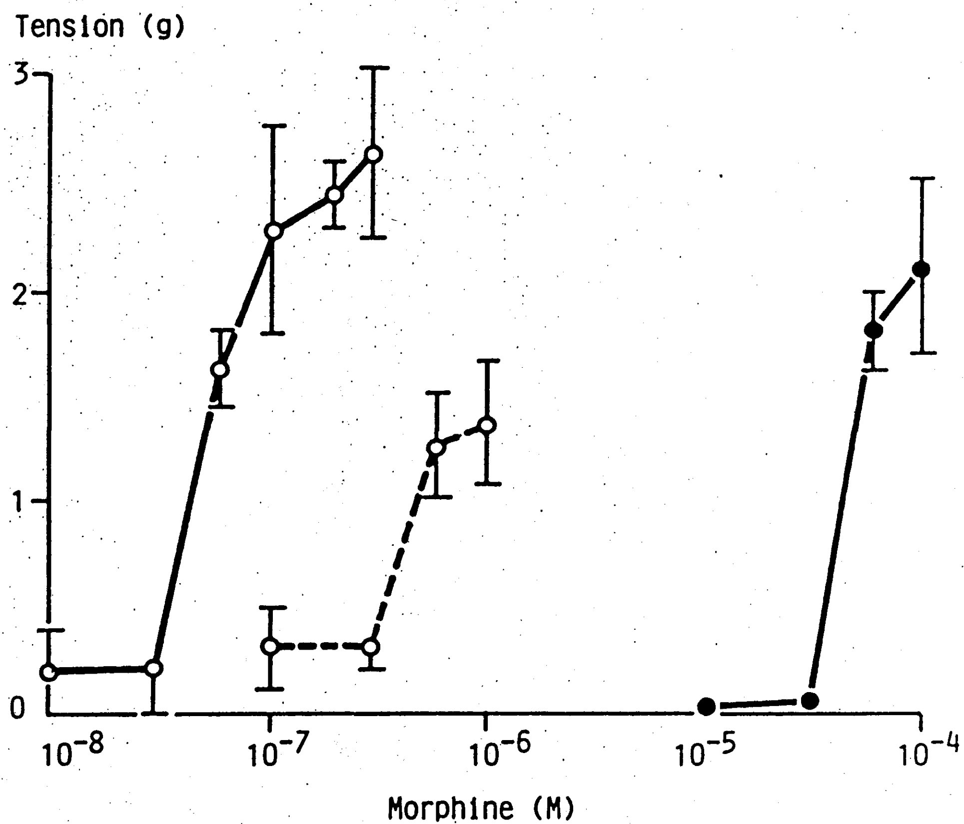


FIGURE 5 Effect of methysergide and naloxone on the contraction of the rat colon induced by morphine. o———o, control response, o-----o, response in the presence of methysergide (3×10^{-7} M), ●———●, response in the presence of naloxone (3×10^{-7} M). Values (mean $\pm$ S.E.M.), n = 5.

Sensitivity of the rat colon to different agonists and the mean force (g) developed to the agonists.

Agonist	EC_{50} Mean $\pm$ (S.E.M.) ($\times 10^{-8}$ M)	p Value*	Max response (g) Mean $\pm$ (S.E.M.)	p Value*
Morphine	5.63 ± 1.40		3.18 ± 0.27	
DAGO	0.45 ± 0.09	<0.01	4.60 ± 0.67	NS
Leucine Enkephalin	0.01 ± 0.03	<0.01	5.40 ± 0.47	<0.01
Methionine Enkephalin	5.29 ± 3.00	NS	3.30 ± 0.30	NS
5-Hydroxytryptamine	67.00 ± 8.70	<0.001	6.28 ± 0.66	<0.001
Carbachol	9.45 ± 2.29	NS	7.27 ± 1.03	<0.01
Substance P	0.70 ± 0.11	<0.01	3.57 ± 0.50	NS

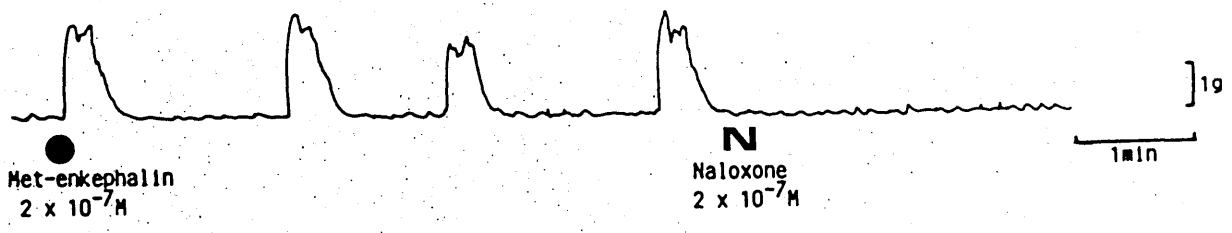
Each value is the mean of 8 observations. The p values refer to comparisons between the responses obtained with the drugs listed and morphine.

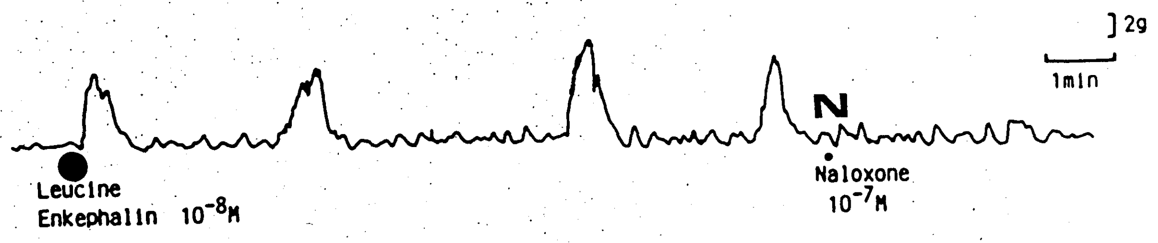
'NS' indicates that there is no significant difference between the EC_{50} or maximum response obtained with a particular drug and the EC_{50} value on maximum response obtained with morphine.

TABLE 1A

This table compares the motility of the intact isolated colon (control) with the motility of the colon in the presence morphine (10^{-6} M), TTX (2×10^{-6} M) or apamine (10^{-7} M) (Drug). This table also shows the effects of saline, atropine (10^{-6} M), methysergide (10^{-6} M) and naloxone (10^{-6} M) on the waves of contraction produced by morphine, TTX or apamine. The p values in the "Drug" column refer to comparisons between "Drug" and "Control". The p values in all other columns (saline, atropine, methysergide and naloxone) refer to comparisons between each of these columns and the "Drug" column.

Drug	Control	Drug	Saline	Atropine (10^{-6} M)	Methysergide (10^{-6} M)	Naloxone (10^{-6} M)	Mean Parameter calculated over 10 m
Morphine (10^{-6} M)	0.37 ± 0.37 (n = 8)	5.57 ± 1.40 (n = 8) $P<0.01$	5.87 ± 2.09 (n = 8) NS	5.22 ± 1.27 (n = 9) NS	6.37 ± 1.22 (n = 8) NS	0.83 ± 0.40 (n = 8) $P<0.05$	Frequency of waves (± SEM)
	0.24 ± 0.24 (n = 8)	2.30 ± 0.45 (n = 7) NS	2.49 ± 0.37 (n = 7) NS	2.40 ± 0.40 (n = 7) NS	2.29 ± 0.40 (n = 8) NS	0.65 ± 0.21 (n = 8) $P<0.01$	Tension of waves in (± SEM)
	2.00 ± 2.00 (n = 9)	23.87 ± 4.23 (n = 8) $P<0.001$	30.50 ± 5.10 (n = 8) NS	24.50 ± 3.62 (n = 8) NS	19.29 ± 2.94 (n = 7) NS	2.89 ± 1.60 (n = 9) $P<0.001$	Area under waves in m (± SEM)
TTX (2×10^{-6} M)	0.42 ± 0.30 (n = 7)	4.17 ± 0.40 (n = 6) $P<0.001$	3.50 ± 0.76 (n = 6) NS	4.28 ± 0.42 (n = 7) NS	3.83 ± 0.17 (n = 6) NS	4.80 ± 0.73 (n = 5) NS	Frequency of waves (± SEM)
	0.20 ± 0.20 (n = 5)	2.38 ± 0.66 (n = 6) $P<0.05$	2.98 ± 0.73 (n = 6) NS	2.60 ± 0.35 (n = 7) NS	2.61 ± 0.40 (n = 7) NS	2.94 ± 0.52 (n = 8) NS	Tension of waves in (± SEM)
	2.00 ± 2.00 (n = 5)	22.28 ± 2.18 (n = 7) $P<0.001$	24.57 ± 1.39 (n = 7) NS	23.14 ± 1.70 (n = 7) NS	20.43 ± 2.16 (n = 7) NS	23.43 ± 1.94 (n = 7) NS	Area under waves in m (± SEM)
Apamin (10^{-7} M)	0.43 ± 0.30 (n = 7)	6.17 ± 1.05 (n = 6) $P<0.01$	7.00 ± 1.59 (n = 6) NS	7.43 ± 0.20 (n = 7) NS	6.20 ± 1.65 (n = 5) NS	6.70 ± 0.86 (n = 5) NS	Frequency of waves (± SEM)
	0.17 ± 0.17 (n = 7)	0.58 ± 0.12 (n = 7) NS	0.69 ± 0.13 (n = 4) NS	0.58 ± 0.13 (n = 5) NS	0.68 ± 0.05 (n = 5) NS	0.70 ± 0.04 (n = 5) NS	Tension of waves in (± SEM)
	1.00 ± 2.24 (n = 7)	21.46 ± 5.57 (n = 5) $P<0.01$	24.83 ± 5.47 (n = 5) NS	17.40 ± 4.42 (n = 5) NS	15.67 ± 1.20 (n = 6) NS	16.16 ± 3.22 (n = 6) NS	Area under waves in m (± SEM)





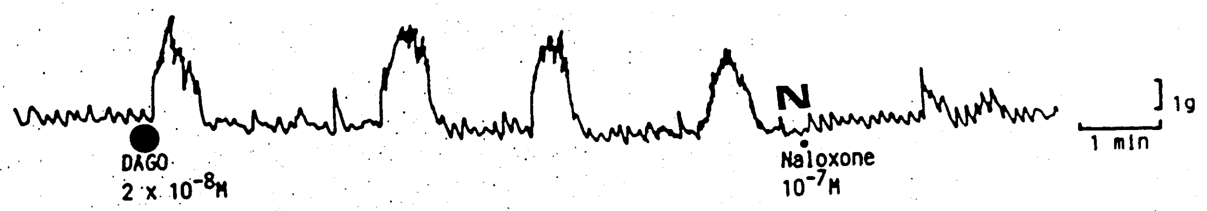


FIGURE 6 Excitatory effect of opioid peptides in the rat colon. Met-enkephalin (2 x 10^{-7} M), leu-enkephalin (10^{-8} M) and DAGO (2 x 10^{-8} M) all produced naloxone-sensitive rhythmic contractions in the rat colon. Each opioid peptide was added to the organ bath at and remained in contact with the tissue until the end of the experiment. Naloxone added at N in each experiment either abolished or reduced the size of these contractions.

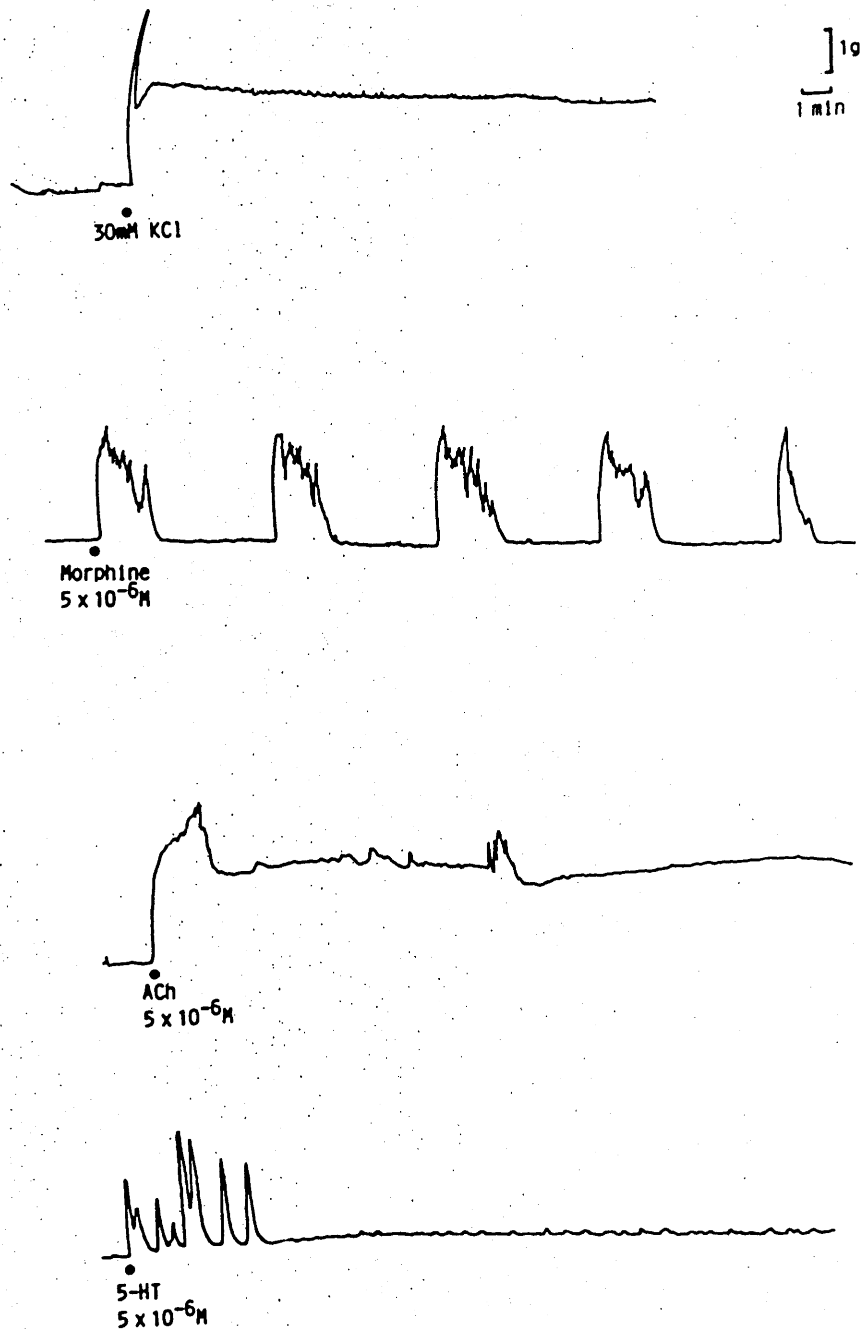


FIGURE 7 Comparison of the responses of the isolated colon to potassium chloride (KCl, 30 mM), morphine (5 x 10^{-6} M), acetylcholine (ACh, 5 x 10^{-6} M) and 5-hydroxytryptamine (5-HT, 5 x 10^{-6} M). The responses of the colon to morphine were quite unlike the responses to KCl, ACh and 5-HT.

briefly, after which the colon relaxed to its initial resting tension despite the continued presence of 5-HT. Thereafter the tissue was unresponsive to 5-HT.

1.4 Effects of temperature on morphine-induced rhythmic contractions

Between 30-38°C temperature changes had little effect on the occurrence and characteristics of morphine-induced rhythmic contractions. Lowering the temperature of the bathing medium from 37°C to 26°C reduced the frequency and increased both the amplitude and duration of morphine-induced contractions (Fig. 8). Increasing the temperature above 38°C generally increased the frequency and decreased the amplitude and duration of the waves.

1.5 Effect of Ca^{++} removal

The ability of morphine and the opioid peptides to produce contractions in the rat colon was reduced in Ca^{++}-free medium. The addition of SKF 525A (10^{-5} M), which blocks Ca^{++} influx (Kalsner, Nickerson & Boyd, 1970), diltiazem or verapamil (10^{-7}-10^{-5} M) reduced the size of the rhythmic waves of contraction (Fig. 9).

2. Analysis of the mechanism of opiate-induced waves of rhythmic contractions in the isolated colon

Two hypotheses proposed to explain the mechanism of the rhythmic waves of contractions seen in the rat colon (Fig. 10) were examined.

The 5-HT/ACh hypothesis was tested in three ways:-

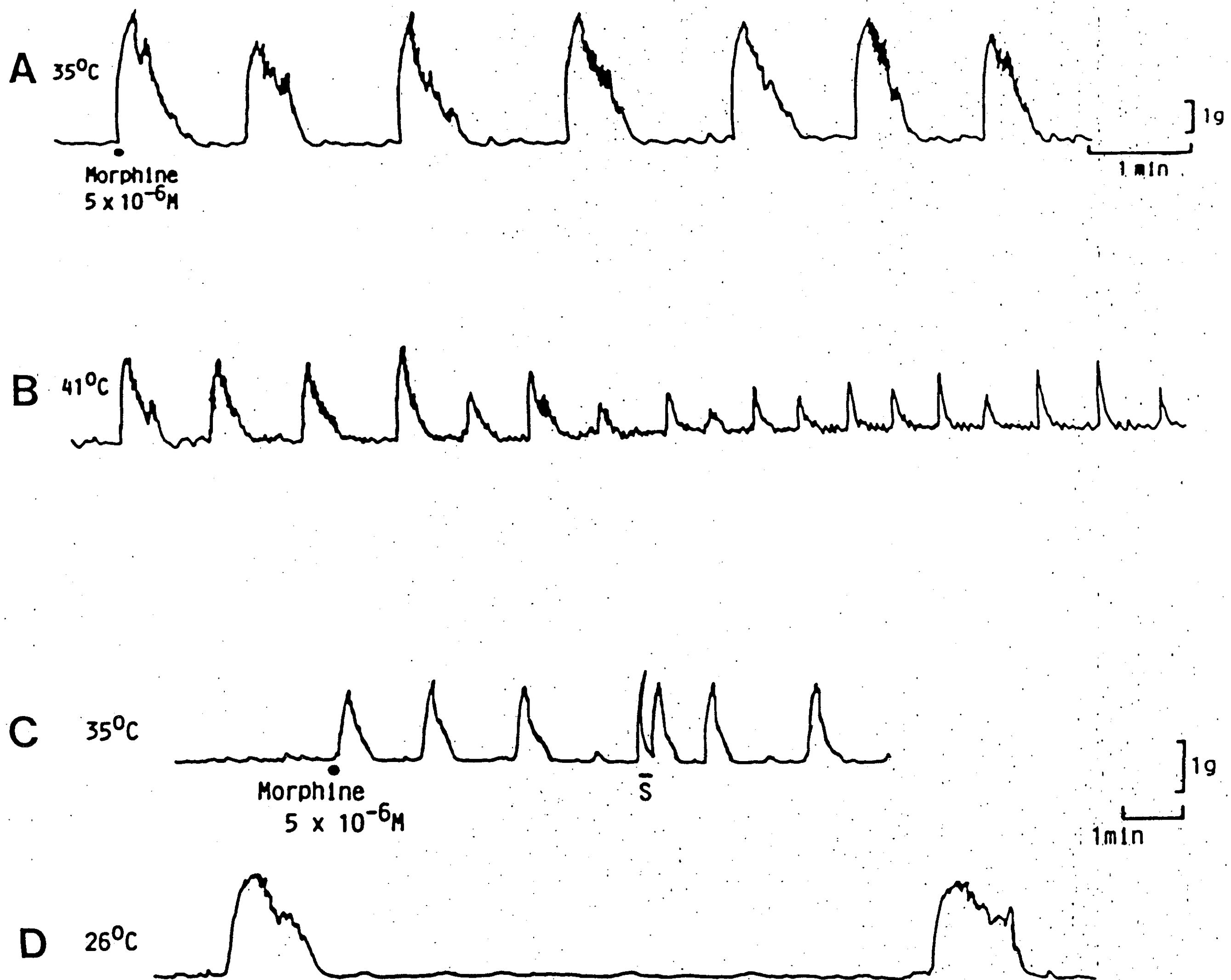


FIGURE 8 Effect of temperature on the amplitude, duration and frequency of morphine (5×10^{-6} M)-induced rhythmic waves of contractions in the rat colon. The first two panels (A and B) are from one experiment. Increasing the temperature from 35°C to 41°C increased the frequency and reduced the amplitude of the contractions. The two lower panels (C and D) are from a different experiment. Reducing the temperature from 35°C to 26°C reduced the frequency and increased the amplitude of the contractions. In trace C field stimulation at S (10 Hz, pulse width 0.5 msec) during a morphine-induced contraction produced an inhibition for the duration of the period of stimulation.

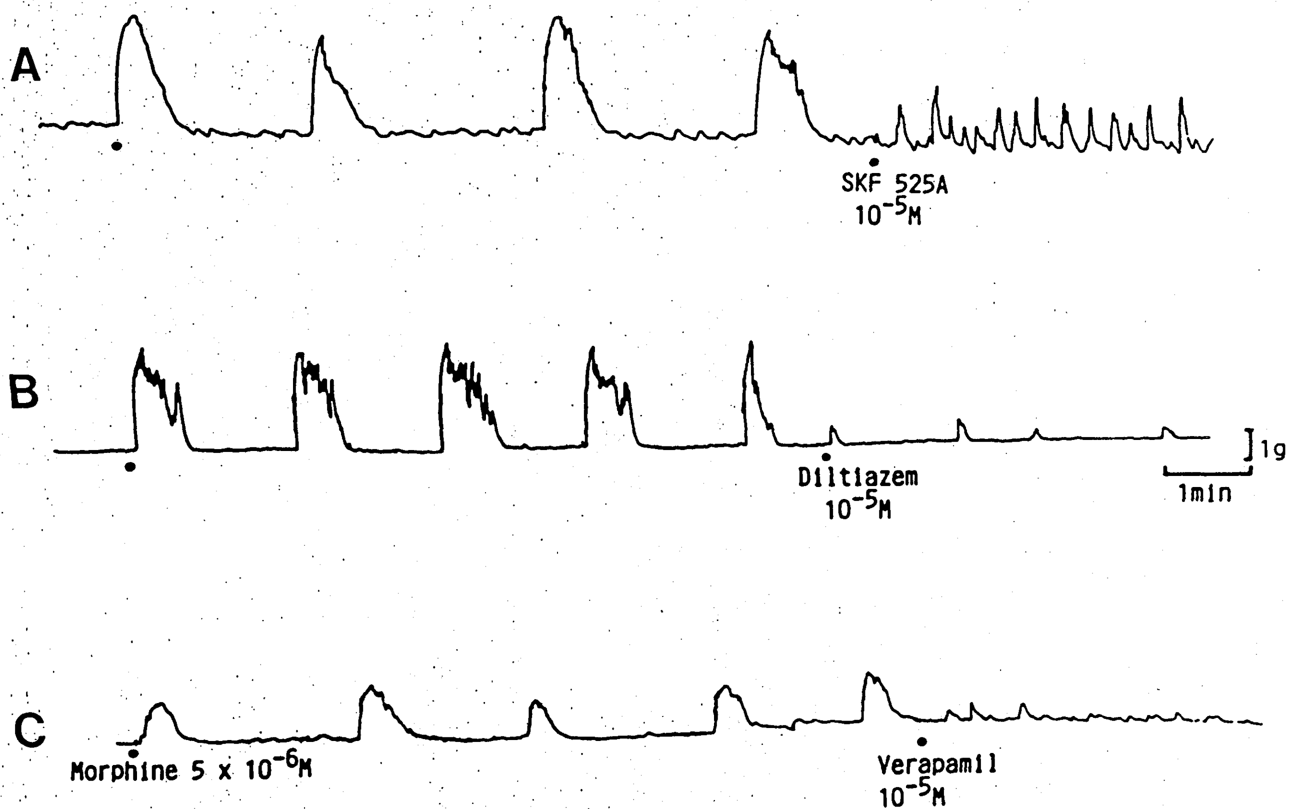


FIGURE 9 Effect of calcium antagonists on morphine-induced rhythmic contractions SKF 525A (10^{-5} M), diltiazem (10^{-5} M) and verapamil (10^{-5} M) reduced the size of the rhythmic waves of contractions produced by morphine (5×10^{-6} M). Traces A, B and C are from different experiments but the same calibration applied to all three traces.

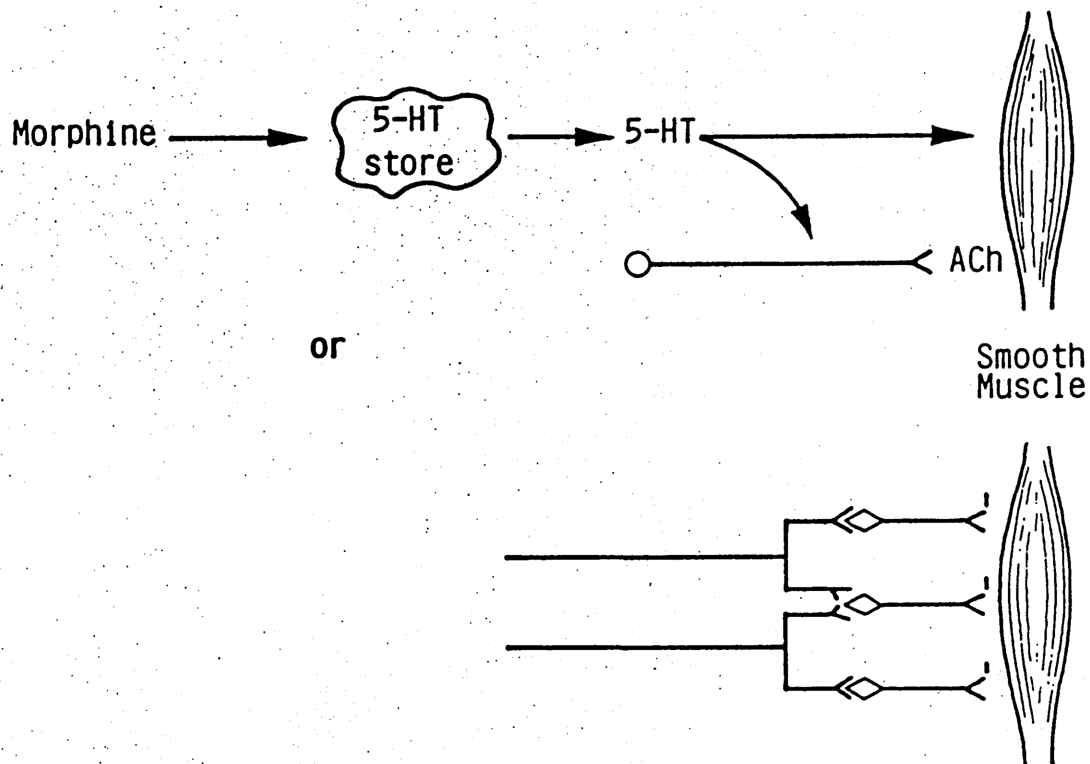


FIGURE 10 Models of possible mechanisms of the excitatory effect of morphine on the rat colon. Morphine releases intestinal 5-HT which then acts directly on the smooth muscle and indirectly through ACh release to cause muscle contraction or morphine by inhibiting an intrinsic spontaneously-active inhibitory neurone, unmasks the inherent myogenic activity of the smooth muscle.

(a) 5-HT autodesensitisation

(b) Depletion studies

(c) Use of antagonists of 5-HT and other drugs

2.1 5-HT hypothesis

2.1a 5-HT autodesensitisation

The effects of repeated exposure of the rat colon to increasing concentrations of 5-HT on responses to opiates was investigated (Fig. 11). These experiments examined the effect of adding the same high concentration of 5-HT repeatedly to the colon without washing out the previously-administered 5-HT. 5-HT (5×10^{-6} M) was added three times and with each administration, the size of the response diminished. When the concentration of 5-HT was increased to 5×10^{-5} M, a response could still be obtained but the addition of a higher concentration of 5-HT now produced no response.

At this point, with 5-HT still in the bath, morphine (5×10^{-6} M) caused an immediate contraction which was followed by further contractions and relaxations. These contractions were inhibited by naloxone (10^{-6} M).

2.1b Depletion studies

Effects of PCPA or reserpine pretreatment

Pretreatment of rats with PCPA reduced the 5-HT content of the colon. Figure 12 shows the 5-HT content of colons from control, PCPA- and reserpine-pretreated rats. PCPA was effective in reducing the 5-HT content of the rat colon but reserpine was less effective. Despite the marked effect of PCPA on the 5-HT content of the colon, morphine still

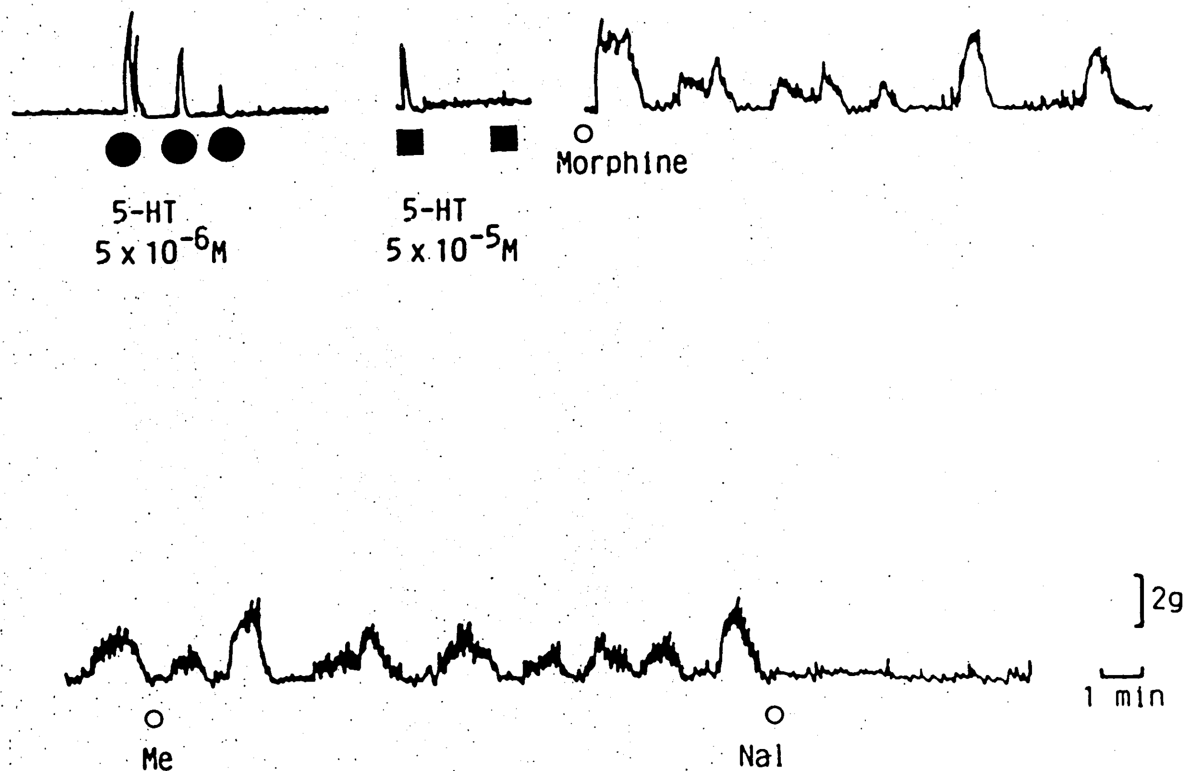


FIGURE 11 Excitatory effect of morphine in colon desensitised to 5-hydroxytryptamine (5-HT). Desensitisation was produced by repeated application of increasing concentrations of 5-HT. 5-hydroxytryptamine (● 5-HT, 5×10^{-6} M) was added three times without washing out the preceding dose. A higher concentration of 5-HT (■ 5×10^{-5} M) was again added. The second cumulative addition of 5-HT (■ 5×10^{-5} M) no longer produced any response. Without washing out the 5-HT, addition of morphine (5×10^{-6} M) produced rhythmic waves of contractions which persisted in the presence of methysergide (Me, 10^{-5} M). These waves of contraction were apparently inhibited by naloxone (Nal, 5×10^{-6} M). Both panels are from the same experiment.

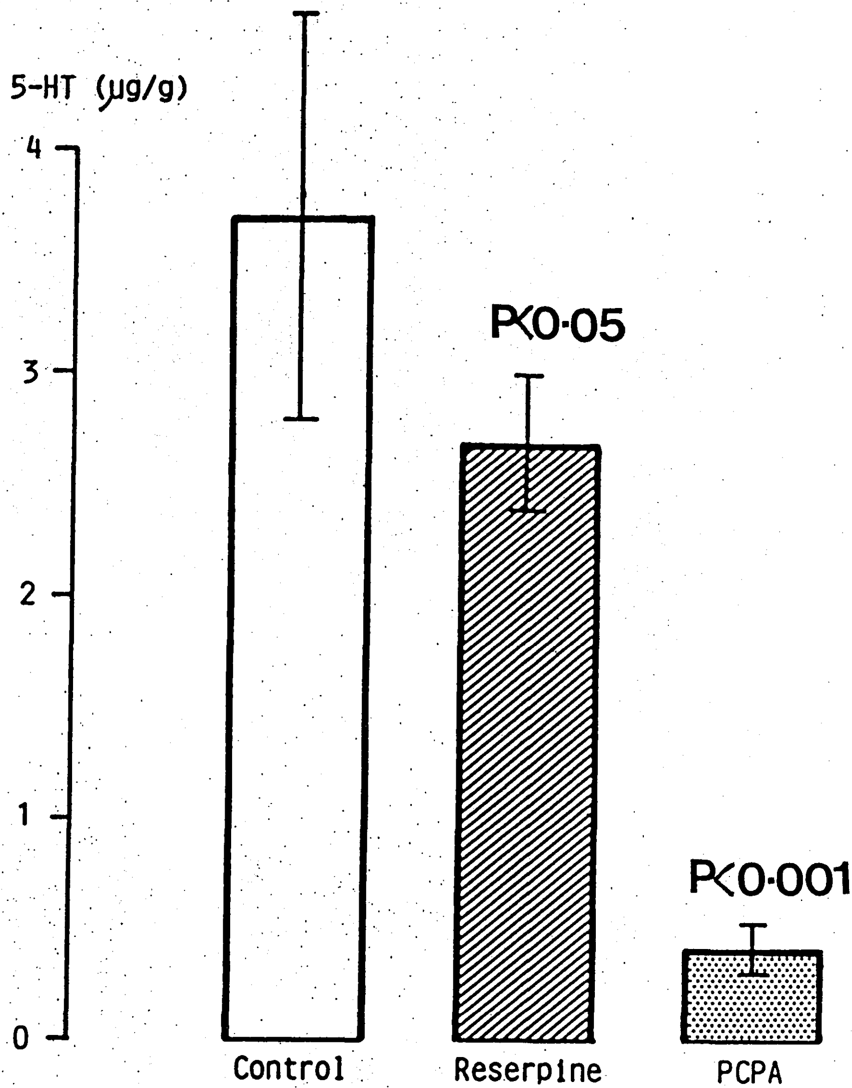


FIGURE 12 Effect of reserpine- or PCPA-pretreatment on the 5-HT content of the rat colon. Each histogram is a mean of 5 observations. The I-bars represent the standard error of the mean. The p values refer to comparisons with the control.

caused such colon to contract and these rhythmic waves of contractions were inhibited by naloxone (10^{-6} M) (Fig. 13).

2.1c Effects of 5-HT and other drugs

(a) Methysergide

The rhythmic waves of contractions produced by opioids were unaffected by methysergide (10^{-5} M) in control tissues (Figs. 15, 16) or in tissues rendered subsensitive to the effect of 5-HT by the process of autodesensitisation (Fig. 11).

(b) Adrenergic and cholinergic antagonists

The rhythmic waves of contraction produced by morphine and the opioid peptides were unaffected by both the cholinergic antagonist atropine (10^{-6} M) and the adrenergic antagonists phentolamine (10^{-6} M) and propranolol (10^{-6} M) (Figs. 14, 15, 16).

(c) Quinidine

Quinidine (10^{-5} M), which non-specifically blocks the effect of purinergic nerves in some tissues (Burnstock, 1972), did not affect the waves of rhythmic contractions produced by morphine.

2.2 The tonic inhibitory nerve hypothesis

The possibility that morphine might remove a tonic inhibitory influence that normally suppresses myogenic activity was considered. If this hypothesis is correct, then rhythmic waves of contraction should not only be produced by opioids, but also by other drugs that inhibit neuronal mechanisms or inhibit inhibitory mechanisms in smooth muscle.

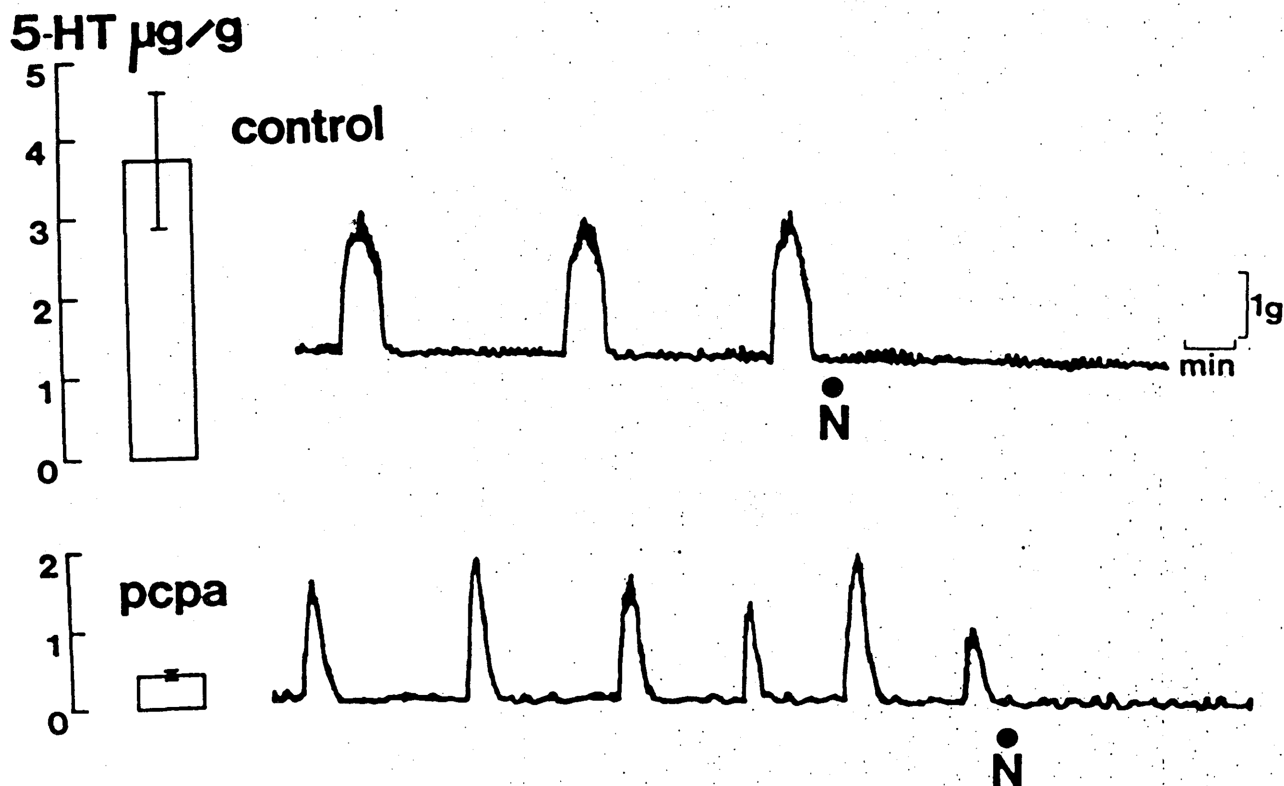


FIGURE 13 Excitatory effect of morphine on PCPA-pretreated colon. The upper panel shows the 5-HT content of the control colon and the rhythmic waves of contractions produced by morphine (5×10^{-6} M) in such a colon. The contractions were antagonised by naloxone (10^{-6} M) added at **N**. The lower panel shows the 5-HT content of a PCPA-pretreated colon and the naloxone (10^{-6} M)-sensitive contractions produced by morphine (5×10^{-6} M) in such a colon.

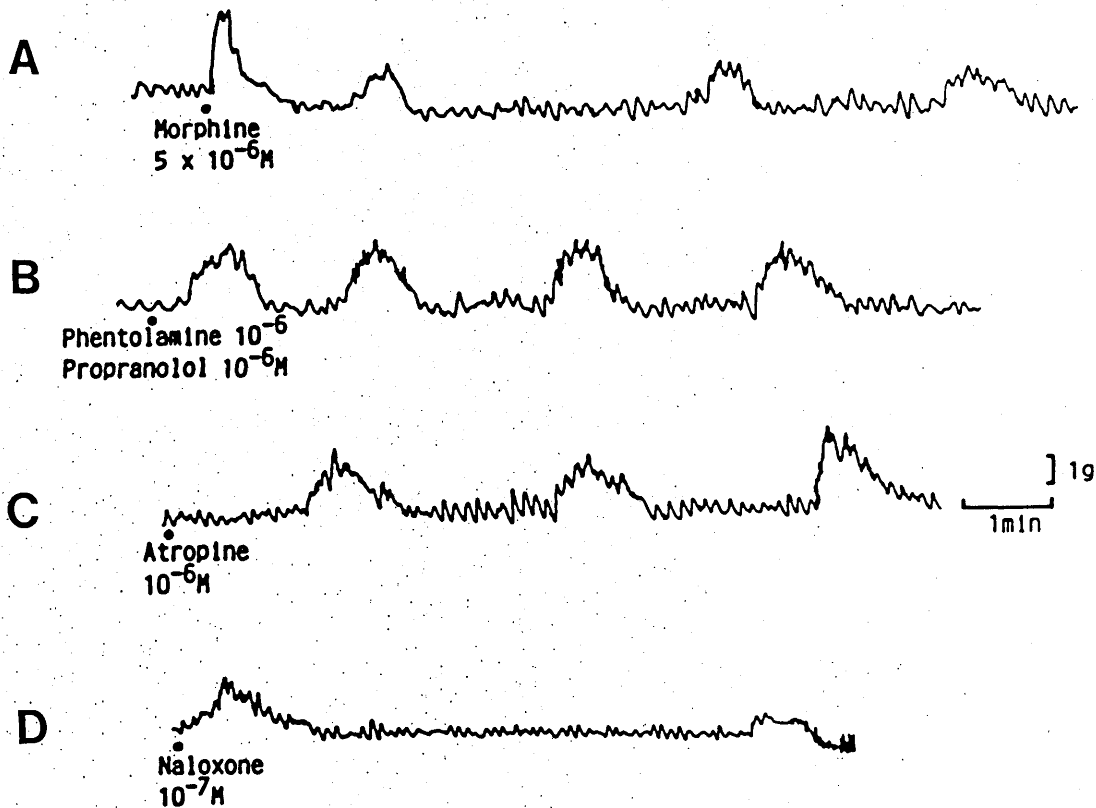


FIGURE 14 Effect of adrenergic and cholinergic antagonists on morphine-induced rhythmic contractions in the isolated colon. Morphine (5×10^{-6} M) produced waves of contractions (**A**). These were not inhibited by propranolol (10^{-6} M) and phentolamine (10^{-6} M) (B). The cholinergic antagonist atropine (10^{-6} M) did not abolish morphine-induced waves of contractions (**C**). Naloxone (10^{-7} M) inhibited the waves of contractions (D). Traces **A**, **B**, **C** and **D** are from the same experiment.

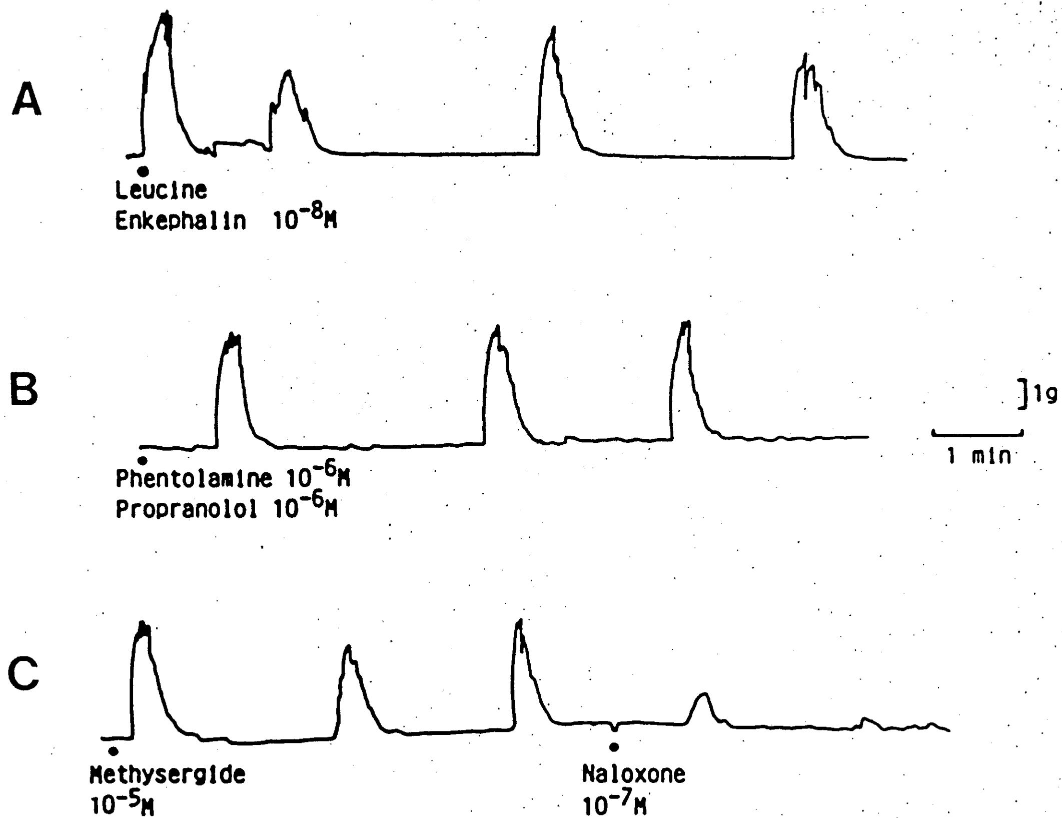


FIGURE 15 Effect of adrenergic and 5-HT antagonists on contractions produced by leucine enkephalin in the colon. Leucine enkephalin (10^{-8} M) produced rhythmic waves of contractions in the colon (**A**). These contractions were unaffected by both propranolol (10^{-6} M) and phentolamine (10^{-6} M) (**B**). The 5-HT antagonist methysergide (10^{-5} M) did not affect these waves of contractions (**C**). Naloxone (10^{-7} M) inhibited the rhythmic contractions (**C**). Traces **A**, **B** and **C** are from the same experiment.

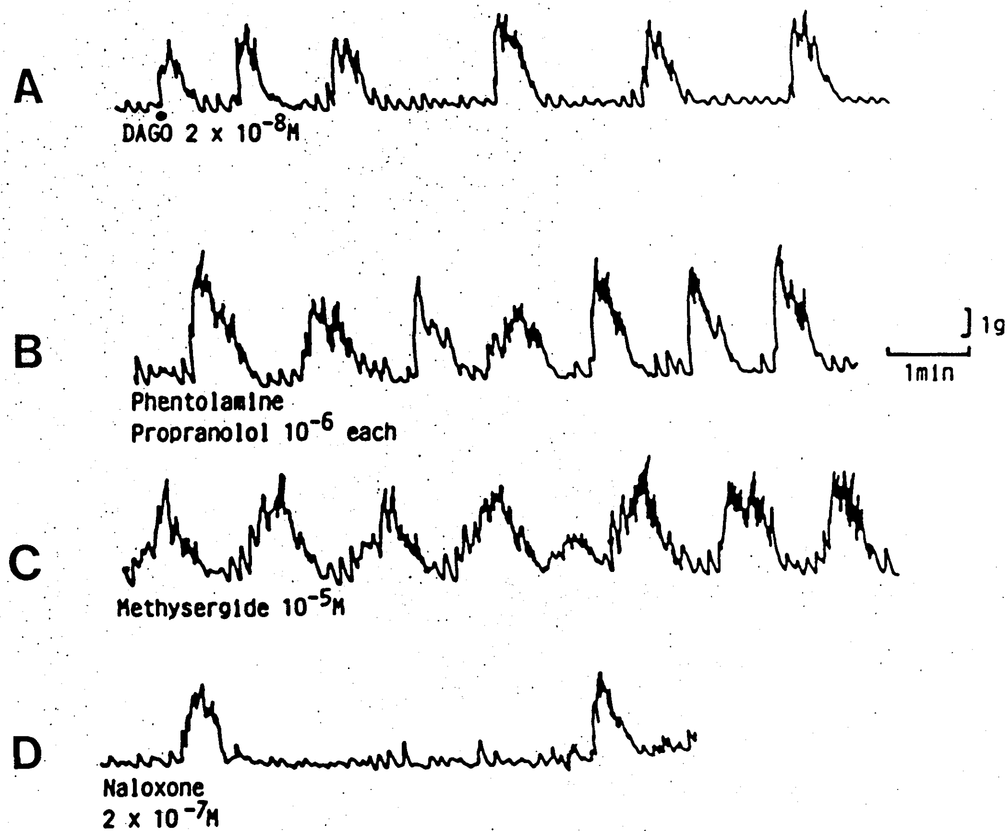


FIGURE 16 Effect of antagonists on the rhythmic waves of contractions produced by DAGO. DAGO (2×10^{-8} M) produced rhythmic waves of contractions in the isolated colon of the rat (A). The combination of phentolamine (10^{-6} M) and propranolol (10^{-6} M) did not inhibit the contractions (B). Instead, the frequency of the contractions increased after these drugs. Methysergide (10^{-5} M) did not inhibit the contractions (C) but the frequency of the waves of contractions was reduced by naloxone (2×10^{-7} M). **A, B, C** and **D** are consecutive traces from the same experiment.

Such drugs might be expected to have an excitatory effect similar to that of morphine.

Two drugs are of interest:-

(a) TTX which blocks sodium conductance and consequently abolishes the nerve action potential

(b) Apamin which inhibits inhibitory mechanisms that depend on increased potassium permeability in smooth muscle

2.2a <u>Response of the colon to apamin or TTX</u>

Apamin (10^{-8} M) or TTX (10^{-6} M) produced rhythmic contractions similar to those produced by morphine and the opioid peptides (Fig. 17). The rhythmic contractions continued for as long as the drug remained in contact with the tissue. The opiate antagonist naloxone (5×10^{-6} M) did not affect contractions produced by apamin or TTX (Table 1a).

Comparison of the rhythmic contractions produced by apamin or TTX with those produced by opioids revealed that the amplitude and wavelength of the contractions produced by a single drug varied in different tissues. However, the durations of the waves of contractions produced by apamin or TTX were remarkably similar to those produced by the opioids (Fig. 17).

3. <u>The innervation of the rat colon</u>

The results obtained with both TTX and apamin suggested that there is an inhibitory mechanism in the rat colon which could be NANC or adrenergic. The nature of this inhibitory mechanism and the innervation of the colon was investigated by electrical field stimulation of the isolated colon.

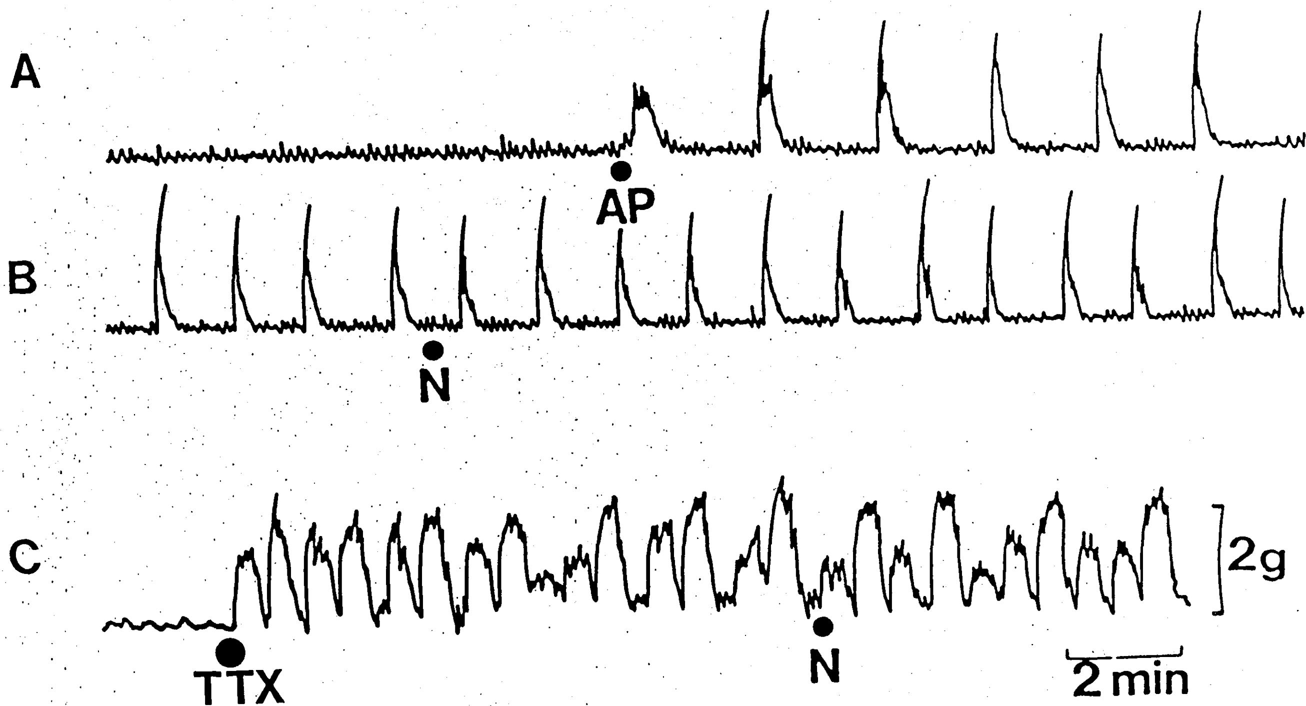


FIGURE 17a Comparison of the excitatory effects in the rat isolated colon of apamin (10^{-8} M) added at AP in trace **A** and TTX (10^{-6} M) in trace **C.** The rhythmic waves of contractions produced by either apamin or TTX were unaffected by naloxone (5×10^{-6} M) added at **N** in traces **B** and **C.** The first two panels (**A** and **B**) are from the same experiment. The bottom trace (**C**), was from a different experiment.

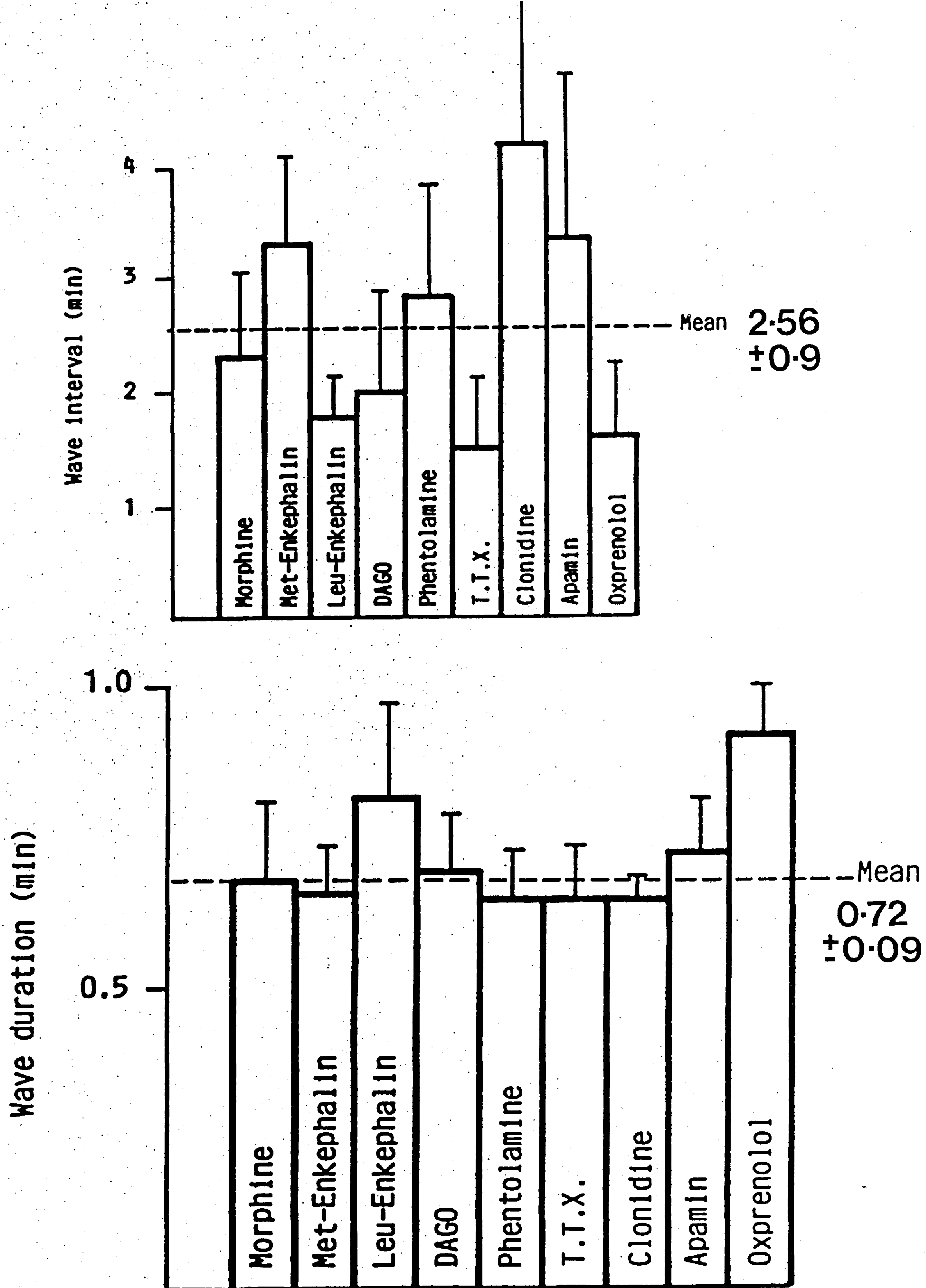


FIGURE 17b Comparison of the wave duration and wave interval produced by different drug treatments (mean ± S.D.). The mean wave durations were more similar than the wave intervals. n = 9 in each experiment.

3.1 Responses of the rat colon to electrical field stimulation

Field stimulation of isolated segments of rat colon with supramaximal voltage, pulses of 0.5 ms duration for periods of up to 10 seconds produced complex responses which varied according to the tone of the preparation and the frequency of stimulation (Figs. 18, 20). At frequencies of stimulation below 5 Hz, there was either no response or a small inhibitory response or occasionally an excitatory response. Stimulation at frequencies above 5 Hz produced during stimulation a contraction which was not well-maintained. Following stimulation, a powerful post-stimulus contraction occurred at all frequencies but was greater at lower frequencies.

3.2 Effect of drugs on responses of the rat colon to electrical field stimulation

3.2a Atropine

A typical field stimulation-induced response in the presence of atropine is shown in (Fig. 18). Atropine (5×10^{-6} M) reduced the contractile responses previously observed at high frequencies of stimulation.

3.2b Apamin

Apamin (5×10^{-8} M) potentiated the field stimulation-induced motor responses particularly those seen at higher frequencies of stimulation (Fig. 19).

3.2c Morphine

Morphine (5×10^{-6} M) reduced or abolished the motor responses to nerve stimulation without affecting the post-stimulation contraction

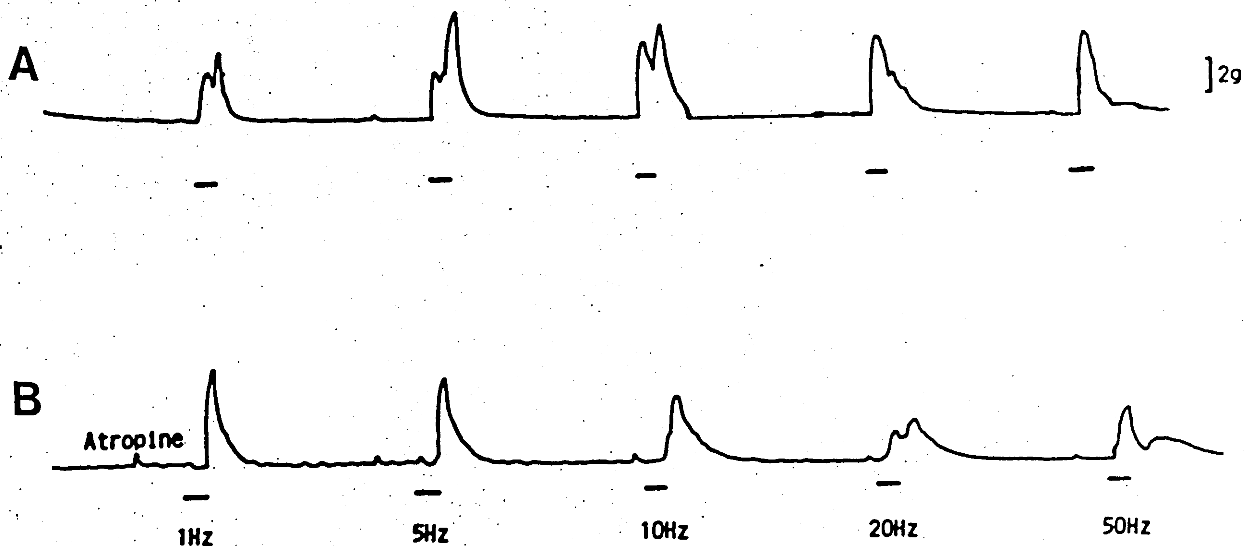


FIGURE 18 The response of the rat colon to electrical field stimulation and the effect of atropine on these responses. (A) Motor responses were obtained to electrical field stimulation (frequency as indicated, supramaximal voltage, 0.5 ms pulse width for 10 secs). Following stimulation there was a post-stimulation contraction, especially at frequencies between 1-10 Hz. (B) In the presence of atropine (5 x 10^{-6} M) motor responses to field stimulation were inhibited.

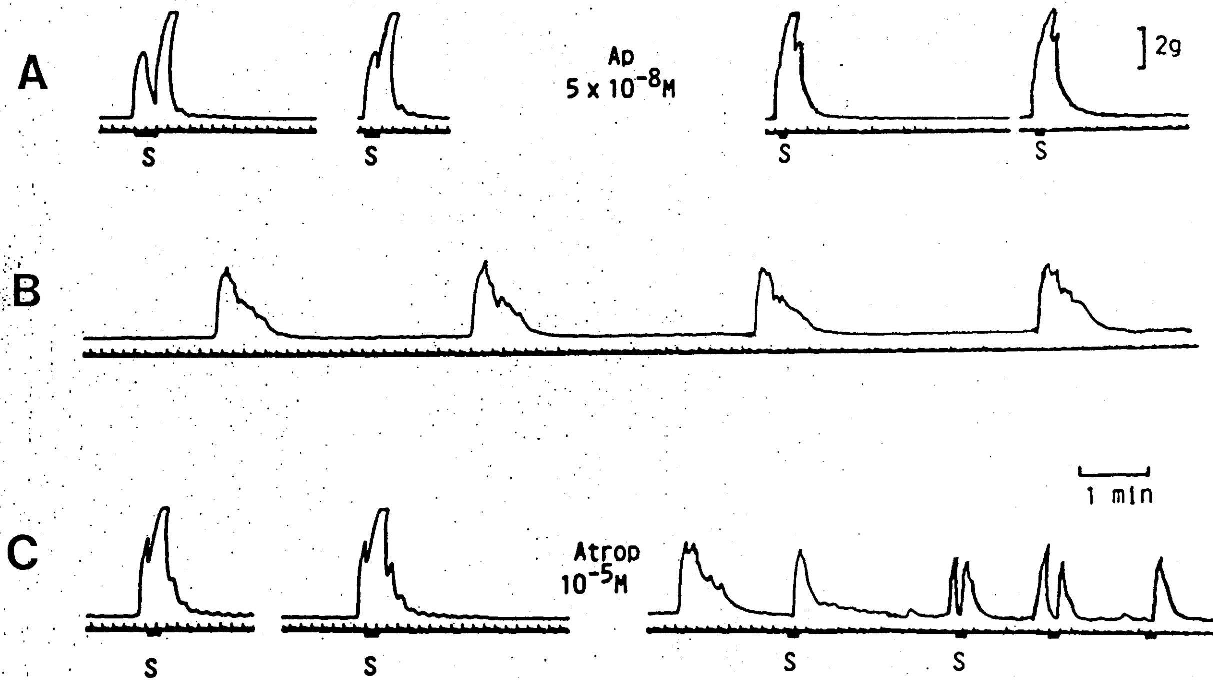


FIGURE 19 Effect of apamin on the isolated colon of the rat. In trace **A** apamin (AP, 5×10^{-8} M) potentiated the motor response to electrical field stimulation (10 Hz, 0.5 ms for 10 sec) applied at S. Trace **B** shows rhythmic waves of contraction produced by apamin. Electrical field stimulation at the peak of an apamin-induced contraction produced a slight inhibitory motor response in trace **C**. The addition of atropine (10^{-5} M) converted this small inhibitory response into a well-maintained inhibition and also inhibited the motor response to nerve stimulation. Traces **A**, **B** and **C** are from the same experiment.

(Fig. 20). The inhibitory response observed at low frequencies of stimulation became more pronounced.

3.2d 6-OHDA pretreatment

6-OHDA pretreatment potentiated the motor response to field stimulation. This effect of 6-OHDA was more pronounced at low frequencies of stimulation (Fig. 21).

3.3 Effects of drugs on post-stimulus contractions

The post-stimulus contraction obtained following the cessation of nerve stimulation was not abolished by the cholinergic antagonist atropine (5 x 10^{-6} M), the adrenergic antagonist guanethidine (10^{-5} M), propranolol (10^{-5} M) and phentolamine (10^{-5} M) (Figs. 18 and 26). In view of the possible involvement of prostaglandins in this post-stimulus contraction (Burnstock *et al*, 1975), the effects of the water soluble prostaglandin synthetase inhibitor flurbiprofen were investigated. Flurbiprofen (10^{-5} M) had no effect on the post-stimulus contractions obtained following intramural nerve stimulation at 5 Hz for 10 secs (Fig. 22).

3.4 Inhibitory response of the rat colon to electrical stimulation

The resting colon develops little maintained tone on its own and, hence, the inhibitory response to electrical field stimulation was usually very small. When the tone increased during spontaneous rhythmic contractions, electrical field stimulation at the peak of a wave produced an inhibition. Spontaneously-generated waves of rhythmic contractions offer the best opportunity for studying the inhibitory response, uncomplicated by the presence of drug. The occurrence of

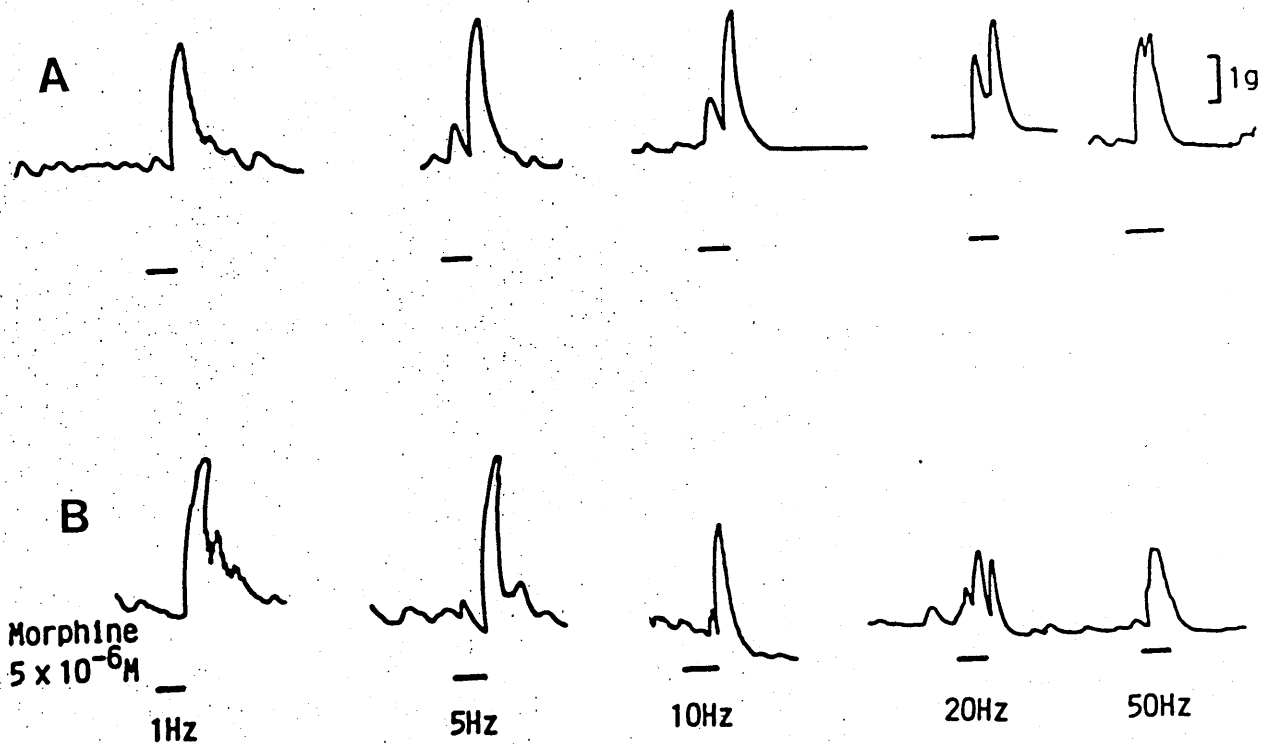


FIGURE 20 Effect of morphine on the responses of the rat colon to electrical field stimulation. Trace **A** shows the responses of the colon to field stimulation in the absence of morphine; during stimulation contractile responses were obtained, followed by a post-stimulus contraction. In trace B in the presence of morphine (5×10^{-6} M), contractile responses produced during stimulation were reduced. Traces **A** and B are from the same experiment.

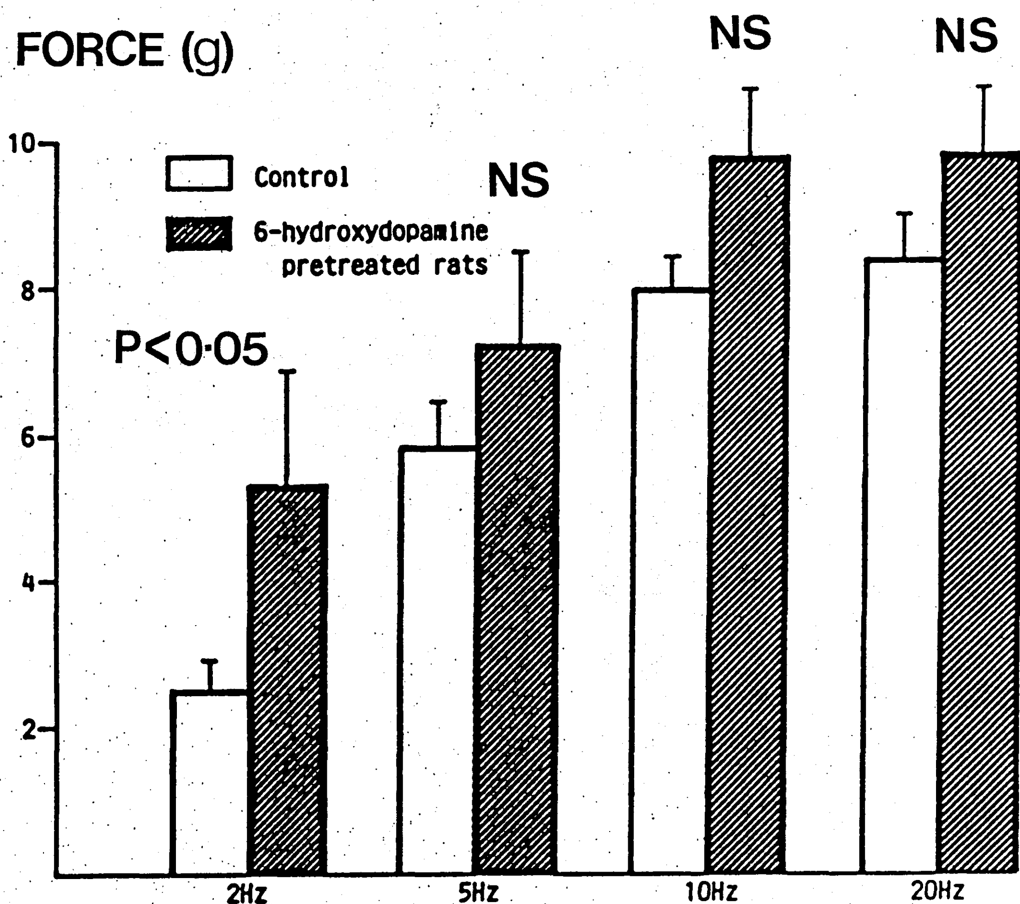


FIGURE 21 Effect of 6-hydroxydopamine pretreatment on the motor response of the colon to electrical field stimulation (100 pulses, 0.5 ms, supramaximal voltage, frequency as indicated). 6-OHDA pretreatment potentiated the motor response to field stimulation especially at low frequency. Each histogram is a mean of 6 observations. The I bars represent the S.E.M. The p value refers to the comparison between the responses of colon from 6-OHDA pretreated rats and responses of the control colon at the same frequency of stimulation.

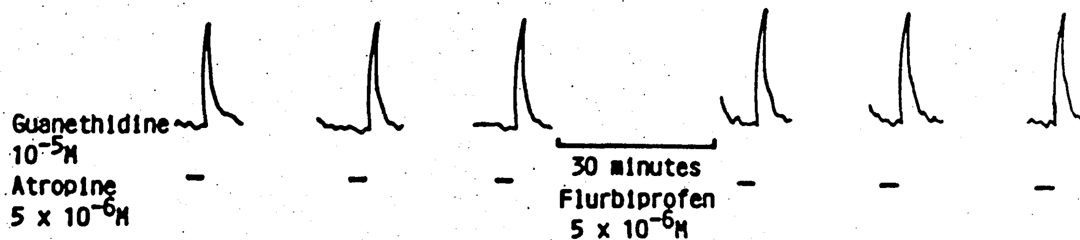


FIGURE 22 The effect of flurbiprofen on the post-stimulus contraction of the rat colon to electrical field stimulation. Post-stimulus contractions obtained following electrical field stimulation (5 Hz, 0.5 ms for 10 sec) in the presence of guanethidine (10^{-5} M) and atropine (5 x 10^{-6} M) was unaffected by prior incubation with flurbiprofen (5 x 10^{-6} M) for 30 minutes. The post-stimulus contraction (mean $\pm$ S.E.M.) after flurbiprofen was 102 ($\pm$6) percent of the response obtained before flurbiprofen and was not significantly different from the control.

these waves was, however, rare. Advantage was also taken of the rhythmic waves of contractions produced by various drugs. The colon was stimulated by electrical field stimulation at the peak of these waves of contractions. In this way the nature of the inhibitory response was studied.

3.4a Investigation of the possible existence of an NANC mechanism in the rat colon

In the presence of both guanethidine (10^{-5} M) and atropine (2×10^{-6} M) to block adrenergically- and cholinergically-mediated responses respectively, electrical field stimulation at the peak of a morphine-induced wave produced a well-maintained inhibition during stimulation, with a return to original (elevated) tone when stimulation ceased (Fig. 23). The optimum frequency of this NANC inhibitory response was less than 5 Hz.

3.4b Effects of drugs on the NANC inhibitory response

(a) Reserpine and 6-OHDA pretreatment

Both reserpine and 6-OHDA were used to deplete the colon of noradrenaline. Comparison of the fluorescence in tissues from control and from 6-OHDA-pretreated rats confirmed the sparse adrenergic innervations of the rat colon and the effectiveness of these treatments in eliminating catecholamines from the rat colon. In such 6-OHDA-pretreated tissues, stimulation between morphine-induced contractions produced a powerful motor response. Field stimulation applied at the peak of a morphine-induced wave produced a brief, small inhibiton, which was followed during stimulation by a powerful contraction. The absence of a well-maintained inhibitory response at the peak of a contraction

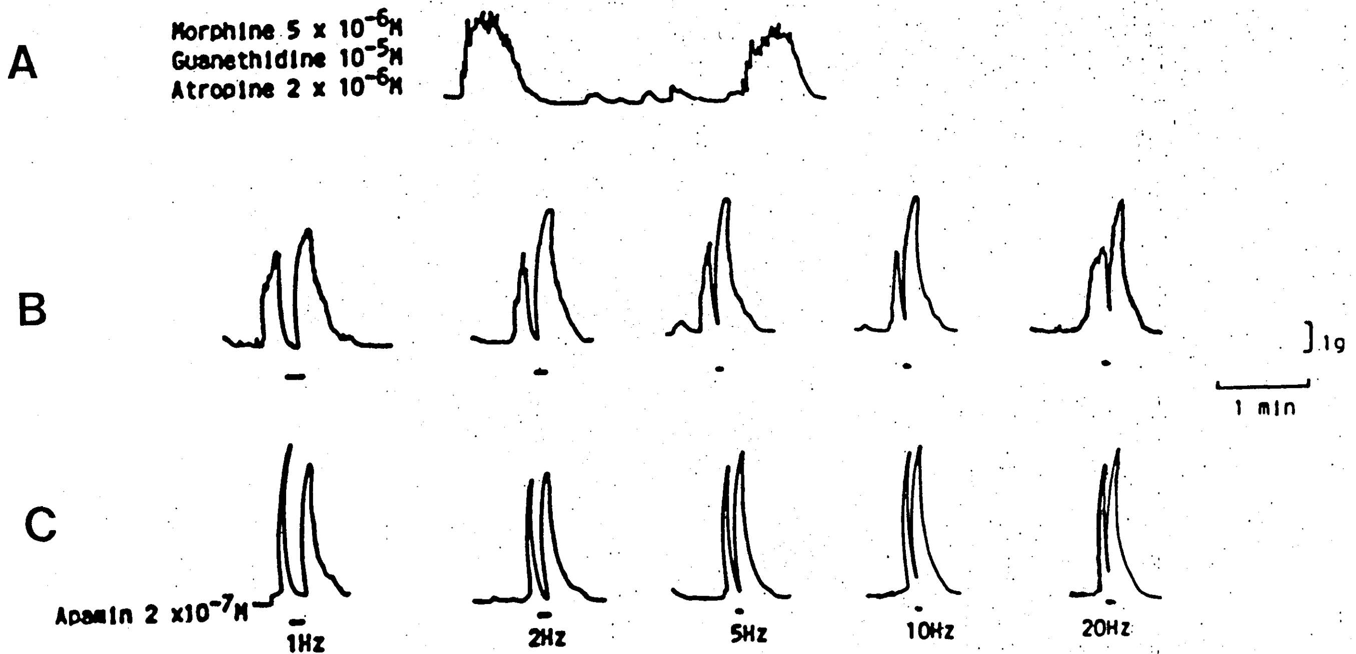


FIGURE 23 Effect of apamin on the non-adrenergic, non-cholinergic inhibitory response to electrical field stimulation of the isolated colon (10 pulses, 0.5 ms, frequency as indicated). In traces **B** and **C** inhibitory responses were obtained to electrical field stimulation applied at the peak of morphine (5×10^{-6} M)-induced waves in the presence of both guanethidine (10^{-5} M) and atropine (2×10^{-6} M). The optimum frequency of stimulation was less than 5 Hz. In trace **C** apamin (2×10^{-7} M) did not inhibit these inhibitory responses. Traces **A**, **B** and **C** are from the same experiment.

might suggest that the inhibitory responses was adrenergic, since it had apparently been reduced by 6-OHDA pretreatment.

However, the addition of atropine (10^{-6} M), which blocked the effect of the released ACh and of exogenously administered ACh (Fig. 24) revealed that the inhibitory response persisted in these 6-OHDA-pretreated tissues.

This inhibitory response also persisted in reserpine pretreated tissues (Fig. 24).

(b) Effect of TTX

TTX (10^{-6} M) produced waves of contraction in the colon and abolished motor responses to electrical field stimulation (Fig. 25) without any effect on the inhibitory response. This inhibitory response was reduced by increasing concentrations of TTX (3×10^{-6} M) (Fig. 25). Generally three times the concentration required to inhibit the motor response was required to reduce the inhibitory response of the colon to field stimulation.

(c) Effect of haemolysate, apamin and methylene blue on NANC inhibitory response

1. Haemolysate derived from lysed red blood cells inhibits inhibitory responses of the bovine retractor penis muscle to field stimulation and to certain agonists (Bowman & Gillespie, 1982). Haemolysate also potentiated the response of perfused rabbit ear artery and the rat anococcygeus muscle to NA (Wylie, 1981). Oxyhaemoglobin is responsible for these effects of the haemolysate (Bowman, Gillespie & Pollock, 1982).

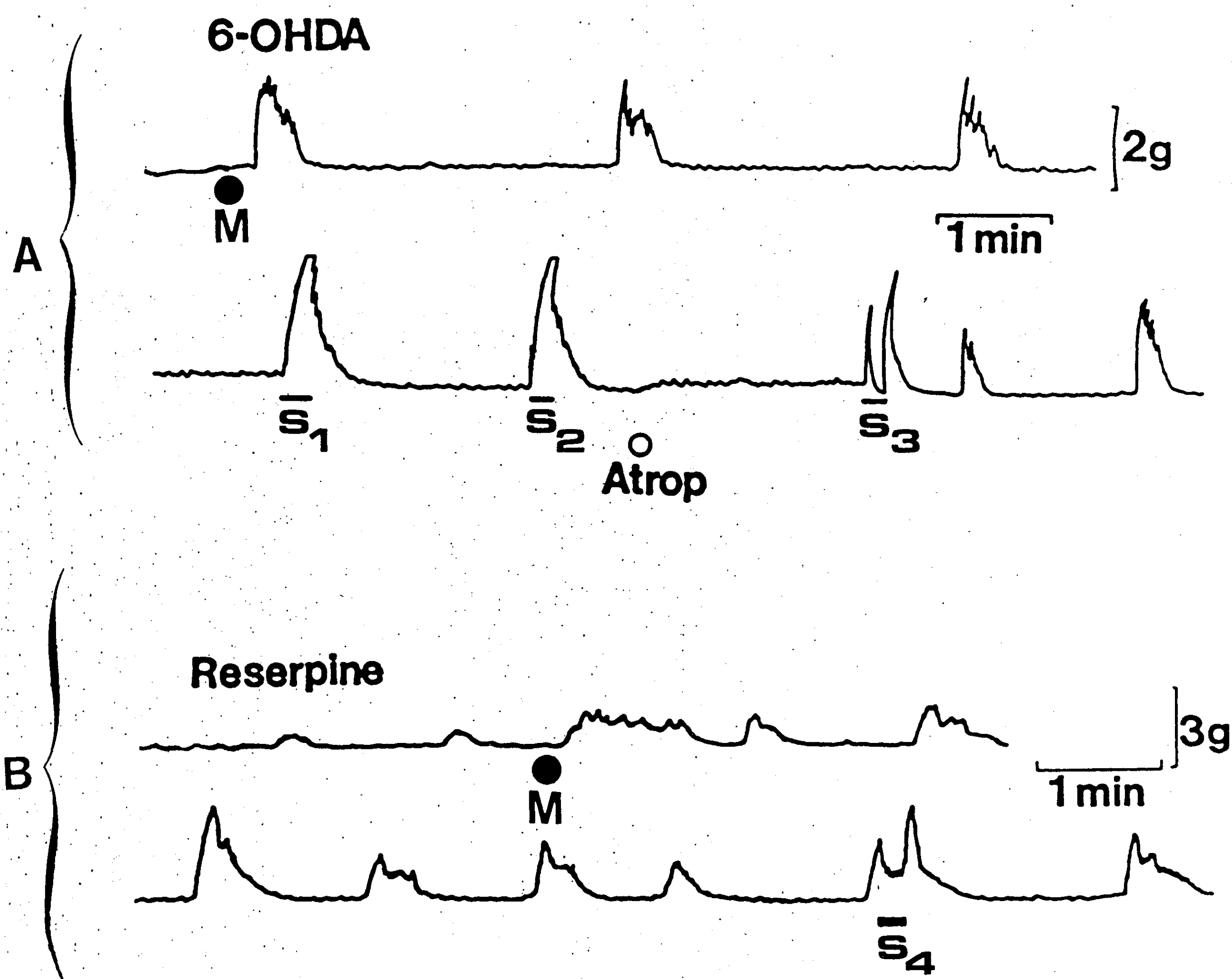


FIGURE 24 Effect of 6-hydroxydopamine and reserpine pretreatment on the inhibitory response to nerve stimulation. In trace **A** addition of morphine (**M**, 5 x 10^{-6} M) produced rhythmic waves of contractions in the colon. Field stimulation (S_1) between waves of contraction produced a motor response. Field stimulation (S_2) at the peak of a morphine-induced contraction produced a slight inhibitory response followed by a motor response. Addition of atropine (**Atrop**, 5 x 10^{-6} M) converted this small inhibitory response to field stimulation into a well-maintained inhibition (S_3). Trace **B** shows that in colon from reserpinised rats, morphine (**M**, 5 x 10^{-6} M) produced rhythmic waves of contractions. Stimulation at the peak of a contraction (S_4) still produced an inhibitory response.

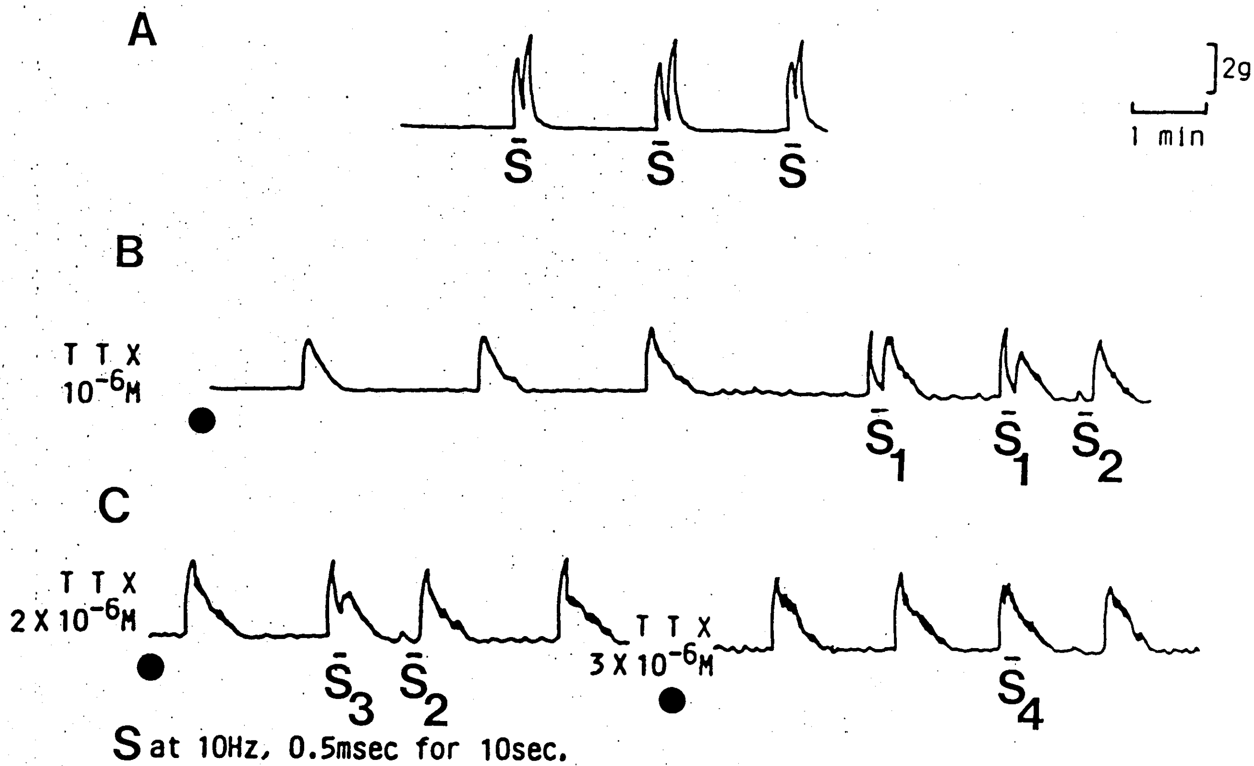


FIGURE 25 Effect of TTX on the isolated colon of the rat.

(**A**) Electrical field stimulation of the isolated colon (10 Hz, 0.5 ms for 10 secs) produced reproducible motor response (at S). (**B**) TTX (10^{-6} M) caused rhythmic waves of contractions. Electrical field stimulation applied at the peak of a TTX-induced contraction (at S_1) produced an inhibition but field stimulation between waves of contraction (at S_2) now produced no contraction during stimulation but when stimulation ceased, there was powerful post-stimulus contraction. (**C**) Increasing the concentration of TTX to 2 x 10^{-6} M reduced the inhibitory response to field stimulation (at S_3) and increasing the concentration of TTX to 3 x 10^{-6} M inhibited the response to field stimulation applied at the peak of a TTX-induced contraction (at S_4). **A**, **B** and **C** are from the same trace.

Haemolysate did not inhibit the inhibitory responses to field stimulation applied at the peak of a morphine-induced contraction (Fig. 26).

2. Methylene blue

Cyclic guanosine mono-phosphate (cGMP) may be involved in neurogenic relaxation of some smooth muscle (Bowman & Drummond, 1984). The effect of methylene blue, which blocks the activation of guanylate cyclase (Ignarro & Kadowitz, 1985), was, therefore, tested on the field stimulation-induced inhibitory response obtained in the rat colon at the peak of a wave of contraction. Methylene blue (3×10^{-5} M) did not inhibit the NANC inhibitory response to field stimulation at frequencies between 1 and 10 Hz (Fig. 27).

3. Apamin

Apamin (2×10^{-7} M) did not inhibit the inhibitory response to field stimulation applied at the peak of a morphine-induced contraction (Fig. 23).

4. Adrenergic mechanism as possible mediator of tonic inhibitory influence affected by the opiates

The possibility that an adrenergic mechanism was responsible for the inhibitory tone removed by morphine to reveal myogenic activity, was examined using both the adrenoceptor antagonists and clonidine.

4.1 Responses of the isolated colon to adrenergic receptor antagonists and neurone blocking drugs

Various adrenergic receptor antagonists and neurone-blocking drugs had excitatory effects similar to those of the opiates.

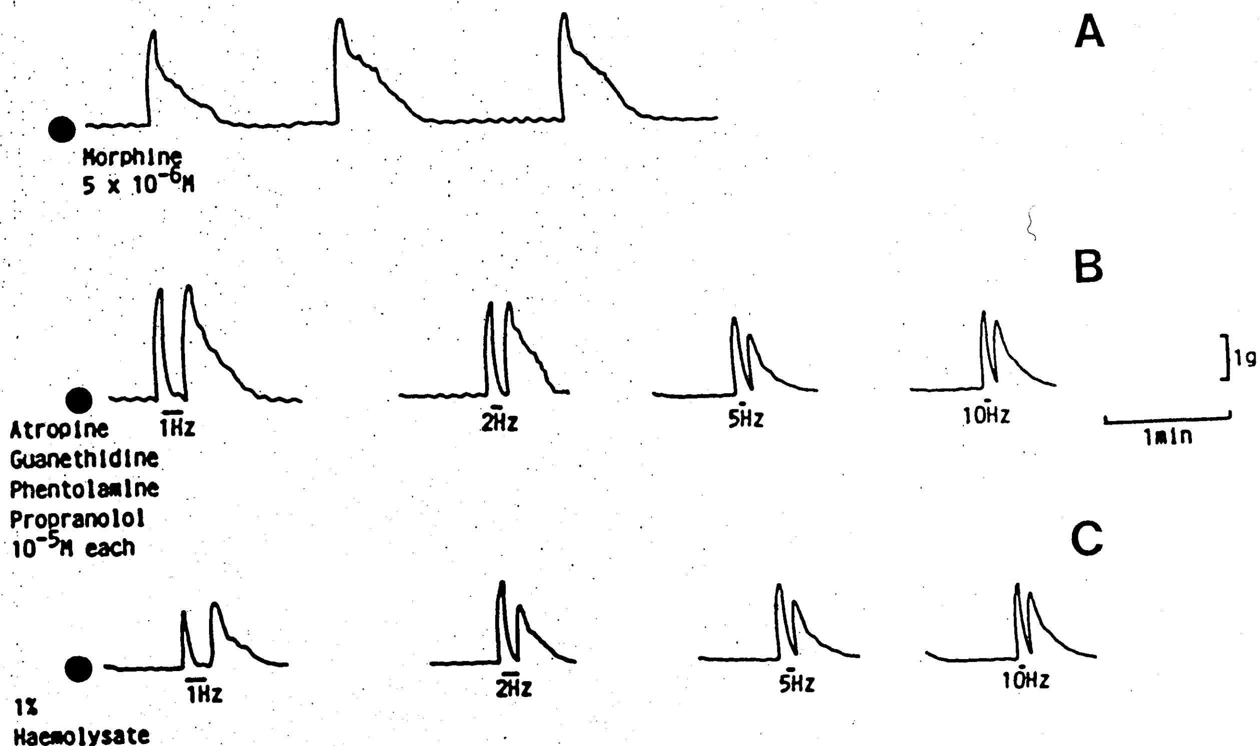


FIGURE 26 Effect of haemolysate on the non-adrenergic non-cholinergic inhibitory response of the isolated colon to electrical field stimulation (10 pulses, 0.5 ms, frequency as indicated). In traces **B** and **C** inhibitory responses were obtained to electrical field stimulation at the peak of a morphine (5×10^{-6} M)-induced contraction in the presence of atropine, guanethidine, phentolamine and propranolol (10^{-5} M) each. In trace **C** 1% haemolysate had no effect on the inhibitory responses. Because the size of the inhibitory response depended upon the size of the contraction, upon which the field-stimulation-induced contraction was superimposed, inhibitions in the presence and absence of haemolysate were expressed as percentages. Inhibitory responses (mean $\pm$ S.E.M.) to stimulation at 2 Hz in the presence of haemolysate were $98 \pm 7\%$ (n = 6) of the control response previously obtained in the absence of haemolysate. Traces **A**, **B** and **C** are from the same experiment.

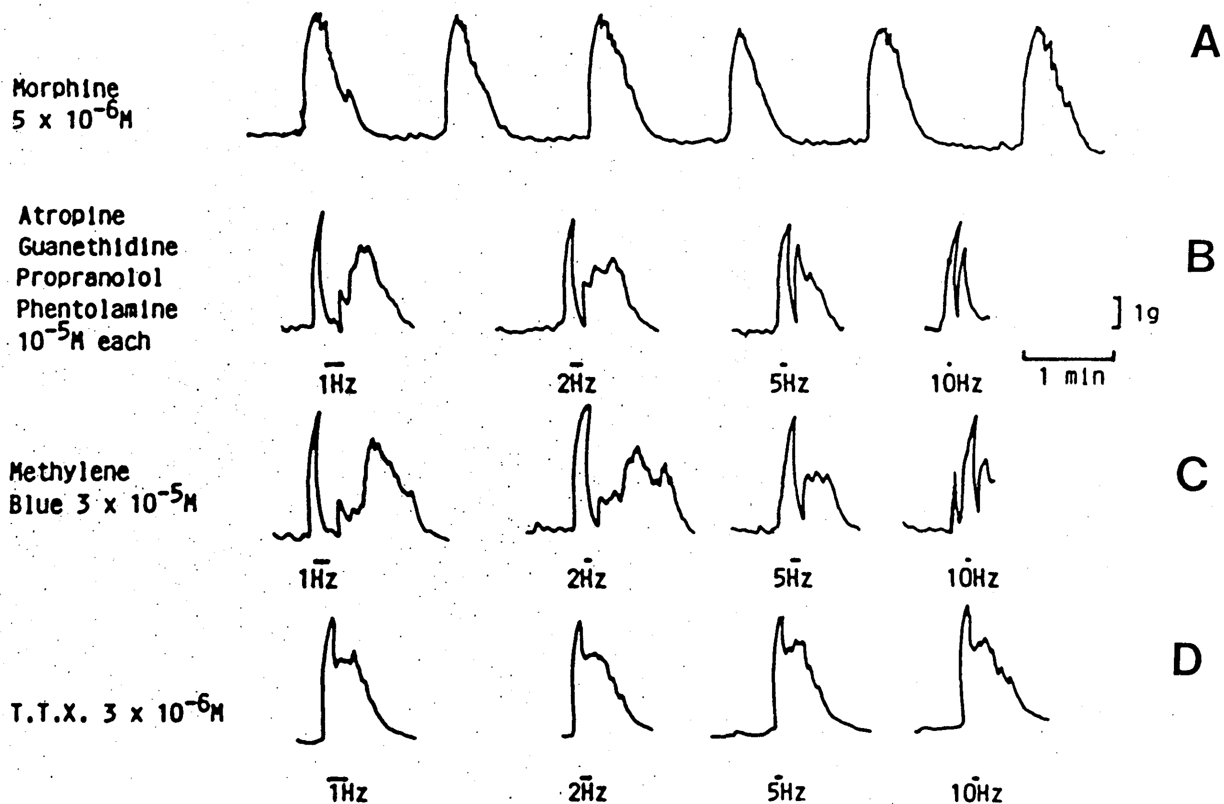


FIGURE 27 Effect of methylene blue on the non-adrenergic, non-cholinergic inhibitory response of the isolated colon to electrical field stimulation (10 pulses, 0.5 ms, frequency as indicated). In traces **B** and **C** inhibitory responses were obtained to electrical field stimulation at the peak of morphine (5×10^{-6} M)-induced contraction in the presence of atropine, guanethidine, propranolol and phentolamine (10^{-5} M) each. In trace **C** methylene blue (3×10^{-5} M) did not inhibit these inhibitory responses. In trace **D** the inhibitory responses were reduced but not abolished by TTX (3×10^{-6} M). Traces **A, B, C** and **D** are a continuation of the same experiment.

For example, colon from reserpine-pretreated rats showed a 22% increased incidence of spontaneous rhythmic activity (results were analysed by the chi-square test, $P<0.01$). The addition of the α-adrenoceptor antagonist phentolamine (10^{-5} M) to a control colon caused it to contract rhythmically. The effect of phentolamine was unaffected by yohimbine (10^{-7} M). The specific α_1-adrenoceptor antagonist prazosin did not produce rhythmic waves in the colon. Either propranolol (10^{-5} M) or oxprenolol (10^{-5} M) also produced regular waves of contraction (Fig. 28).

4.2 Effect of clonidine

Clonidine (2×10^{-8} M) caused the colon to contract usually immediately and this contraction was followed by regular waves of contraction and relaxation (Fig. 29). This excitatory effect of clonidine occurred at low concentrations and were unaffected by naloxone (10^{-6} M) and the specific α_1-adrenoceptor antagonist prazosin (10^{-6} M). In contrast, the excitatory effect of clonidine was readily inhibited by a low concentration of the specific α_2-adrenoceptor antagonist yohimbine (10^{-7} M) (Fig. 29).

4.3 Effect of tyramine on apamin-induced waves

Rhythmic waves of contractions produced by apamin were reduced in size and frequency by the indirect sympathomimetic tyramine (10^{-6} M). This effect of tyramine (10^{-6} M) was antagonised by propranolol (5×10^{-6} M) (Fig. 30).

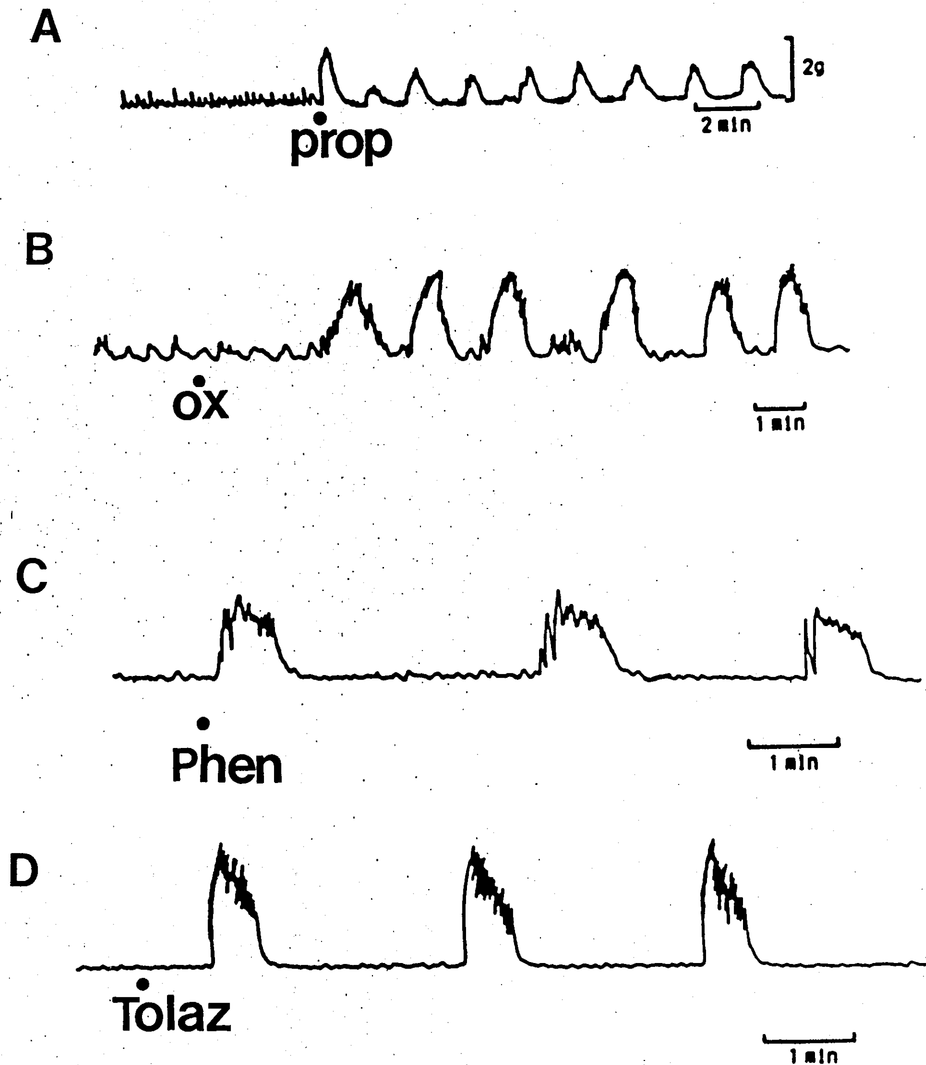


FIGURE 28 Excitatory effect of adrenergic antagonists on the rat isolated colon. (A) Propranolol (Prop, 10^{-5} M), (B) Oxprenolol (OX, 10^{-5} M), (C) Phentolamine (P, 10^{-5} M) and (D) Tolazoline (Tolaz, 10^{-5} M) all produced rhythmic waves of contractions in the colon. Traces A, B, C and D are from different experiments but the tension calibration applies to all four traces.

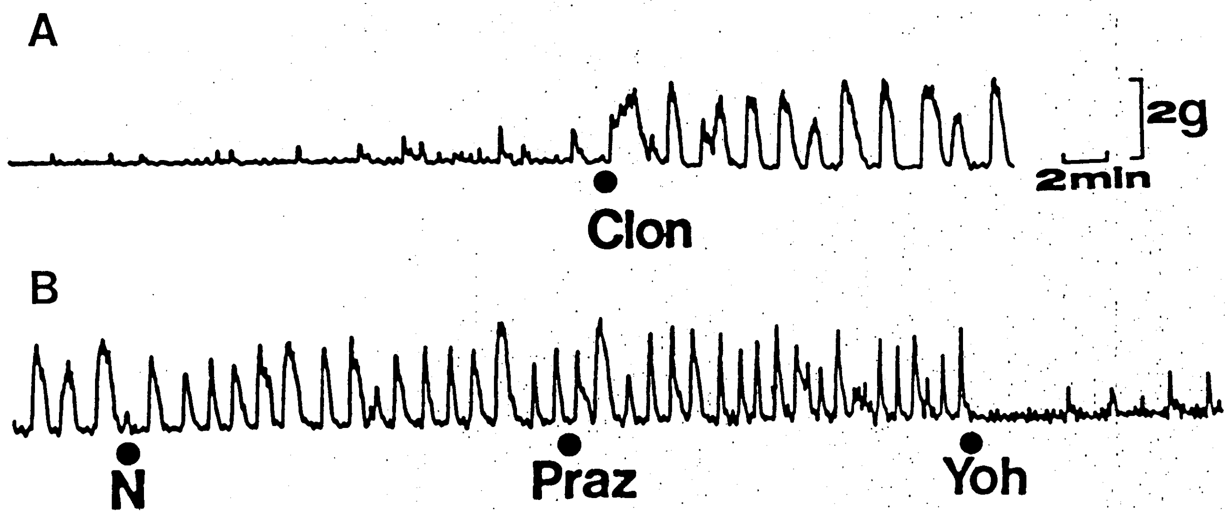


FIGURE 29 Excitatory effect of clonidine on the isolated rat colon. Clonidine (Clon, 2 x 10^{-8} M) produced rhythmic waves of contractions. These contractions were not inhibited by naloxone (N, 10^{-6} M) or prazosin (Praz, 10^{-6} M) but were inhibited by yohimbine (Yoh, 10^{-7} M). Trace B is a continuation of trace A.

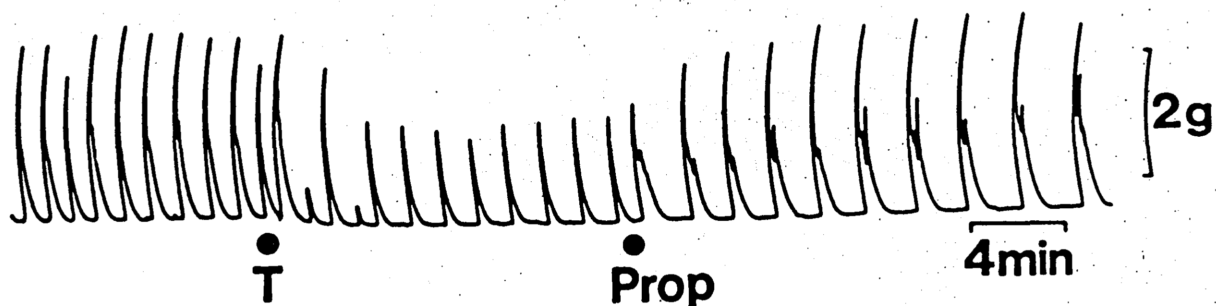


FIGURE 30 Inhibitory effect of tyramine (T, 10^{-6} M) on apamin-induced rhythmic contractions. The inhibitory effect of tyramine was antagonised by the β-adrenoceptor antagonist propranolol (Prop, 5 x 10^{-6} M).

HPLC and radioactive (^3H) experiments

At the end of the last section the possibility was considered and results were presented to suggest that the inhibitory mechanism affected by morphine might be adrenergic in nature. Implicit in this suggestion is the supposition that this adrenergic inhibitory mechanism is still tonically active *in vitro* hours after the preparation has been set up. The primary objective of the HPLC study was, therefore, two-fold:-

i) To determine whether such a tonic release of noradrenaline occurred

ii) To examine the effects of drugs producing rhythmic waves of contraction on such a tonic release.

As a prelude to these experiments and in an effort to become familiar with the experimental techniques involved, an attempt was made to demonstrate spontaneous and electrical field stimulation-induced release of noradrenaline from both the guinea-pig and the mouse vasa deferentia. That such releases occur is well-documented (Macrae, 1983; Gillespie & Macrae, 1983). The noradrenaline and dopamine content of the guinea-pig, rat and mouse vas deferentia as well as the rat colon were also measured.

Factors affecting chromatogram separation

A typical chromatogram is shown in Figure 31. At the working electrode potential of +0.7V a mixture containing 100 pg each of dihydroxyphenylglycol (DOPEG), noradrenaline (NA) and dopamine (DA) was detected and separated. DOPEG and NA eluted very near the solvent front, while DA was retained for a longer period on the column.

The relationship between concentration and signal was linear in the range of from 50-300 pg (Fig. 32). At higher concentrations a linear relationship also existed but the slope differed.

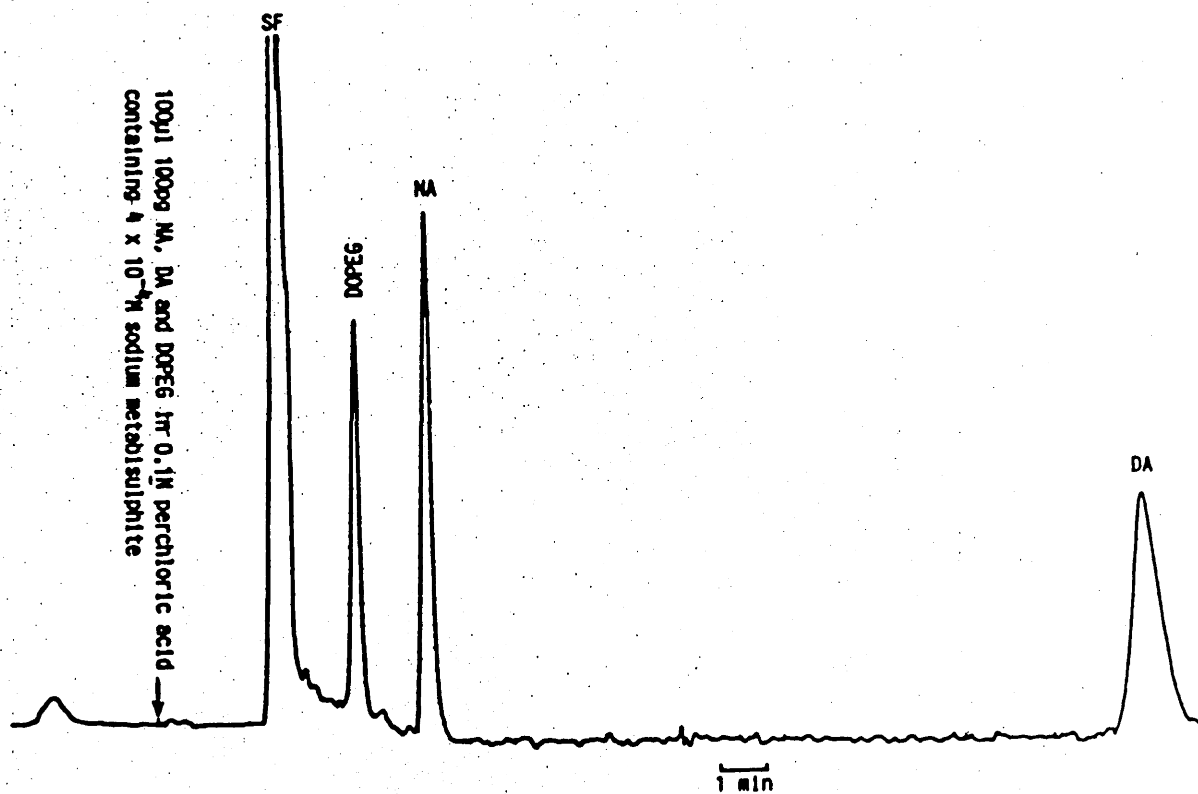


FIGURE 31 Chromatographic resolution of a standard mixture of dihydro-oxyphenylglycol (DOPEG), noradrenaline (NA) and dopamine (DA) solvent front (SF). Mobile phase: 100 mM phosphate buffer pH 3.2. Electrode potential was + 0.7 V vs Ag/AgCl reference electrode. 100 pg each of the standard was injected.

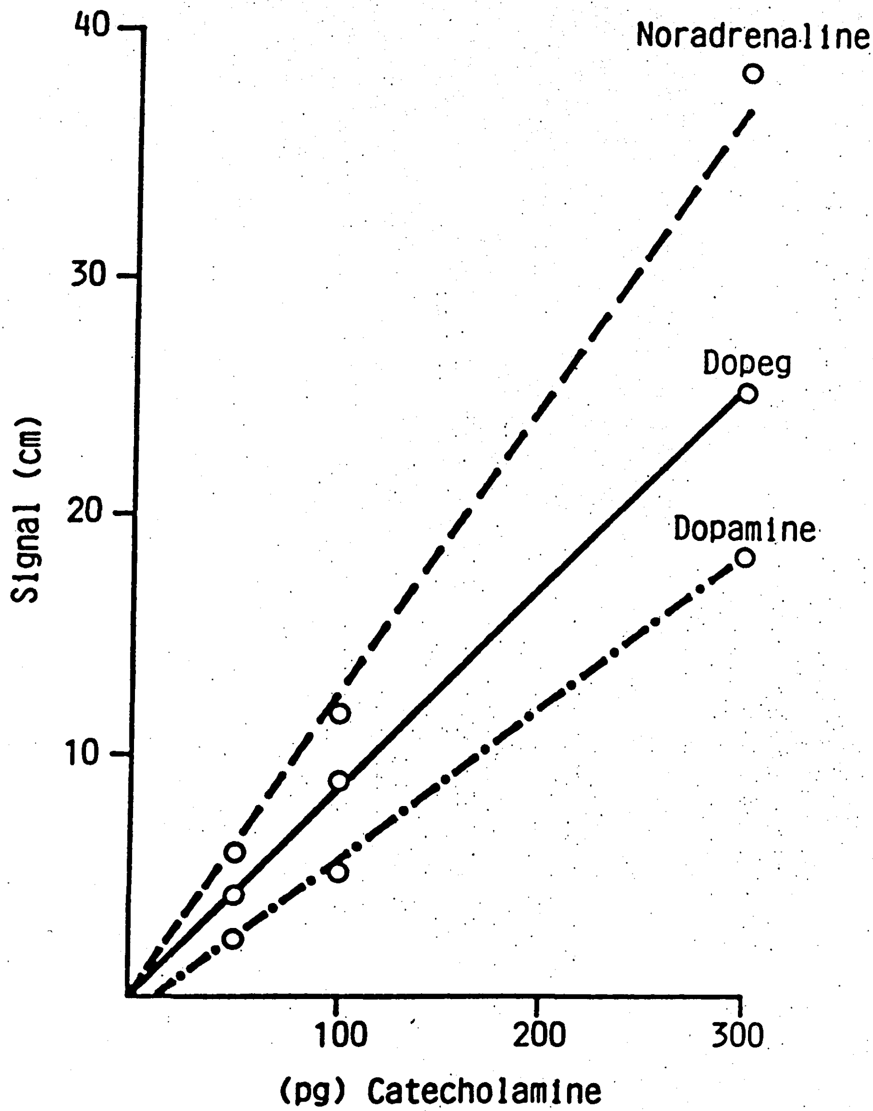


FIGURE 32 Standard graph for noradrenaline, dihydroxyphenylglycol and dopamine. 50-300 pg each of standard was injected. See Methods for details of chromatographic conditions.

As the column aged, its retention capacity decreased and separation became difficult and incomplete. Table 2 shows the effect of adjusting the composition of the mobile phase on retention time of NA and DA. A decrease in either pH, phosphate ion or methanol concentration or an increase in concentration of the ion-pairing agent (octanesulphonic acid) increased the retention time and improved separation.

Effects of working electrode potential

Figure 33 shows the voltammograms of NA, DOPEG, DA and NM. Peak signals occurred at +0.55V for NA, DOPEG and DA. Voltages higher than +0.7V increased the signal-to-noise ratio and the extent of interfering compounds. At working electrode potentials greater than 0.4V, the signal obtained for an equal amount of NA, DOPEG, DA was highest for NA. At potentials between 0.3V and 0.4V, the DA signal was higher than the NA signal. No signal was obtained for NM at working electrode potentials of less than 0.6V.

1.1 The catecholamine content of the vasa deferentia and the rat colon

The catecholamine content of the rat, mouse and guinea-pig vasa deferentia and the rat colon are shown in Table 3. The noradrenaline content of the rat colon was small in comparison with the densely innervated vasa. In addition, no dopamine was detected in the rat colon. Despite the differences in the noradrenaline content of the vasa, their dopamine contents were very similar.

1.2 Spontaneous release of noradrenaline from the guinea-pig vas deferens

Spontaneous overflow of noradrenaline was detected in stop-flow samples collected and analysed at the beginning of experiments (Fig. 34).

The effect of changes in flow rate and the composition of the mobile phase on noradrenaline and dopamine retention time in a single experiment.

	Mobile phase	Flow Rate	Noradrenaline retention time	Dopamine retention time
a	100 mM phosphate buffer	1.5 ml/min	2 min 3 secs	3 min 54 secs
	10% methanol pH 3.2	1.0 ml/min	3 min 6 secs	5 min 44 secs
b	100 mM phosphate buffer	1.0 ml/min	3 min 45 secs	9 min 44 secs
	5% methanol pH 3.2	0.8 ml/min	5 min 10 secs	13 min 47 secs
		0.5 ml/min	9 min 6 secs	24 min 10 secs
c	100 mM phosphate buffer pH 3.2 10% methanol	0.5 ml/min	not determined	7 min 45 secs
d	50 mM phosphate buffer pH 3.2 10% methanol	0.5 ml/min	not determined	12 min 15 secs

a and b were done separately from c and d

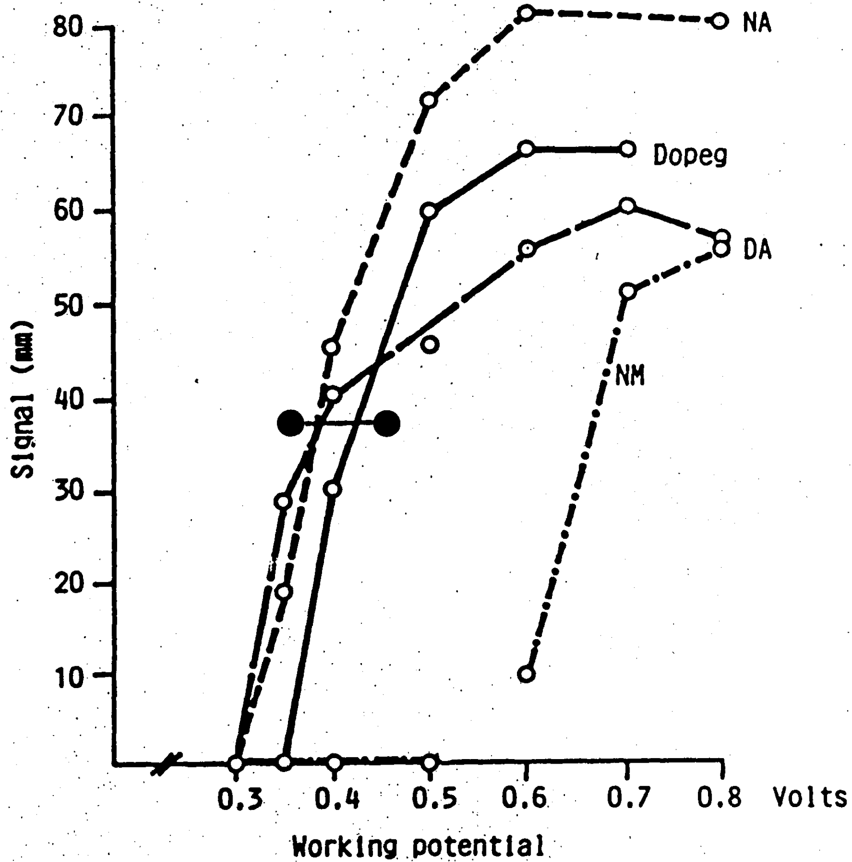


FIGURE 33 Effects of detector electrode potential variation on the detection of catecholamines and metabolites. 100 pg each of noradrenaline (NA), dihydroxyphenylglycol (DOPEG), dopamine (DA) and normetanephrine (NM) were injected. ●—● The crossing over of NA and DA signals extended to 0.45V.

TABLE 1

Catecholamine content of rat, mouse and guinea-pig vasa deferentia and the rat colon. Mean ± S.E.M., n = 7.

Tissue	Content (ug/gm)			
	NA (± SEM)	p Value	DA (± SEM)	p Value
Rat vas deferens	8.20 ± 1.06		0.64 ± 0.16	
Mouse vas deferens	6.90 ± 0.71	NS	0.65 ± 0.17	NS
Guinea-pig vas deferens	13.57 ± 3.84	NS	0.60 ± 0.15	NS
Rat colon	0.06 ± 0.01	<0.001	ND	-

The p values refer to comparisons between the contents of NA and DA in the mouse vas deferens, guinea-pig vas deferens, rat colon and the content of these amines in the rat vas deferens.

ND means none detected.

To eliminate the possibility that this spontaneous release was due to non-specific leakage caused by adrenergic nerves during preparation (Macrae, 1983), at least 45 minutes was allowed between set up and collection of the stop-flow sample. No dopamine was detected in the spontaneous sample.

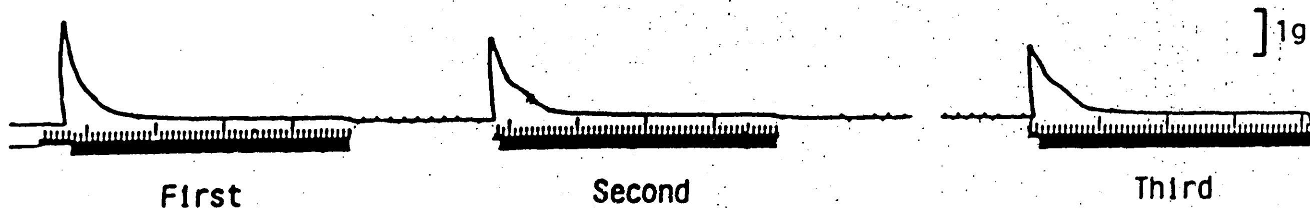
1.3 Noradrenaline overflow evoked by electrical stimulation in the guinea-pig vas deferens

Electrical stimulation at a frequency of 20 Hz (4,800 stimuli) produced an increase in noradrenaline overflow from the guinea-pig vas deferens (Fig. 34). The histogram showed that with repeated stimulation the evoked-release of noradrenaline declined gradually from the 1st period. The mechanical responses to nerve stimulation are also shown and it is clear that both the initial contractile response and the slow secondary component of the mechanical response also declined with repeated periods of stimulation.

1.4 Spontaneous and electrical stimulation-evoked release of NA, DA and DOPEG from the mouse vas deferens

Before the experiments described for the mouse vas deferens were performed the chromatography conditions were investigated and optimised to make the system capable of detecting the NA metabolites which were mainly DOPEG and normetanephrine (NM) (see Methods). However, NM was not adsorbed on to alumina, thus only DOPEG, NA and DA, which were adsorbed and could be eluted, were analysed.

Unlike the guinea-pig vas deferens, where spontaneous and electrical field stimulation-evoked release of NA was consistently demonstrated and followed a pattern, the results obtained with the mouse



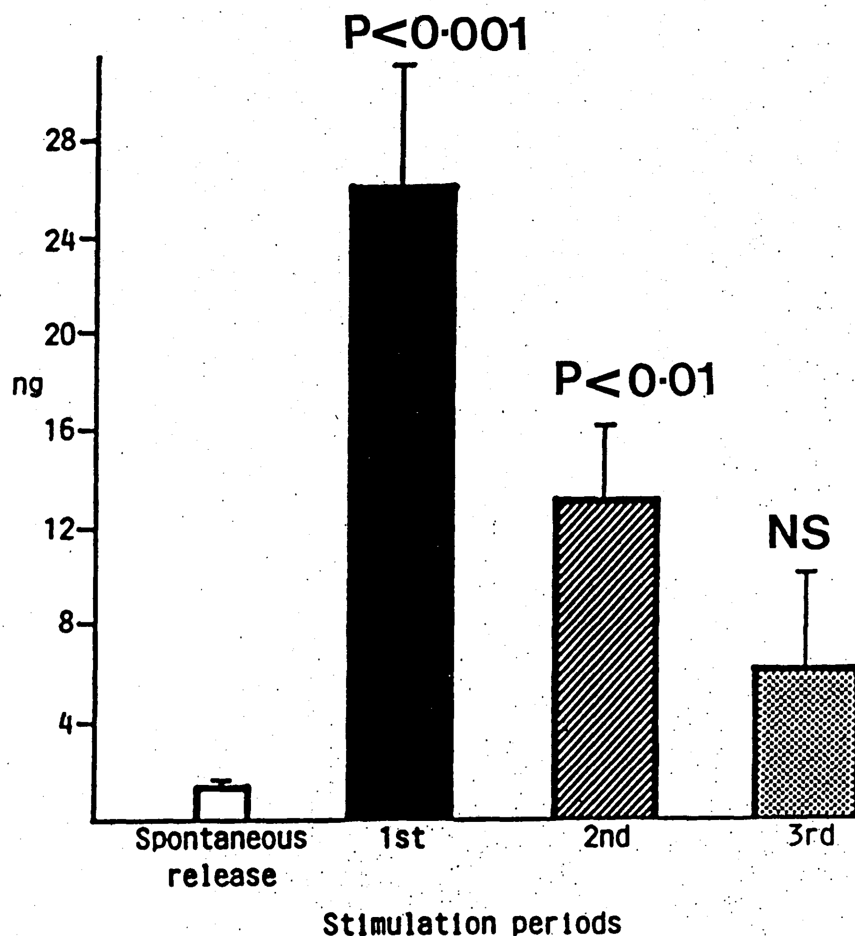


FIGURE 34 Mechanical response of the guinea-pig vas deferens to 3 periods of transmural electrical stimulation (4,800 pulses, 1 ms, 20 Hz, supramaximal voltage) and the overflow of noradrenaline evoked by stimulation. Both the mechanical response and the overflow of noradrenaline evoked by stimulation decreased from the 1st period. Means $\pm$ S.E.M., n = 8. P values refer to comparisons between the amount of NA released during each period of stimulation with the amount of NA released spontaneously.

vas deferens following electrical stimulation (20 Hz, 4,800 pulses) varied from one experiment to another not only in the quantities of catecholamines released but also in the proportion of their release. The various patterns of release are enumerated below.

(a) In some experiments no spontaneously-released catecholamine was detected nor was there any release as a result of field stimulation, despite the fact that the vas contracted in response to stimulation.

(b) Only DOPEG was detected both during nerve stimulation and spontaneous release, the proportion of DOPEG being higher during spontaneous release than during nerve stimulation (Table 4).

(c) Noradrenaline was the only major catecholamine detected. However, unlike the guinea-pig vas deferens where the electrical stimulation-evoked release of NA was greater than the spontaneous release, and decreased from the first stimulation period to the third, no clear pattern was seen. In fact, the amount of NA released during the third stimulation period was more than the first and second period (Table 4b).

(d) In some experiments both DOPEG and NA were released spontaneously. However, during the first and second stimulation, no NA was detected. In addition, similar amounts of NA were released spontaneously and during electrical field stimulation (Table 4C and D).

1.5 Effect of phenoxybenzamine (PB) on the pattern of NA, DA and DOPEG released in the mouse vas deferens

The effect of PB, a potent pre- and post-junctional α-adrenoceptor antagonist, which also blocks both uptake 1 and 2 mechanisms, was examined on the transmitter overflow in the mouse vas deferens. The pattern of results varied from one preparation to another.

Spontaneous and stimulation-induced catecholamine overflow from the mouse vas deferens.

	Stimulation period	DOPEG (ng)	NA (ng)	DA (ng)
A	1st spontaneous release	1.59	ND	ND
	1st stim.	0.36	ND	ND
	2nd stim.	ND	ND	ND
	3rd stim.	0.28	ND	ND
	2nd spontaneous release	0.28	ND	ND
B	1st spontaneous release	ND	0.42	ND
	1st stim.	ND	0.67	ND
	2nd stim.	0.17	0.67	ND
	3rd stim.	ND	0.86	ND
	2nd spontaneous release	ND	0.58	ND
C	1st spontaneous release	0.24	0.12	ND
	1st stim.	0.24	ND	ND
	2nd stim.	ND	ND	ND
	3rd stim.	0.16	0.12	ND
	2nd spontaneous release	0.16	0.12	ND
D	1st spontaneous release		ND	0.31
	1st stim.		0.90	0.20
	2nd stim.		ND	ND
	3rd stim.		ND	ND
	2nd spontaneous release		ND	ND

A, B, C and D were separate experiments.

ND means none detected.

(a) In experiments where no amine was detected either as a result of spontaneous release or as a result of field stimulation, the addition of PB (10^{-5} M) after the first stimulation and thirty minutes before the second stimulation, caused an increase in the overflow of both NA and DA (Table 5A).

(b) In other experiments where DOPEG, NA and DA were detected as a result of spontaneous release and as a result of nerve stimulation, PB (10^{-5} M) added after the second stimulation period increased the amount of NA released but decreased the amount of DOPEG released. There was an increase in the amount of DA released during the third stimulation, followed by a decrease (Table 5B and C). Insufficient data was available to permit a statistical analysis to be performed or any conclusion to be reached other than that the results were very variable.

(c) No increase in spontaneous overflow and only a slight increase in field stimulation-induced overflow was apparent following the addition of PB (10^{-5} M) (Table 5D).

1.6 Effect of PB on the mechanical response to nerve stimulation in the mouse vas deferens

The response of the mouse vas deferens to electrical field stimulation (4,800 pulses at 20 Hz) consisted of two components. First, there was a rapidly-developed response and, secondly, a slower, more prolonged contraction when stimulation was continued for several minutes. This second component disappeared almost completely after about 1 minute and was replaced by a low level of maintained tone (Fig. 35). PB (10^{-5} M) inhibited both components of the mechanical response irrespective of the pattern of overflow obtained after phenoxybenzamine.

Effect of phenoxybenzamine on spontaneous and stimulation induced catecholamine overflow from the mouse vas deferens.

	Stimulation period	DOPEG (ng)	NA (ng)	DA (ng)
A	1st spontaneous release		0.10	ND
	1st		0.26	ND
	2nd*		0.63	0.01
	3rd*		0.53	ND
	2nd spontaneous release*		0.10	ND
B	1st spontaneous release	ND	ND	ND
	1st	0.46	0.20	ND
	2nd*	0.39	0.30	ND
	3rd*	ND	0.35	ND
	2nd spontaneous release*	ND	ND	ND
C	1st spontaneous release	0.60	0.45	0.79
	1st	0.50	0.40	0.47
	2nd*	1.0	0.20	0.67
	3rd*	0.50	1.0	4.05
	2nd spontaneous release*	0.35	0.78	0.32
D	1st spontaneous release	ND	ND	ND
	1st	ND	0.20	ND
	2nd*	ND	0.26	ND
	3rd*	ND	0.37	ND
	2nd spontaneous release*	ND	ND	ND

* Experiments done in the presence of phenoxybenzamine (10^{-5} M).

A, B, C and D were separate experiments.

ND means none detected.

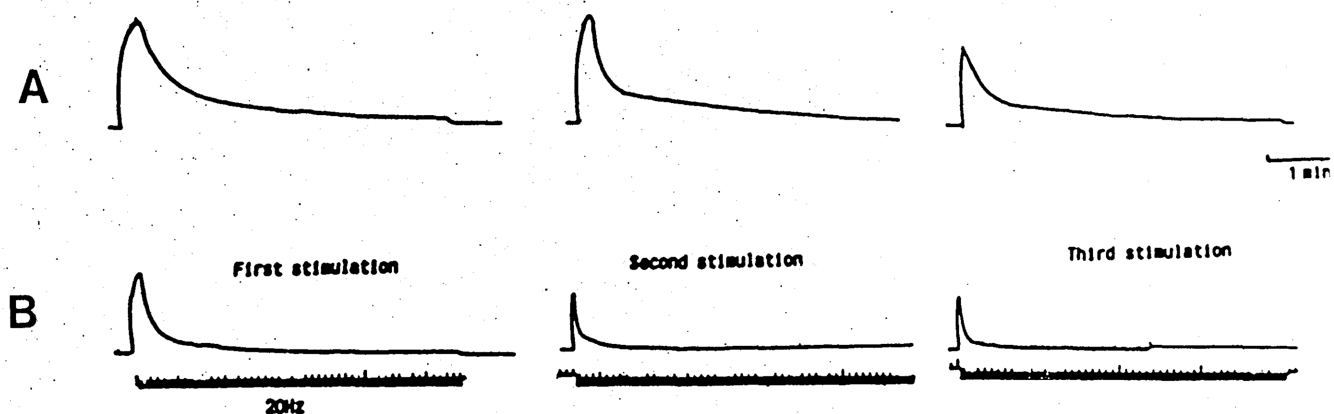


FIGURE 35 Effect of phenoxybenzamine (10^{-5} M) on the mechanical response of the mouse vas deferens to electrical transmural stimulation (4,800 pulses at 20 Hz, 1 ms). **A** shows the response of the vas deferens to 3 periods of stimulation. The initial component of the motor response and the low level secondary component declined during successive stimulations. **B** shows the effect of phenoxybenzamine which was present during the second and third period of stimulation. Phenoxybenzamine accelerated the decline of the initial component of the motor response to field stimulation. **A** and **B** are from different experiments.

1.7 Spontaneous release of NA from the rat colon

Examination of Table 4 reveals the low content of NA in the rat colon. The primary objective of the HPLC study was to determine whether NA was released spontaneously in segments of the rat colon. In the experiments where this was attempted, no spontaneous release could be demonstrated. This was not surprising since the NA content of the tissue was low.

2.1 Tritium accumulation in the rat colon

The time course of the accumulation of (^{3}H)-NA was determined in the isolated colon by incubating the tissue with 444 KBq/ml (^{3}H)-NA for time intervals varying from 30 sec to 1 hr. Accumulation was linear up to 20 minutes, thereafter remaining fairly constant (Fig. 36).

In order to study the relationship between (^{3}H) accumulation and substrate concentrations, accumulation was measured after 2 minutes incubation with various substrate concentrations. The accumulation of (^{3}H)-NA was saturable (Fig. 37).

2.2 Efflux of tritium from the rat colon

Rat colon was incubated in 444 KBq/ml (^{3}H)-NA for 20 minutes and then set up for mechanical recording. Analysis of the Krebs' solution collected at 10 minute intervals showed that over the first 40 minutes there was a fast efflux of loosely bound label from the tissue and a second, slow efflux probably related to spontaneous release of (^{3}H)-NA from nerve endings. There was little mechanical activity in the absence of drugs throughout the collecting period (Fig. 38).

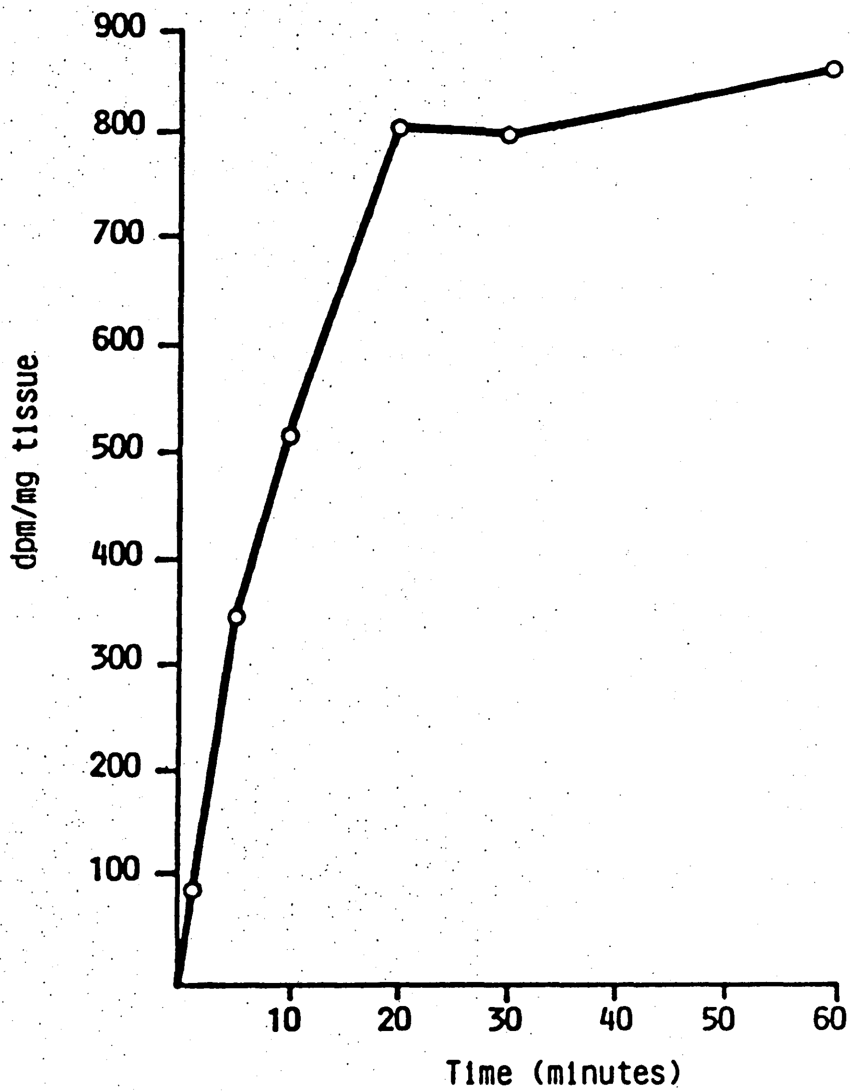


FIGURE 36 Time course of accumulation of (^{3}H)-NA into the rat colon. Accumulation expressed as disintegrations per minute (dpm) per mg tissue. Duration of incubation in minutes.

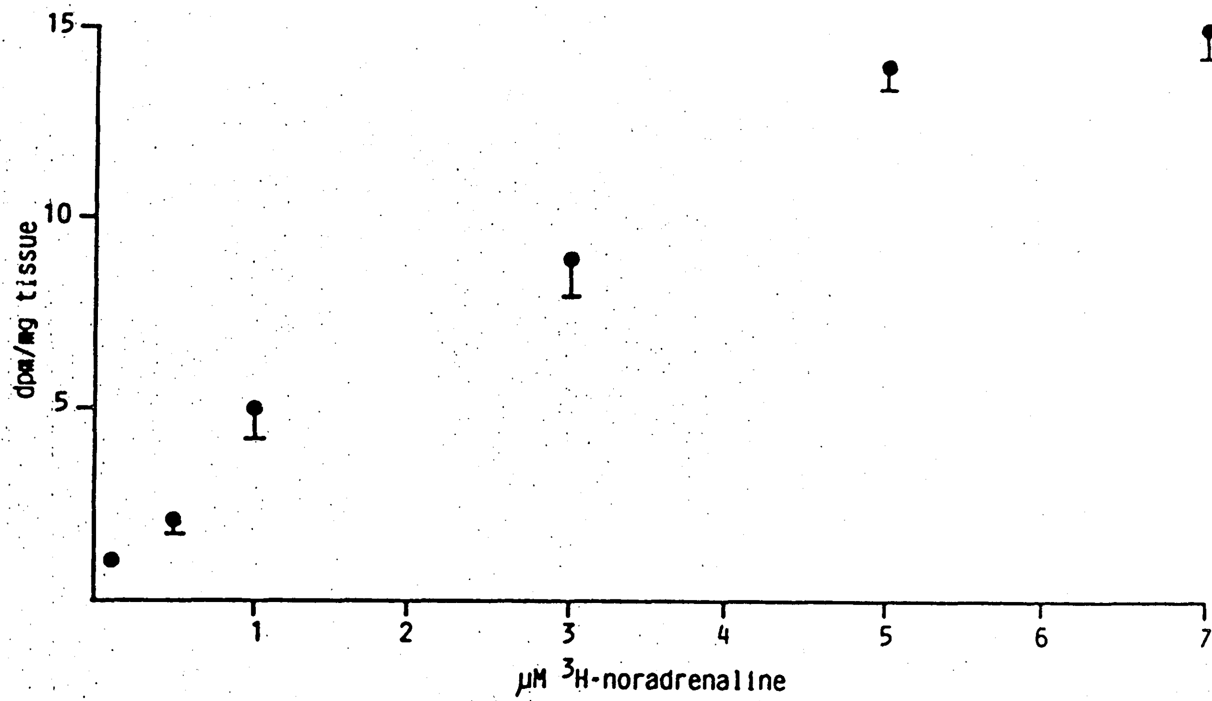


FIGURE 37 Uptake of (^{3}H) by the rat colon. The colon was exposed to different concentrations of (^{3}H)-NA for 2 minutes, washed briefly in a large volume of ice-cold Krebs' blotted dry and thereafter counted for radioactivity. Each point is the mean ($\pm$ S.E.M.) of at least 8 observations.

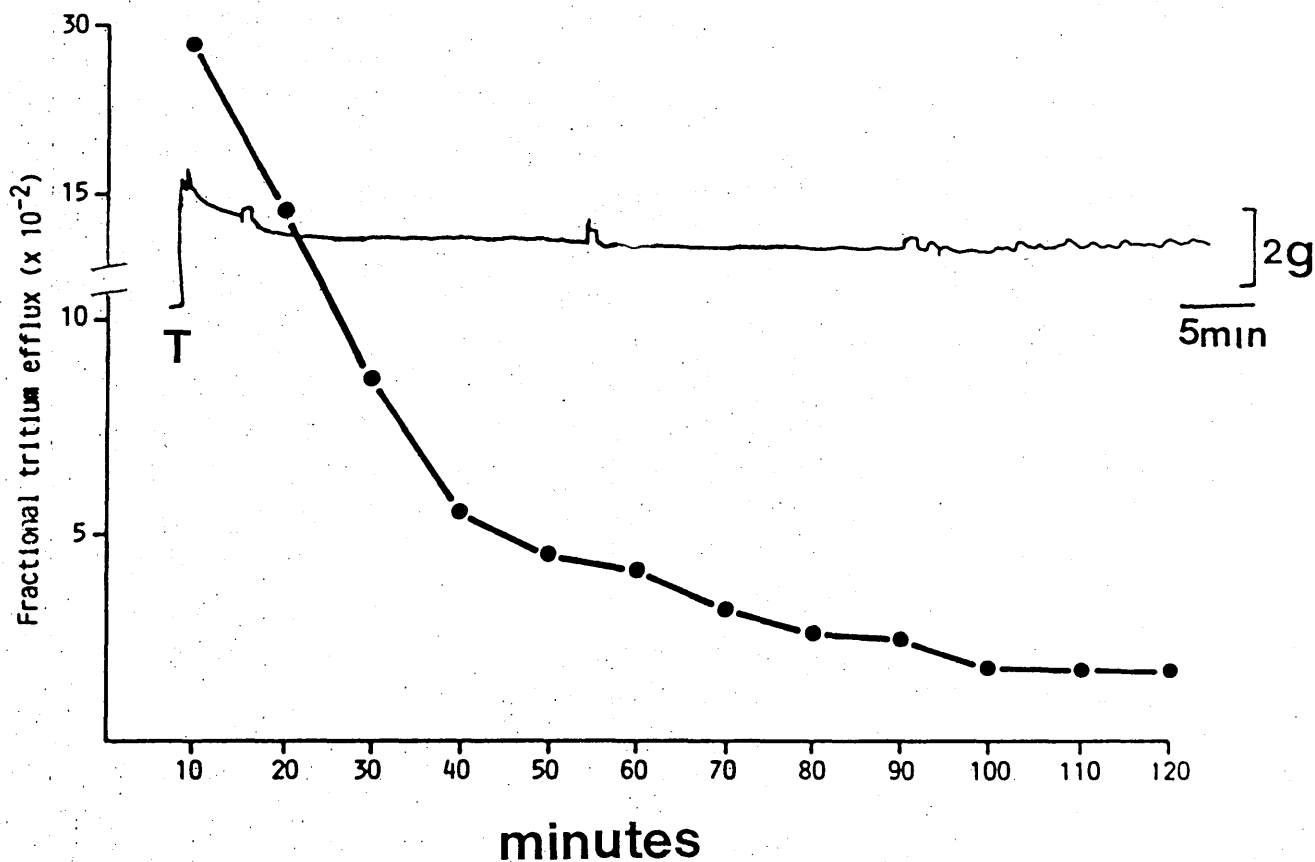


FIGURE 38 Efflux of ^{3}H from the isolated rat colon after prelabelling with (^{3}H)-noradraneline. Ordinate: fractional tritium efflux. Abscissa: time after preloading with (^{3}H)-NA in minutes. Colon was incubated for 20 minutes at 37°C with 444 KBq (^{3}H)-NA and thereafter washed in a large volume of Krebs' solution before being set up for mechanical recording. The initial resting force was set to 2 g at T. The Krebs' bathing the colon was replaced every 10 minutes and counted for radioactivity. Superimposed on the efflux curve is a trace showing the low level of mechanical activity in the colon.

2.3 Effect of drugs on tritium efflux and mechanical response of the rat colon

This study examined the effects of drugs, which produced rhythmic waves of contraction in colon, on the efflux of (^{3}H). The Krebs' solution used for efflux was changed after 90 minutes to one containing appropriate concentration of agonists.

Morphine (5×10^{-6} M), clonidine (2×10^{-8} M) and TTX (10^{-7} M) produced rhythmic waves of contraction without having any effect on the efflux of (^{3}H) (Figs. 39, 40 and 41).

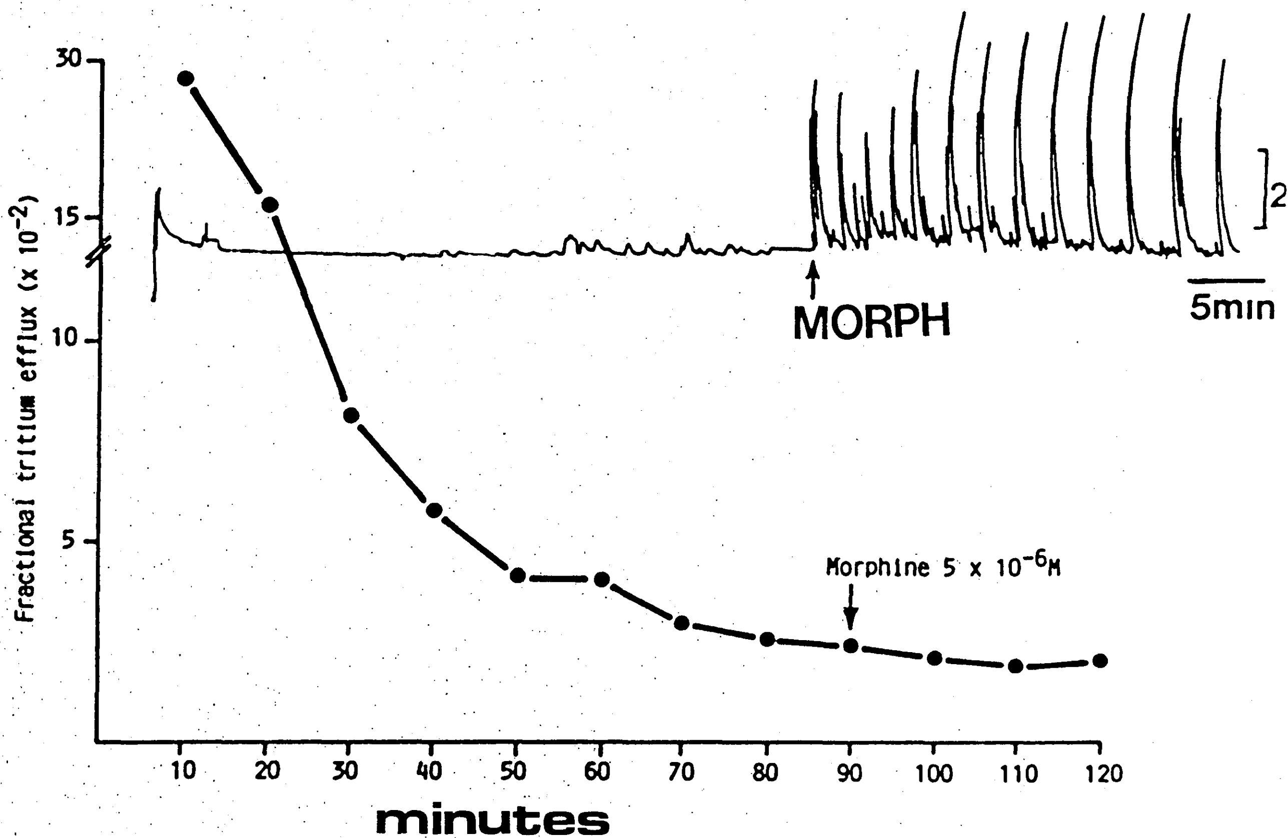


FIGURE 39 Effect of morphine on the efflux of tritium from the rat colon and mechanical activity of the colon. Experimental conditions were as described for Fig. 38 except that the normal Krebs' solution was changed to one containing morphine (5×10^{-6} M) at the 90th minute. Morphine produced rhythmic waves of contractions without affecting the tritium efflux.

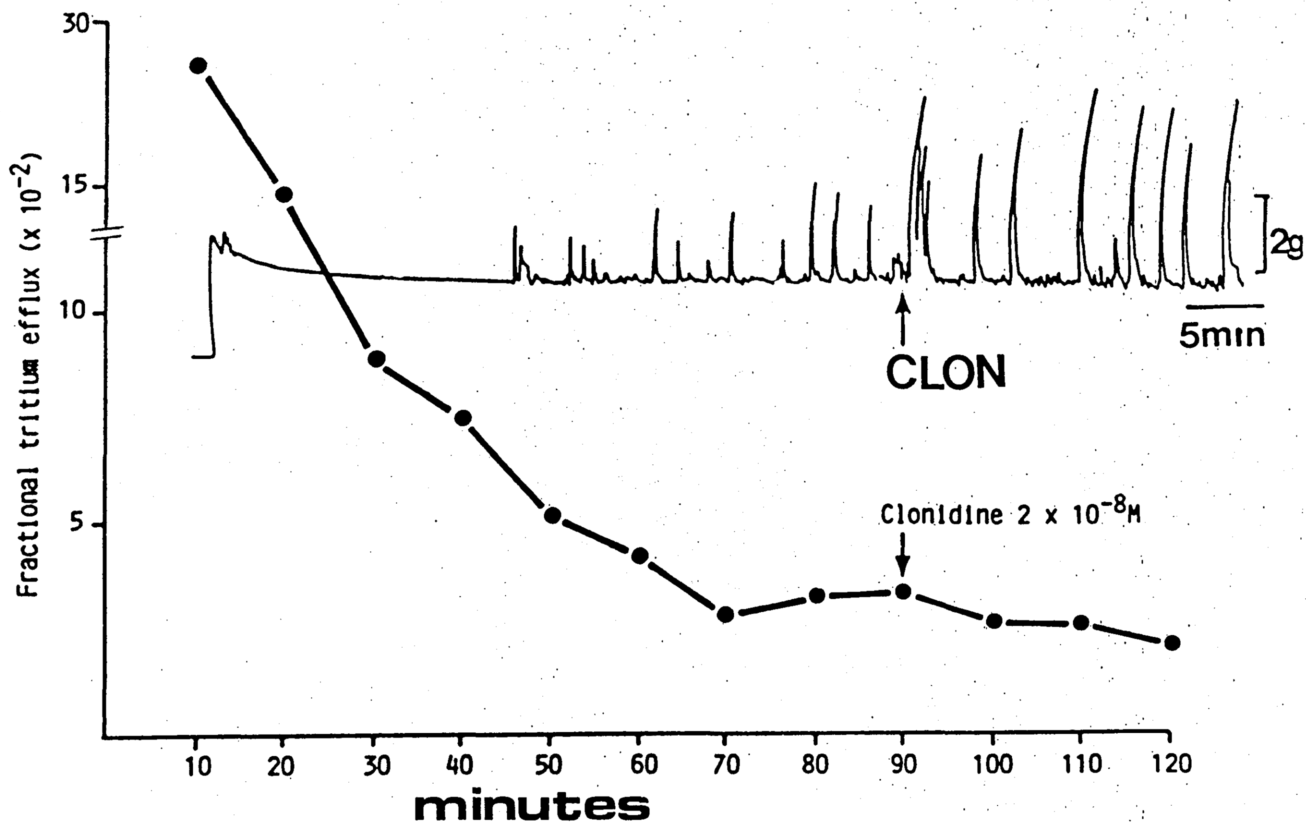


FIGURE 40 Effect of clonidine on the efflux of tritium from the rat colon and the mechanical activity of the colon. Experimental conditions were as described for Fig. 38 except that the normal Krebs' solution used was changed to one containing clonidine (2 x 10^{-8} M) at the 90th minute. Clonidine produced rhythmic waves of contractions without affecting tritium efflux.

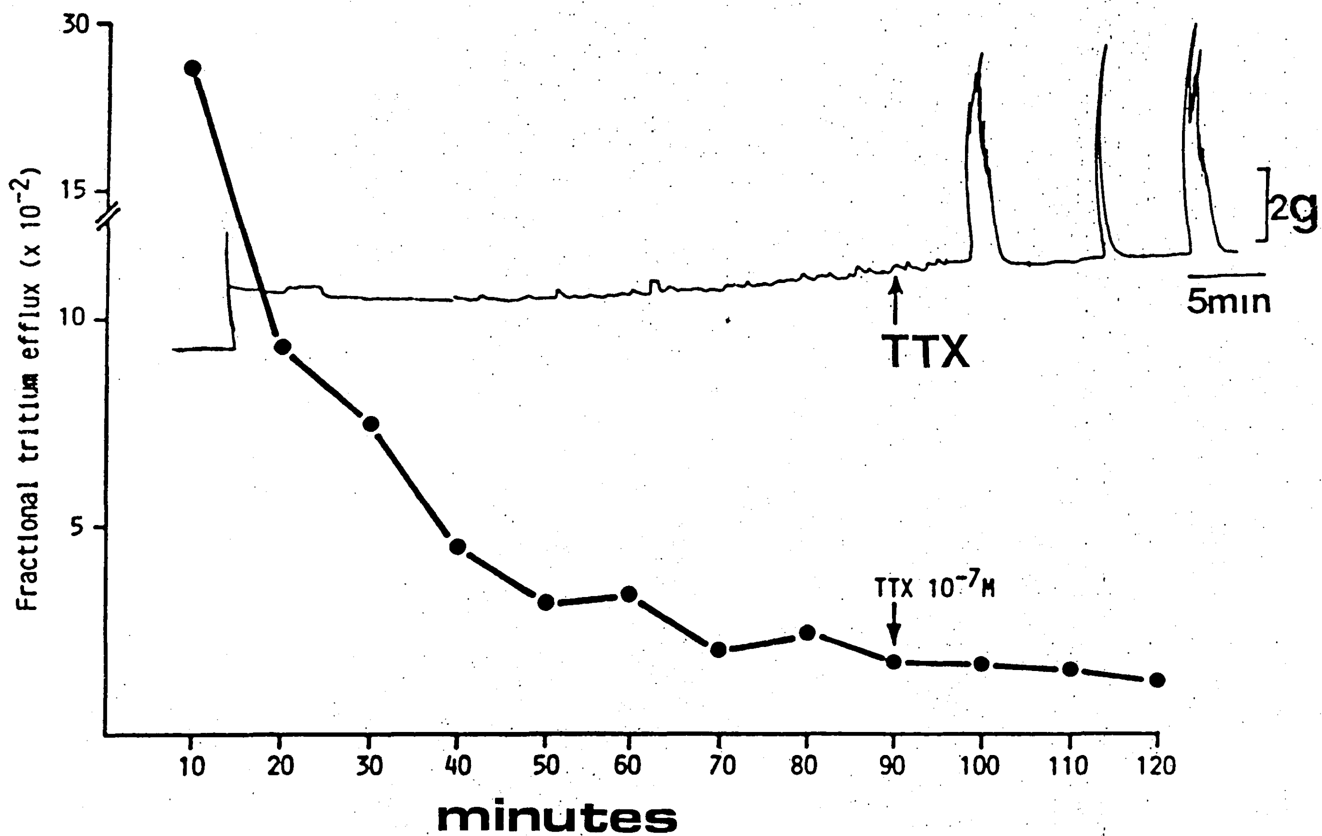


FIGURE 41 Effect of TTX on the efflux of tritium from the rat colon and the mechanical activity of the colon. Experimental conditions were as described for Fig. 38 except that the normal Krebs' solution was changed to one containing TTX (10^{-7} M) at the 90th minute. TTX produced waves of rhythmic contractions without affecting tritium efflux.

DISCUSSION

The effects of morphine and the opioid peptides on the gut motility are still incompletely understood. The aim of this study was, first, to investigate the effects of the opioids on the intestine. Secondly, to elucidate some of the mechanisms and the possible neuronal basis of these effects. In particular, this study examined the possible involvement of 5-HT and ACh in these effects (Burks, 1973, 1976; Huidobro-Toro & Way, 1981) and the possible existence of a tonically active, non-adrenergic non-cholinergic inhibitory mechanism (Gillan, 1977; Gillan & Pollock, 1980; Scheurer <u>et al</u>, 1981). Electrical field stimulation of the isolated intestine was also employed to provide further evidence about the role of excitatory and inhibitory nerves in these responses.

The preparation used for this investigation was the isolated colon of the rat. The colon was chosen primarily because (a) morphine as well as the enkephalins have excitatory effects on this tissue (Gillan & Pollock, 1980; Nijkamp & Van Ree, 1980; Boura & Olley, 1981; Scheurer <u>et al</u>, 1981) and (b) since specific opioid receptors and also enkephalins are present in the gastro-intestinal tract (Manara & Bianchetti, 1985) and are located in or near the myenteric plexus (Hughes, Kosterlitz & Smith, 1977). Moreover, since the enkephalins are released in response to nerve stimulation (Schulz, Wuster, Simantov, Snyder & Hertz, 1977; Corbett, Sosa, McKnight & Kosterlitz, 1980), their physiological significance may be expected to be primarily local and, therefore, apparent <u>in vitro</u>.

The results obtained in this study confirmed some of the results obtained by other workers (Gillan & Pollock, 1980; Nijkamp & Van Ree, 1980; Scheurer <u>et al</u>, 1981) that morphine and the opioid peptides

produce rhythmic contractile activity in the terminal colon of the rat, but cannot be explained by any single hypothesis previously postulated. This rhythmic contractile activity is receptor-mediated but the concentration response relationship is difficult to establish. Morphine-induced rhythmic contractions were abolished by the specific opioid antagonist naloxone. While the rhythmic waves of contraction produced by the opioids were "all or none" in nature, the size of the initial response was concentration-related.

The ability of opioids to initiate waves of rhythmic contraction has either been ignored in the past or the experiments were carried out in conditions which minimised the chances of their occurrence (Huidobro-Toro & Way, 1981; Moritoki et al, 1984).

Although the excitatory effect of opioids on the intestine is well-documented, the exact mechanisms by which the opioids produce this effect is far from being clear. This is further complicated by the fact that, in vivo, morphine and the opioid peptides can also influence gastro-intestinal motility by centrally-mediated effects (Parolaro et al, 1977; Burleigh et al, 1981; Burks, 1980). Evidence for central opioid-sensitive sites for inhibition of gastro-intestinal transit comes primarily from animal studies, in which morphine-like drugs were administered directly into the CNS. Results of such studies are of considerable interest, since they provide evidence for the existence of specific action sites within the CNS influencing the gut. However, centrally-mediated actions on bowel by narcotic drugs injected directly into the CNS may not explain entirely their effects on the intestine following systemic administration (Manara & Bianchetti, 1985). Since morphine does not pass the blood brain barrier easily (Oldendorf, Hyman,

Braun & Oldendorf, 1972; Jaffe & Martin, 1980), therapeutic doses cannot be expected to have a predominantly central component on intestinal motility. Recently, the spinal cord has also been considered a potential locus of opioid action in the production of gastro-intestinal motor effects.

Morphine and the opioid peptides administered intrathecally in the spinal cord of mice effectively inhibit the passage of radiolabelled marker through the gastro-intestinal tract (Porrecca & Burks, 1983; Porrecca, Filla & Burks, 1983) but a similar study in the rat did not confirm the spinally-elicited constipating effect of morphine in this species (Vaught, Cowan & Gimerek, 1983). It may be that the rat is a better predictive model for humans than mice because constipation does not seem to occur in patients given morphine to produce spinal analgesia (Zenz, Schappler-Scheele, Neuhaus, Pipeubrock & Hilfrich, 1981; Chirubasik, 1984).

An additional peripheral site, at which opioids might act to produce their anti-transit effects is the intestinal mucosa, where they can affect water and electrolyte reabsorption (Powell, 1981). However, the ability of morphine and related drugs to produce rhythmic contractile activity in isolated intestine eliminates the possibility that the CNS or the extrinsic autonomic innervation are the only sites of action.

A mechanism postulated to explain the stimulant action of morphine on the intestine involves the release of intestinal 5-HT and ACh. From experiments on the small intestine of rat and dog Burks (1973, 1976) and Huidobro-Toro and Way (1981) suggested that morphine released 5-HT

which then stimulated the smooth muscle both directly and indirectly by releasing ACh from cholinergic nerve in the intestinal wall.

This hypothesis was based on (1) the observation that responses to morphine were abolished by the 5-HT antagonists methysergide and cyproheptadine, and reduced by atropine; (2) that responses to morphine were absent in tissues from animals pretreated with reserpine to deplete the 5-HT content of the intestine. In this study, the 5-HT antagonist methysergide non-competitively inhibited the concentration-response relationship to morphine, while it was ineffective against the rhythmic waves of contraction produced by the opioids. In contrast to the non-competitive nature of methysergide antagonism, naloxone competitively inhibited this initial response and abolished the waves of rhythmic contraction produced by morphine. At first sight, the inhibitory effect of methysergide might be taken as an indication of the involvement of 5-HT in this initial response to morphine. The result obtained with 5-HT is complicated by the lack of specific 5-HT antagonist. It bears emphasising that methysergide has a spectrum of effects which become more apparent as its concentration is raised. At concentrations that are effective in blocking neural 5-HT receptors, it is comparably effective in antagonising the contractile effects of carbachol, dimethylphenylpiperazinium and histamine in the intestine (Furness & Costa, 1982). Morphine may be added to the list of diverse drugs whose effects in the intestine are antagonised by methysergide (Nijkamp & Van Ree, 1980; Huidobro-Toro & Way, 1981; this study). It has been suggested that attempts to use this drug in the analysis of the actions of 5-HT on intestinal nerves should profitably be abandoned because of its non-specificity (North, Henderson, Katayama & Johnston, 1980).

Indeed, the ability of another 5-HT antagonist, cyproheptadine, to inhibit morphine-induced contractions, albeit non-competitively (Gillan & Pollock, 1980; Nijkamp & Van Ree, 1980), may not be related to antagonism at the 5-HT receptor site. Cyproheptadine also inhibits the membrane translocation of calcium in smooth muscle by reducing the flow of Ca^{++} ions through voltage-dependent Ca^{++} channels (Lowe, Matthews & Richardson, 1981), hence great care must be taken in the interpretation of the results with these 5-HT antagonists.

In contrast to the equivocal nature of the results with methysergide, the results obtained with PCPA, which depleted the tissue of 5-HT (Koe & Weissman, 1966; Weber, 1969), provided evidence against the involvement of 5-HT in the excitatory effects of opioids in the colon. Since, in colon from PCPA pretreated rats, morphine was still capable of producing naloxone-sensitive rhythmic waves of contractions, it seems unlikely that 5-HT is required for this response. However, since a small residual quantity of 5-HT remained in the colon from PCPA-treated rats, this observation alone does not eliminate the possible involvement of 5-HT in this phenomenon.

This study also confirmed the ineffectiveness of reserpine in depleting the 5-HT content of the rat intestine (Erspamer, 1966b; Carlsson, 1969). However, it is interesting that in an attempt to link the excitatory effect of morphine with the release and effect of 5-HT, reserpine was used to deplete the 5-HT level, although the 5-HT content was not measured. Furthermore, in this study, the ability of morphine to cause the colon to contract rhythmically was unaffected in tissues rendered insensitive to 5-HT by prolonged exposure to high concentration of this amine.

More convincing evidence against the involvement of 5-HT/ACh in the response of the rat colon to morphine was obtained from experiments which demonstrated that the characteristic excitatory effect of opioids could not be mimicked by administered ACh or 5-HT. In addition, atropine did not interfere with the ability of morphine to cause the colon to contract rhythmically. Perhaps the most striking characteristics of the opioid-induced excitatory response were its rhythmicity and persistence, neither of which is compatible with the ACh/5-HT release hypothesis.

These results and conclusions differ from those of Huidobro-Toro and Way (1981), who obtained concentration-related contractions to morphine in the rat colon and concluded that the excitatory effects of opioids were mediated by 5-HT. These workers pointed out that their Long Evans rats were more sensitive to opioids than other strains such as the Wistar rats used in this study. However, it is unlikely that this difference could explain the discrepancies between the results obtained in the two studies. The present results are not consistent with the idea that a 5-HT/ACh mechanism is involved in the effects of opioids in the rat colon. Fontaine and Reuse (1985) recently reached a similar conclusion about the non-involvement of 5-HT and/or ACh in the excitatory response of the mouse colon to opioids.

An alternative explanation of the excitatory effects of opioids is that morphine removes a tonic inhibitory neural influence that normally suppresses myogenic activity so that the contractions produced by morphine are myogenic (Gillan & Pollock, 1980; Scheurer <u>et al</u>, 1981; Moritoki <u>et al</u>, 1984; Fontaine & Reuse, 1985). This hypothesis was based on that proposed by Wood to explain the stimulant action of

atropine, TTX and local aneaesthetic in cat small intestine (Wood, 1972, 1975). From studies of the electrical activity of neurones in Auerbach's plexus and the mechanical activity of the circular layer in cat small intestine, Wood proposed that the circular muscle was maintained in a state of inhibition by the continuous release of an inhibitory transmitter from spontaneously active neurones and that removal of this inhibitory influence unmasked the inherent myogenic activity of the circular muscle. Evidence that blockade of neural activity by atropine, local anaesthetic or TTX (Wood, 1972; Wood & Marsh, 1973; Ohkawa & Prosser, 1972b; Biber & Fara, 1973; Tonini, Secchini, Frigo & Crema, 1974), cold storage (Wood, 1972), surgery (Schiller, Suriyapa, Mutchler & Anderson, 1973) or congenital absence of ganglion cells (Wood, 1973) produce similar effects on the mechanical activity of intestinal muscle were cited in support of this hypothesis (Wood, 1975). Despite the attractiveness of this hypothesis, the continuous release of such an inhibitory transmitter _in vitro_, has not been demonstrated (Daniel & Sarna, 1978). However, if this explanation is correct, then other drugs that interfere with neuronal inhibitory mechanisms should have effects similar to morphine in the colon.

The results obtained in this study with TTX, which abolishes the nerve action potential by blocking sodium conductance (Narahashsi, 1974) and with apamin, which inhibits potassium-dependent inhibitory mechanism in smooth muscle (Banks, Brown, Burgess, Burnstock, Claret, Cocks & Jenkinson, 1979), are consistent with such an explanation. Both TTX and apamin produced regular rhythmic contractions which resembled morphine-induced contractions but were unaffected by naloxone, atropine or methysergide.

The question that arises concerns the possible nature of this inhibitory mechanism that is affected by opioids to reveal myogenic activity. Gillan and Pollock (1989), Scheurer et al (1981) and Moritoki et al (1984) proposed that morphine acts presynaptically to inhibit the final release of an NANC inhibitory transmitter, thereby unmasking intrinsic myogenic activity, whereas Nijkamp and Van Ree (1980) suggested that an adrenergic mechanism could be inhibited by morphine. To clarify which of these transmitters was involved in the tonic inhibitory mechanism, responses of the colon to electrical field stimulation and the effects of drugs on these responses were investigated.

Electrical field stimulation of the colon provided evidence about the nature of the morphine-induced excitatory response and the possible role of inhibitory nerves in this response. Initial experiments confirmed the results of previous workers that there are cholinergic motor nerves in the colon (Stockley & Bennett, 1973; Bennett & Stockley, 1975; Gillan & Pollock, 1980). Field stimulation of the colon produced an excitatory response which was maximal at frequencies between 10 and 20 Hz. This excitatory response was antagonised by atropine, TTX or morphine. Since morphine did not affect responses to administered ACh (Cherubini, Morita & North, 1985), it is likely that morphine acted on presynaptic nerves to inhibit ACh release (Down & Szerb, 1980; North, 1982) either by hyperpolarizing the soma of the enteric neurones (Wood, 1980) or by blocking conduction in nerve cell processes (Morita & North, 1981).

On cessation of stimulation, a post-stimulus contraction was observed. This contraction is thought to be myogenic in origin and to

arise from activation of non-adrenergic inhibitory nerves, which in turn trigger the release of prostaglandins (Burnstock et al, 1975). However, the involvement of prostaglandins in this post-stimulus contraction is controversial. Kadlec, Masek and Seferna (1974) found no evidence of an inhibitory effect of indomethacin on this post-stimulus contraction. Bauer, Matusak, Bezekwa, Benes and Kuriyama (1982) showed that indomethacin dissolved in ethanol markedly depressed the rebound contraction. On the other hand, indomethacin dissolved in a weakly alkaline bicarbonate solution does not inhibit but rather increased the response.

Thus the discrepancies in the literature concerning the role of prostaglandins in the rebound contraction may be the result of different solvents used to dissolve indomethacin. In addition, indomethacin has also been reported to reduce the availability of calcium ions within the muscle cell, independently of prostaglandin synthesis (Northover, 1977). In this study, a water-soluble prostaglandin synthesis inhibitor flubiprofen (Adams & Buckler, 1979) had no effect on the post-stimulus contraction suggesting at least that prostaglandins are not involved in the rebound contraction in the rat colon.

In addition to the cholinergic excitatory motor innervation of the colon, field stimulation at frequencies below 5 Hz caused a small inhibitory response rather than a contraction and this response too was also followed by a post-stimulus contraction. Since this inhibitory response was not abolished by atropine or any adrenergic receptor antagonist or neurone blocking drug or by pretreatment with reserpine or 6-OHDA, it appears that it was neither adrenergic nor cholinergic (NANC) in nature. However, because the rat colon rarely develops and

maintains tone on its own, advantage was taken of the rhythmic waves of contractions produced by various drugs. Further evidence for the existence of an NANC inhibitory response was obtained in experiments in which field stimulation applied at the peak of a morphine-induced contraction, produced an immediate and maintained inhibitory response.

This response was maximal at low frequencies of stimulation and was unaffected by atropine, quinidine, adrenergic receptor- or neurone-blocking drugs. It appears that this response is NANC in nature but there remains doubt about whether it is neurally-mediated, since it was unaffected by concentrations of TTX that readily abolish the cholinergic motor response. Similar doubts have been expressed about the basis of inhibitory NANC response elsewhere (Crossley & Gillespie, 1983). However, it is possible that NANC nerve terminals may be directly stimulated by field stimulation without the need for axonal conduction. There is evidence that this occurs in other tissues (Douglas & Taraskevich, 1980; Salzberg, Obaid, Senseman & Gainer, 1983). Electrophysiological studies indicate that focal depolarization of nerve terminals releases transmitter by a calcium-dependent mechanism even under complete blockade of the inward sodium current by TTX (Katz & Miledi, 1969; Illes & Thesleff, 1978). Alternatively, large electrical currents may open channels distinct from the voltage-sensitive Ca^{++} channels or may even create new routes for the entry of Ca^{++} into nerves (Illes, Meier & Starke, 1984). The difference in sensitivity to TTX may, however, simply reflect the different susceptibility of the cholinergic motor nerves and the NANC inhibitory nerves to TTX blockade.

The possibility of whether morphine and other drugs affect the NANC inhibitory mechanism to produce the myogenic rhythmic contractions can now be considered. Inhibitory responses to electrical field stimulation were obtained at the peak of morphine-induced contractions. This suggests that if morphine causes rhythmic contractions by removing a tonic inhibitory neural influence then it is unlikely to be this NANC inhibitory influence since this NANC response can be more clearly demonstrated when morphine is present and has inhibited ACh release. Previously, when it was suggested that the tonic inhibitory influence removed by morphine might be NANC (Gillan & Pollock, 1980), the paradox of the persistence of this inhibitory response at the peak of an opiate-induced contraction was explained by postulating that morphine acted at a ganglionic site preceding the inhibitory nerve. In such circumstances, the tonic inhibitory influence of the nerve could be removed but the capacity to stimulate the nerve distal to the blockade to produce an inhibitory response would remain (Gillan & Pollock, 1980). However, the fact that morphine depresses non-adrenergic inhibitory response to transmural nerve stimulation in the guinea-pig taenia coli is not compatible with this explanation (Shimo & Ishi, 1978). Alternatively the mechanism of spontaneous transmitter release and the mechanism of electrically-evoked transmitter release may not be equally susceptible to blockade by morphine. In addition, it could be argued that electrical excitation of the inhibitory nerves may release sufficient transmitter to overcome blockade by morphine and effectively suppress the contraction of the muscle.

In this study, experiments with apamin, which, unlike morphine, acts on the smooth muscle directly, makes this interpretation less likely. Apamin caused contractions similar to those produced by

morphine and this observation supports the view that morphine-induced contractions are caused by the inhibition of an inhibitory mechanism that suppresses myogenic activity. However, field stimulation applied at the peak of an apamin induced-contraction produced either no response or only a small inhibition. The ability of atropine to convert this small inhibitory response into a large and well-maintained inhibitory response, suggests that ACh released by field stimulation normally masked the inhibitory response. Thus it appears that NANC transmission survives both morphine and apamin. An important distinction between apamin and morphine is that morphine inhibited motor responses to field stimulation by inhibiting ACh release, whereas apamin potentiated such responses probably by inhibiting a neural mechanism that normally restrains the size of the cholinergic motor response. The study of NANC inhibitory mechanisms is complicated since there are few substances that block responses to stimulation of NANC nerves. Apamin is one such substance and it blocks relaxation of the guinea-pig taenia caeci in response both to NANC stimulation and to ATP (Banks et al, 1979) and to VIP-induced and neurogenic vasodilation in the cat small intestine (Sjoqvist et al, 1980). However, the failure of apamin to block the NANC inhibitory response in this study suggests that the mechanism underlying relaxation of the smooth muscle in rat colon differs from that of the guinea-pig taenia caecum and cat intestine blood vessels. Likewise, apamin failed to block the inhibitory responses of the retractor penis muscle to nerve stimulation (Bowman & Gillespie, 1982).

The role of cyclic GMP in relaxant responses to NANC nerve stimulation has been elucidated only recently (Bowman & Drummond, 1984). It has been proposed that in the bovine penile artery and certain related structures in the rat and the dog there may be a novel,

inhibitory transmitter which on release activates guanylate cyclase in the smooth muscle cells and that it is the cyclic GMP thus formed that mediates neural relaxation (Bowman & Drummond, 1985). Evidence in support of this hypothesis included (a) The rise in cyclic GMP level following stimulation of the NANC inhibitory nerves in the bovine retractor penis muscle, (b) Haemolysate and N-methylhydroxylamine, which blocks guanylate cyclase, also blocked the relaxation and the rise in cyclic GMP produced by inhibitory nerve stimulation, and (c) 8-Bromo-cyclic GMP, which is a stable derivative of cyclic GMP, produced a relaxation that was not blocked by haemolysate (Bowman & Drummond, 1984).

In this study, both methylene blue, which blocks the activation of guanylate cyclase (Ignarro & Kadowitz, 1985) and haemolysate, derived from lysed red blood cells, had no effect on the inhibitory response to NANC nerve stimulation in the rat colon, suggesting that this inhibitory response is similar to that obtained in the NANC inhibition of tracheal ring, the guinea-pig taenia caeci and the anal sphincter, but unlike the neurogenic vasodilation in bovine and canine retractor penis muscles and penile arteries (Bowman, personal communications).

The above discussion strongly suggests the presence in the rat colon of NANC inhibitory responses, which are unaffected by the opioids and apamin and, therefore, are unlikely to be involved in the waves of rhythmic contractions produced by these drugs.

Such a conclusion would be more in accordance with evidence in the literature that in tissues where there are NANC inhibitory nerves, morphine has no effect on the release of the NANC inhibitory transmitter. Thus, morphine has no effect on NANC transmission in the

rabbit rectococcygeus muscle (Ambache, Killick & Zar, 1974), bovine retractor penis muscle (Klinge & Sjostrand, 1974), rat anococcygeus muscle (Tilmisany, 1975), guinea-pig stomach and taenia (Huizinga & Hertog, 1979), guinea-pig ileum (Ito & Tajima, 1980), guinea-pig anal sphincter (Lim, personal communication).

Clearly, if the effect of the drugs producing the rhythmic waves of contraction in the rat colon is to be explained on the basis of an inhibitory effect on transmitter release, then it becomes obvious that neither the cholinergic motor nor the NANC inhibitory nerves are involved and a second tonically-active inhibitory transmitter would have to be proposed. Such a transmitter might be the adrenergic inhibitory mechanism although such an explanation will not be without difficulties. First, the NA content of the colon as measured by HPLC in this study was very low compared with other densely adrenergic innervated tissues such as the vas deferens. However, the tonic inhibition need only be concentrated on few pace-maker cells. Secondly, fluorescence histochemical studies have demonstrated that in the intestinal tract of most mammalian species, the post-ganglionic adrenergic nerves terminate in the region of the ganglion cells of Auerbach's plexus, with few fibres innervating the longitudinal and circular muscle layers directly (Norberg, 1964; Jacobwitz, 1965; Hollands & Vanov, 1965; Furness & Costa, 1978). Thus the effect of this tonically-active adrenergic mechanism might have to be indirect, exerted primarily at the level of intramural ganglion cells. Thirdly, there was little evidence of any significant contribution from the adrenergic nerves in the inhibitory response of the colon to field stimulation since adrenergic neurone and adrenoceptor blockers did not affect the inhibitory response. However, this observation is not unique to the rat colon. Stockley and Bennett (1977) showed that in human taenia coli, which is densely innervated

with non-adrenergic inhibitory fibres, the adrenergic response to nerve stimulation may be masked by the non-adrenergic inhibitory response.

Indirect evidence for the existence of an adrenergic inhibitory mechanism in this study included the potentiation of the cholinergic motor response to nerve stimulation in 6-OHDA-pretreated rats especially at low frequency of stimulation when the "braking" effect of released NA on the motor response might be expected to be maximal. The higher incidence of spontaneous waves of rhythmic contractions in reserpinised colon is also suggestive of such an adrenergic-mediated tonic inhibitory effect. Moreover, the rhythmic waves of contractions produced by apamin were inhibited by the indirectly acting sympathomimetic tyramine. Since this inhibitory effect of tyramine was antagonised by propranolol, it seems likely that this effect was mediated via α-adrenoceptors. It is, therefore, possible that apamin inhibited an α-adrenoceptor-mediated inhibitory mechanism to produce an excitatory effect, which could be overcome by catecholamines acting via α-adrenoceptors.

Evidence supporting such a view was obtained using the α_2-adrenoceptor agonist clonidine, which produced regular rhythmic contractions like those produced by morphine, apamin or TTX. Since the excitatory effect of clonidine was antagonised by yohimbine but not by prazosin, it appears that this action of clonidine was mediated via α_2-adrencoeptor, perhaps located on adrenergic nerve terminals to inhibit transmitter release.

Further evidence that the second, tonically-active inhibitory mechanism might be adrenergic and that it is this mechanism that is readily inhibited by opioids, apamin and TTX, albeit by different

mechanisms and at different sites, was obtained with α-adrenoceptor antagonists and adrenergic neurone blockers. These drugs also produced contractions similar to those produced by apamin, morphine and TTX. In addition, Gillan and Pollock (1980) and Nijkamp and Van Ree (1980) reported the potentiating effect of an α-adrenoceptor antagonist on opioid-induced contractions in the rat intestine.

It is a paradox that the apparent inability of reserpine pretreatment, adrenergic neurone blockers and adrenoceptor antagonists to antagonise the excitatory effect of the opioids (Scheurer et al, 1981; Moritoki et al, 1984; Fontaine & Reuse, 1985) could be taken as evidence for the non-involvement of an adrenergic mechanism in the tonic inhibitory mechanisms affected by the opioids. Additonal blockade of this adrenergic mechanism by drugs affecting adrenergic transmission should theoretically either have no effect or potentiate the response.

The suggestion that the hypothetical tonic inhibitory mechanism affected by these diverse drugs is adrenergic immediately offered the possibility that the inhibitory effect of these drugs on tonically released NA might be investigated. Unlike the NANC inhibitory mechanism, whose transmitter is unknown and, therefore, more difficult to analyse, NA overflow and inhibition of this overflow can be measured. Both HPLC with electrochemical detection and tritium efflux were used in this study to determine whether drugs which had an excitatory action in the colon inhibited NA release.

Liquid chromatographic methods, using either cation exchange or reversed phase chromatography with electrochemical detection have been

used for the determination of catecholamines in both brain and smooth muscle (Felice, Felice & Kissinger, 1978; Wagner, Vatali, Palfreyman, Zraika & Huot, 1982; Maruyama, Oshima & Nakajima, 1980; Macrae, 1983) as well as in plasma samples (Hallman, Fernebo, Hamberger & Johnsson, 1978; Goldstein, Ferneistien, Izzo, Koplin & Keiser, 1981; Eriksson & Persson, 1982). HPLC proved to be a fast and efficient method for separating the amines following their extraction. Problems of amine separation and artefact production were often experienced during this study. Poor separation resulted in either the NA and DOPEG peaks being superimposed on the falling phase of the solvent front or both NA and DOPEG peaks becoming joined together. This resulted in difficulties in measuring peak height which in turn led to inaccuracies in quantifying amine levels.

The occurrence of artefact was successfully controlled by improved methodological practise. Catecholamine separation was improved by altering the composition of the mobile phase. For example, a decrease in phosphate ion, pH and methanol content of the mobile phase caused an increase in retention time and thus improved separation. This also caused an increase in the retention of DA, giving a very lengthy chromatogram. Better separation was thus gained only at the expense of time. The necessity for mobile phase adjustment is clear when one considers the variability between columns (Hansson, Agrup, Rorsman, Rosengren, Rosengre & Edholm, 1979) and the cost of new columns relative to manipulation of the mobile phase. Thus, Goldberg (1982) showed that the retentivity and selectivity of columns with similar packings is not constant due to differences in (a) the material used as the support, (b) the technique used to bond the functional groups to the support, (c) the method used to pack the column, and (d) the amount of end capping to mask unbonded sites.

Additionally, loss of resolution occurs with time as columns deteriorate due to loss of the bonded octadecyl moiety and/or the irreversible binding of molecules to the C-18 moiety. Careful selection, followed by proper care and cleaning of the column, as discussed by Rabel (1980), extended the life and ensured proper performance of the column.

Preliminary experiments were done to measure the catecholamine content of the guinea-pig, rat and mouse vasa deferentia and the rat colon with the HPLC. The values obtained for the catecholamine content of the vasa agreed with some of those reported in the literature (Sjostrand, 1965; Boadle-Biber & Roth, 1975), but were less than the values reported by Blakeley et al (1970), Bell and Gillespie (1981) and Macrae (1983). The reason for the apparent discrepancy between these results is unknown but is unlikely to be related to differences in methodology and strain of animal used since these were similar in the present study and that of Macrae (1983).

Experiments in the guinea-pig confirmed previous findings that spontaneous and electrically-induced release of NA occurred in this tissue and could be modified by drugs (Macrae, 1983; Bell, Gillespie & Macrae, 1984). Unlike the guinea-pig vas deferens, the spontaneous and nerve stimulation-induced release of CA from the mouse vas deferens, varied from one experiment to another. In some experiments there was apparently no CA released spontaneously or as a result of nerve stimulation.

In others, NA was the only major CA detected while in others again NA, DOPEG and DA were all detected. Overall, it was very much easier

to demonstrate amine release in the guinea-pig than in the mouse vas deferens.

With the HPLC study, no spontaneous release of NA and/or metabolite was demonstrated in the rat colon, casting doubt on the NA hypothesis. However, the possibility exists that the inability to demonstrate spontaneous release of NA in the rat colon might simply be a result of the low amine content of this tissue. The amount of NA released may, therefore, be below the detection limit of the HPLC system used.

Efflux experiments with (^{3}H)-NA were performed as an alternative to the HPLC studies. In these experiments the effects of drugs on the motility of the colon and on the efflux of tritium were examined. No effect of drugs on the spontaneous efflect of ^{3}H was demonstrated in the colon despite the fact that these drugs produced rhythmic waves of contraction in the same experiments. The inability of these drugs to affect spontaneous ^{3}H confirmed the results of previous workers that in other systems these drugs do not affect spontaneous ^{3}H efflux (Hughes _et al_, 1975; Vizi, Harsing & Knoll, 1977; Arbilla, Langer & Gonzalez, 1983).

The results of the biochemical studies seem to cast serious doubts on the validity of the hypothesis that NA is the transmitter whose release is tonically inhibited by these drugs. However, failure to demonstrate the tonic inhibition of NA release by the drugs which produce rhythmic waves of contraction _in vitro_ does not eliminate the possibility of the existence of such a mechanism _in vivo_ (Daniel, 1968; Drummond, personal communications). The inability to demonstrate tonic release and its inhibition may simply reflect the limitations of both

the pharmacological tools used and the experimental system studied. For example, the possible existence of TTX-insensitive nerves reduces the value of this drug as a tool to identify neuronal mechanisms (Katz & Miledi, 1968; Illes & Thesleff, 1978).

One possibility that still cannot be excluded is that these drugs may have a direct excitatory action on intestinal smooth muscle. At first sight this seems unlikely since morphine does not modify resting membrane potential in the guinea-pig ileum and mouse vas deferens (Ito & Tajima, 1980), the cat colon (Blanquet, Bouvier & Gonella, 1982) or the rat portal vein (Yamamoto, Hotta & Matsuda, 1984). However, mechanical activation in the absence of electrical activity is known to occur in the rat anococcygeus (Creed, Gillespie & Muir, 1975), cat trachea (Ito & Takeda, 1982), bovine retractor penis (Byrne & Muir, 1984) and rat iris sphincter (Banno, Imaizuma & Watanabe, 1985), and has been described as pharmaco-mechanical coupling (Somlyo & Somlyo, 1968). Indeed, such a direct effect of the opioids has been demonstrated in isolated gastric smooth muscle cells devoid of neural elements (Bitar & Makhlouf, 1982, 1984; Makhlouf, 1985) and was not ruled out in the studies of Gillan and Pollock (1980), Yau (1981) and Moritoki et al (1984).

A direct action has also been suggested to explain the excitatory effect of atropine on chick amnion which is devoid of nerves (Cuthbert, 1963), on oesophageal muscle (Christensen & Lund, 1968) and cat small intestine (Bortoff & Miller, 1975). Furthermore, the excitatory action of local anaesthetics on rabbit uterus (Daniel & Wolowyk, 1966), rat vas deferens (Hay & Wadsworth, 1982), cat small intestine (Bortoff & Muller, 1975) and of tetrodotoxin on cat sphincter of Oddi (Persson, 1971) are all thought to involve direct effects on the smooth muscle.

The question then arises as to whether this excitatory action of the opioids on the terminal colon of the rat observed *in vitro* is related to the anti-diarrhoeal effect of opiates. Analysis of sites of drug action *in vitro* is slightly simpler than *in vivo* because often only smooth muscle and axons of intrinsic neurones are present. However, one must be cautious in interpreting *in vitro* data to explain the possible local functional role of endogenous opioids in animals under physiological conditions or to explain the origin of the constipating action of morphine. For instance, how does local application of a substance to the isolated intestine influence its effects and are these effects different from those of systemically-administered drugs *in vivo*? Does removal of other controlling mechanisms as a consequence of isolation of the preparation show up responses to opioids normally absent and/or of little significance in the intact animal (Manara & Bianchetti, 1985)?

Despite these obvious limitations, however, the use of *in vitro* studies has provided valuable information on the pharmacology of opioids. It may be argued that the rhythmic peristalsis of the colon is destroyed by the burst of fast excitatory waves elicited by the opiates or that the opiates increase the proportion of non-propulsive segmenting contraction of the gut, leading to a slowing of the intestinal transit. In a recent review, Manara and Bianchetti (1985) provided evidence to support the hypothesis that the primary site of morphine's constipating action is peripheral in both the intestinal smooth muscle and mucosa. However, a central action *in vivo* may contribute to this effect of opiates (Burks, 1980; Galligan & Burks, 1983; Porreca, Cowan, Raffa & Tallarida, 1983). Thus it seems possible that the constipation produced by morphine has local and central sites of action.

In conclusion, the results of this study confirmed the complexity of the effects of drugs on the isolated colon of the rat and provided evidence about the nature of innervation of this tissue. The results do not agree with the hypothesis that morphine produces its excitatory effect in the rat colon solely by the release of 5-HT and ACh.

This study has also provided evidence inconsistent with the proposition that morphine and other drugs may produce their excitatory action by inhibiting NANC inhibitory mechanisms. A direct effect on smooth muscle by these drugs was not ruled out. The similarity between the effects of the opioids, the adrenoceptor antagonists, the adrenergic neurone blockers, clonidine, apamin and TTX suggested that the actions of these drugs might be mediated through adrenergic neurones albeit by different mechanisms, but the biochemical studies failed to confirm this.

The results of this study make it clear that no impregnable unifying hypothesis is available to explain the mechanism of the excitatory action of the diverse groups of drugs producing rhythmic contractile activity in the colon. It is more likely, therefore, to be a combination of direct excitatory effect on smooth muscle and an indirect neurally-mediated effect, both of which are capable of functioning together or independently of each other.

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PUBLICATIONS

Various aspects of the work presented in this thesis have been presented at conferences or submitted for publication. Reprints where available may be found inside the back cover of this thesis. The references are:-

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