Br. J. Pharmac. (1986), **88**, 307–313

Morphine attenuates cholinergic nerve activity in human isolated colonic muscle

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1 The action of morphine on cholinergic nerves in human sigmoid taenia coli muscle strips (taenia) was investigated using a radiolabelling technique.

2 Basal release of tritiated material from taenia was increased by electrical field stimulation (EFS). This increase was tetrodotoxin (3.14 μM)-sensitive and calcium-dependent. Analysis of basal and stimulated release of tritiated material indicated that evoked release (i.e. stimulated minus basal) is almost entirely due to an increase in [^{3}H]-acetylcholine ([^{3}H]-ACh) output.

3 Evoked release of [^{3}H]-ACh was dependent on the current strength and could be greatly reduced by exposing taenia to hemicholinium (34.8, 87.0 μM) before and during incubation with [^{3}H]-choline (4 μCi ml^{-1}, 15 Ci mmol^{-1}).

4 Spontaneous activity, muscle tone and the motor response of taenia to EFS were unaffected by morphine.

5 Evoked, but not basal, release of tritiated material was inhibited by morphine (1.32–13.20 μM) in a concentration-dependent manner. The inhibition of release was frequency-dependent and naloxone (0.28 μM)-sensitive.

6 The possible relationship between the effects of morphine on cholinergic nerves in taenia muscle and its actions *in vivo* are discussed.

Introduction

The effects of morphine on the human large intestine are well documented; colonic propulsion and peristalsis are reduced or abolished, tone is increased to the point of spasm and the tone of the anal sphincter is greatly augmented (Plant & Miller, 1928; Painter & Truelove, 1964; Rab Chowdhury & Lorber, 1977). Despite this, studies using human tissue *in vitro* have failed to demonstrate any evidence of such a spasmogenic effect of morphine (Bennett, 1975; Burleigh, 1983; Burleigh *et al.*, 1984). This apparent lack of effect may be due to several factors: (a) morphine may not act directly on colonic smooth muscle cells, (b) the unpredictable and changing spontaneous motility of human taenia muscle strips (Bucknell & Whitney, 1966) or a complex response to electrical field stimulation of intrinsic nerves (Stockley & Bennett, 1974) may obscure an effect of morphine on responses to stimulation of intrinsic cholinergic nerves, (c) all human tissue has been obtained from major bowel resections and the opioid drugs administered before or during the operation may prevent responses to subsequent exposure of the tissue to morphine, (d) there is evidence that the gastrointestinal effects of morphine may partly result from actions on the central nervous system (Parolaro *et al.*, 1977; Stewart *et al.*, 1978; Burleigh *et al.*, 1981).

The aim of the present investigation was to determine whether morphine can influence intrinsic cholinergic nerve activity in isolated preparations of human sigmoid taenia coli. We have used a radiolabelling technique devised by Wikberg (1977) to assess activity of cholinergic nerves.

Methods

Macroscopically normal strips of human sigmoid taenia coli (taenia) consisting of muscularis externa without attached mucosa and submucosa, were obtained from specimens of colon resected for local malignancy. Taenia were incubated for 60 min in 2 ml of Krebs solution containing 4 μCi ml^{-1} [^{3}H]-choline, the fluid was kept at 37°C and gassed with 5% CO_2 plus 95% O_2. The taenia were then superfused at 2.0–2.2 ml min^{-1} with Krebs solution containing hemicholinium-3 (HC-3; 34.8 μM). Incubation and superfusion of individual taenia preparations was carried out in the same small volume bath (1.5 ml)

fitted with vertical platinum wire electrodes, 0.5 mm diameter to allow electrical field stimulation of the tissue from a constant current stimulator (Hugo Sachs). After 90 min of superfusion the taenia were subjected to electrical field stimulation and again, unless otherwise stated, 48 min after completion of the first stimulation period. The various parameters of stimulation are indicated in the results section. Motor responses were recorded with SRI isotonic transducers and displayed on a Rikadenki potentiometric pen recorder. Muscle strips were placed under a load of 0.5 g. Superfusion fluid was collected every 4 min and 0.5 ml aliquots added to 5 ml of Packard 'Scintillator 299™' scintillation fluid for counting in a Packard Tricarb 300CD liquid scintillation spectrometer. At the end of the experiment the taenia were weighed, dissolved in hyamine hydroxide and a sample prepared for liquid scintillation counting. Efficiency of counting was determined by the automatic external standard channels ratio method and counted to an accuracy of 5% of 2 sigma error.

Radioactivity in all samples of superfusion fluid was computed in terms of disintegrations per min per mg of tissue according to the formula:

$$\text{Radioactivity per sample} = \frac{\text{d.p.m.} \times \text{FR} \times \text{T}}{\text{W} \times \text{V}}$$

where d.p.m. = disintegrations per min (this value supplied by spectrometer), FR = superfusion flow rate, T = sample collection time, W = taenia muscle strip weight, and V = volume of sample added to scintillation fluid.

For each period of electrical field stimulation 3 sample collections were made before (basal release) and 5 during and after the stimulation (stimulated release). The release of radioactive ^{3}H-labelled (tritiated) material produced by stimulation, i.e. evoked release, was calculated from the differences between the 'calculated' basal release and the stimulated release. Calculated basal release was obtained by fitting a regression line through observed basal values collected before the two periods of stimulation. The total evoked release was expressed as a fraction of the radioactivity present in the tissue at the beginning of the stimulation period. Muscle strips were subjected to two periods of stimulation per experiment and the fractional release of the second stimulation was expressed as a ratio of the first (this value is described as the 'fractional release ratio'). This procedure takes account of variation between tissues in the absolute amounts of radioactivity released (Rand *et al.*, 1982).

The following procedure was adopted for extraction and separation of acetylcholine (ACh) and choline released under basal and stimulated conditions. Physostigmine (0.31 μM) was added 20 min before the third basal collection to preserve ACh. After removal of 0.5 ml aliquots for liquid scintillation counting, samples were stored at 4°C with carrier (50 μl of Krebs containing ACh 3 mM plus choline 3 mM). Extraction and thin layer chromatography (t.l.c.) was essentially by the method of Marchbanks & Israel (1971). For each taenia muscle strip one basal sample (3rd collection) and one stimulated sample, that with the greatest radioactive content, were taken for analysis of [^{3}H]-ACh and [^{3}H]-choline content.

Recovery of ACh and choline was estimated by running known amounts of [^{14}C]-ACh and [^{3}H]-choline through the same extraction and separation procedures. Extraction recovered 54.6 ± 0.9% of [^{14}C]-ACh (6729 ± 107 d.p.m., $n = 8$) and 53.1 ± 1.0% of [^{3}H]-choline (5795 ± 105 d.p.m., $n = 8$). The t.l.c. percentage recovery for [^{14}C]-ACh was 71.9 ± 5.2% ($n = 8$) and for [^{3}H]-choline was 37.0 ± 2.7% ($n = 8$). Under these conditions 1% of [^{14}C]-ACh standard appeared in the choline spot and 2% of [^{3}H]-choline standard appeared in the ACh spot. Analysis of basal and stimulated samples of superfusion fluid indicated that the evoked release of tritiated material resulting from field stimulation is almost entirely due to an increase in [^{3}H]-ACh release. For basal samples 119 ± 41 d.p.m. was found in the choline spot and 65 ± 24 d.p.m. in the ACh spot. For stimulated samples 129 ± 47 d.p.m. was found in the choline spot and 124 ± 20 d.p.m. in the ACh spot.

Results are expressed as mean ± s.e.mean. Statistical significance between means was assessed using the Mann-Whitney U test or Wilcoxan matched-pairs signed-ranks test (for effects of tetrodotoxin and morphine on basal release of tritiated material). A probability value of less than 0.05 was taken to represent a significant difference.

Drugs used were: tetrodotoxin, physostigmine sulphate, acetylcholine chloride, hyoscine hydrobromide, morphine sulphate, naloxone hydrochloride, ethylene glycol tetra-acetic acid (EGTA), hemicholinium-3, [^{3}H]-choline chloride (specific activity 15 Ci mmol^{-1}, Amersham Int. plc) and [^{14}C]-acetylcholine chloride (specific activity 55.3 m Ci mmol^{-1}). Composition of the Krebs solution was (mM): Na^+ 140, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 122, HCO_3^- 25, SO_4^{2-} 1.0, PO_4^{2-} 1.2, glucose 11.5.

Results

Stimulus-response relationship and underlying cholinergic component of motor response of taenia to field stimulation

The responses of taenia to increasing stimulus current strength of electrical field stimulation (EFS) are shown in Figure 1. The response to EFS (1 Hz, 1 ms, 60 pulses

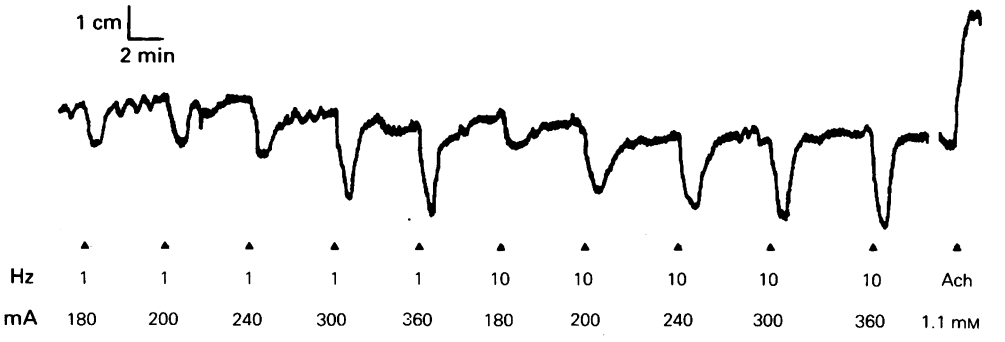

Figure 1 Responses of taenia muscle from human sigmoid colon to electrical field stimulation (1 ms for 1 min). The muscle strip was stimulated at 1 or 10 Hz using a range of current strengths. Finally a maximum contraction was obtained to acetylcholine (ACh, 1.1 mM). In contrast to the responses illustrated in Figure 4 no after-contraction was observed, such variability is not unusual.

at 200 mA) was abolished by tetrodotoxin (TTX; 3.14 μM). In the presence of physostigmine (0.31 μM), biphasic responses were converted to contractions and the contractions were potentiated. These contractions were reduced, blocked or converted to relaxations by hyoscine (2.28 μM). With some taenia, concentration-response curves were obtained to ACh. Contractions to ACh were also potentiated by physostigmine; ED_{50} in the absence of physostigmine 5.00 ± 2.24 μM, ED_{50} in the presence of physostigmine 1.14 ± 0.42 μM ($n = 3$).

Depression of transmitter release by tetrodotoxin or removal of calcium ions

Taenia were stimulated twice with EFS (10 Hz, 1 ms, 480 pulses at 200 mA). The fractional release of radioactivity from the second stimulation period was expressed as a ratio of the release from the first, i.e. the fractional release ratio (see Methods). The effect of TTX (3.14 μM) on the fractional release ratio was evaluated by adding the drug to the superfusion fluid 15 min before the second period of EFS. Removal of calcium ions was made 30 min before the second stimulation period by omitting calcium chloride from the Krebs fluid and adding the calcium chelating agent EGTA (0.1 mM). Release of tritiated material by nerve stimulation was virtually abolished by TTX and greatly reduced by calcium ion deprivation (Figure 2). In contrast to its effects on evoked release, TTX caused a small, non-significant ($P > 0.05$, $n = 8$) reduction of basal release.

Dependence of transmitter release on strength of electrical field stimulation

Taenia were subjected to EFS (10 Hz, 0.5 ms, 480 pulses) at varying current strengths. For this experiment it was only necessary to examine the release of radioactive material by the first stimulation period. Release of tritiated material increased with strength of stimulation. At 140 mA, fractional release was $1.1 \pm 0.5 \times 10^{-3}$, at 220 mA $5.9 \pm 0.8 \times 10^{-3}$ and at 300 mA $7.8 \pm 1.1 \times 10^{-3}$ ($n = 5$ for each value).

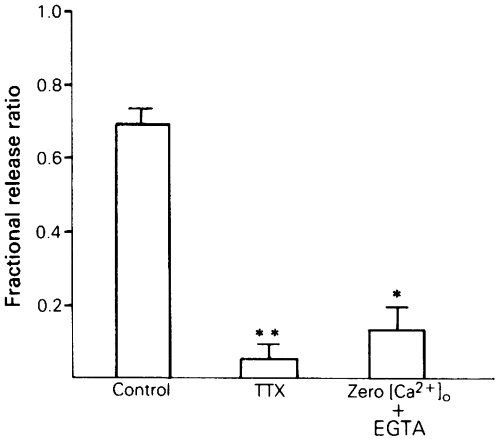

Figure 2 Depression of release of tritiated material by tetrodotoxin (TTX, 3.14 μM) and removal of calcium ions (plus addition of EGTA 0.1 mM). Taenia muscle strips were subjected twice to electrical field stimulation (10 Hz, 1 ms, 480 pulses at 200 mA). Fractional release of tritiated material for the 2nd stimulation period was expressed as a ratio of fractional release of tritiated material for the 1st period of stimulation (fractional release ratio). Addition of TTX or removal of Ca^{2+} was carried out after the 1st stimulation period. Columns are mean values with vertical lines indicating s.e.mean, $n = 4$–6. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from control value.

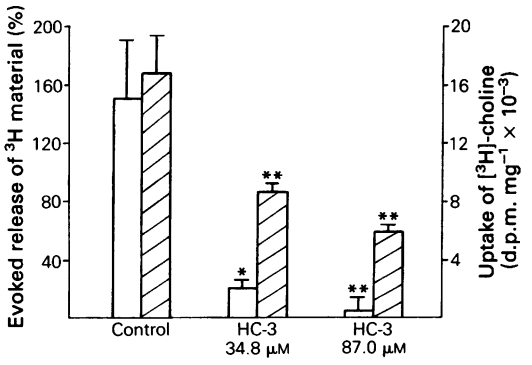

Figure 3 Action of hemicholinium (HC-3) on uptake (d.p.m. mg^{-1} tissue) of [^{3}H]-choline by taenia (hatched columns) and on release of tritiated material evoked by electrical field stimulation (10 Hz, 1 ms, 480 pulses at 200 mA; open columns). Evoked release was expressed as a % of calculated basal release. HC-3 (34.5 and 87.0 μM) was in continuous contact with the taenia starting 45 min before incubation with [^{3}H]-choline. Columns are mean values ± s.e.mean, $n = 4-8$. $^*P < 0.05$; $^{**}P < 0.01$, significantly different from control value.

Effect of hemicholinium preincubation on subsequent tissue uptake of [^{3}H]-choline and evoked transmitter release by electrical field stimulation

Taenia were continually exposed to HC-3, during and for a 45 min period immediately before, incubation with [^{3}H]-choline. Normally HC-3 was added to the Krebs fluid only after the 60 min incubation period with [^{3}H]-choline had been completed. Since HC-3 depressed tissue accumulation of [^{3}H]-choline its effects on release of tritiated material resulting from nerve stimulation are best expressed in terms of evoked release as a percentage of simultaneous (calculated) basal release, rather than as evoked release as a fraction of the total radioactivity remaining in the tissue (fractional release). Hemicholinium-3 (34.8 and 87.0 μM) produced a dose-related decrease in both tissue uptake of [^{3}H]-choline and release of tritiated material evoked by nerve stimulation, the latter effect appeared more marked (Figure 3). Hemicholinium-3 did not affect the motor response to EFS (10 Hz, 1 ms, 480 pulses at 200 mA).

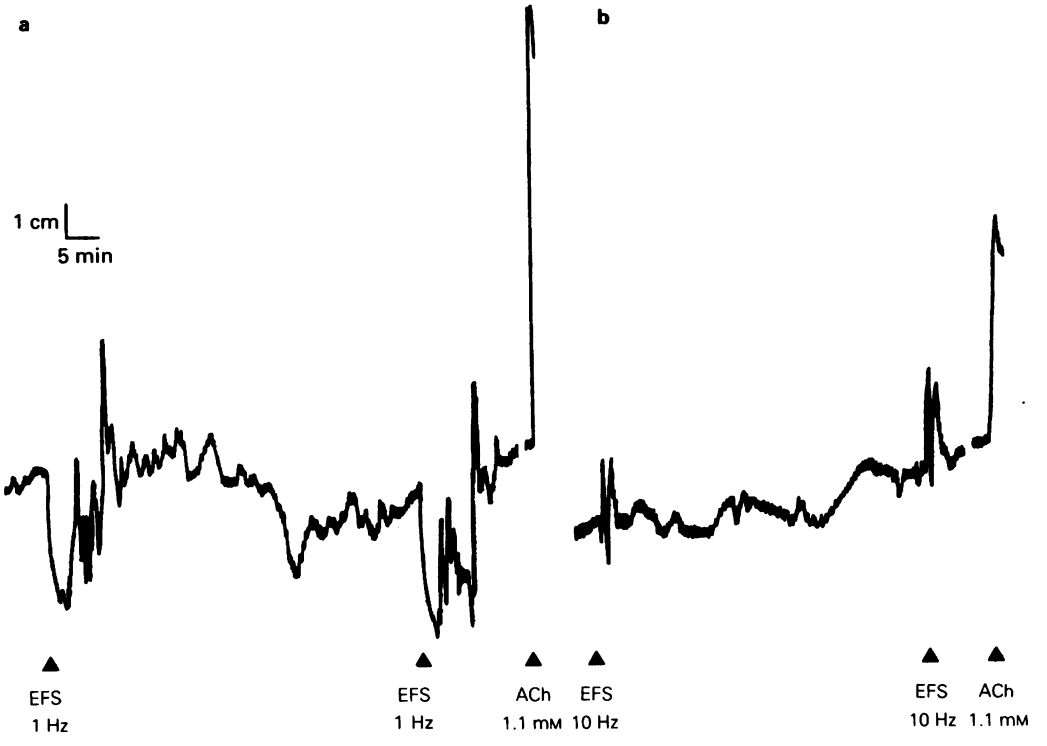

Figure 4 Effect of morphine on response of taenia muscle, from human sigmoid colon, to electrical field stimulation (EFS: 1 ms, 480 pulses at 200 mA). Muscle strips were stimulated at a frequency of 1 Hz (a), or 10 Hz (b). Both were exposed to morphine (1.32 μM) during and 4 min before the 2nd stimulation period. Finally a maximum contraction to ACh (1.1 mM) was obtained.

Effect of morphine on smooth muscle and cholinergic nerve activity of taenia

Taenia were exposed to morphine (0.13, 1.32 and 13.20 μM) 4 min before and during the 2nd stimulation period. The drug had no effect on spontaneous motor activity of the taenia or motor responses to EFS (Figure 4). There was, however, a reduced output of tritiated material from stimulated cholinergic neurones. This effect was concentration-dependent and only attained significance at the lower frequency of stimulation (Figure 5). Basal release was not significantly affected by morphine (1.32 μM, $P > 0.05$, $n = 23$).

The fractional release ratio for taenia stimulated at 200 mA (1 ms, 1 Hz, 480 pulses) was 0.82 ± 0.04 ($n = 8$). This value increased significantly to 1.38 ± 0.14 ($n = 6$) when the current strength of the 2nd period of stimulation was raised to 300 mA ($P < 0.01$). Despite the increase in current strength morphine (1.32 μM) still reduced the release of tritiated material giving a fractional release ratio of 0.99 ± 0.12, although the reduction did not achieve a degree of statistical significance ($P > 0.05$, $n = 9$).

Addition of naloxone (0.28 μM) 30 min before the beginning of the 2nd stimulation period (1 ms, 1 Hz, 480 pulses at 200 mA) significantly reduced the inhibitory effect of morphine (13.20 μM) on release of tritiated material ($P < 0.05$, $n = 5$). The fractional release ratio with both naloxone and morphine present was 0.74 ± 0.07 compared to 0.42 ± 0.13 ($n = 5$) with morphine only. In the presence of naloxone (1.10 μM) alone the fractional release ratio was 1.09 ± 0.18 ($n = 7$) which was not significantly different from the control value of 0.86 ± 0.06 ($P > 0.05$, $n = 8$).

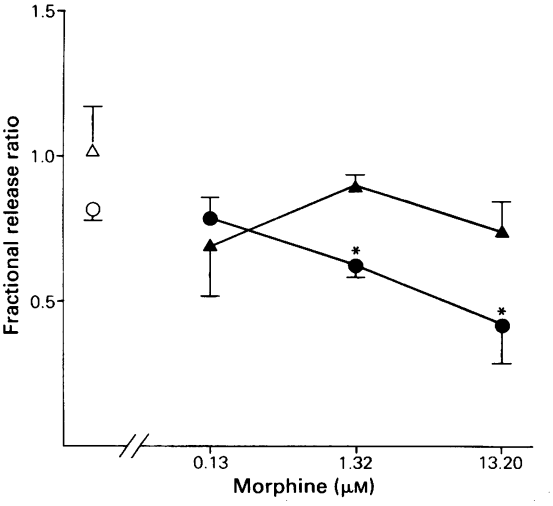

Figure 5 Concentration-response curves for effect of morphine on release of tritiated material from human taenia muscle strips. Strips were stimulated twice (1 ms, 480 pulses at 200 mA) at frequencies of 1 Hz (●, $n = 5$–10) or 10 Hz (▲, $n = 5$). Fractional release of tritiated material for the 2nd stimulation period was expressed as a ratio of fractional release of radioactivity for the 1st period of stimulation. Muscle strips were exposed to morphine for 4 min before and during the second stimulation period. Control experiments are indicated by open symbols. *$P < 0.05$, significantly different from control value.

Discussion

Conventional techniques using isotonic recordings of human taenia coli muscle strip (taenia) motility have confirmed previous observations that electrical field stimulation (EFS) of taenia activates cholinergic nerves (Crema *et al.*, 1968; Stockley & Bennett, 1974). Thus physostigmine enhanced contractions which were subsequently reduced or abolished in the presence of hyoscine and abolished by TTX.

Consistent demonstration of cholinergic contractions of taenia to EFS is only possible in the presence of physostigmine. As physostigmine has been shown to obscure neuromodulatory effects of opioid receptor stimulation (Vizi *et al.*, 1984) release of radioactive ACh was used as an indicator of cholinergic nerve activity. The technique was devised by Wikberg (1977) who showed that radiolabelling techniques could substitute for bioassay in the quantitative detection of acetylcholine release.

The following observations indicated that the increased output of tritiated material evoked by EFS was largely due to ACh release from cholinergic nerves: first, analysis of the tritiated material released by EFS showed it to consist mainly of [^{3}H]-ACh. Similar observations have been made by other groups of workers using radiolabelling techniques (Wikberg, 1977; Vizi *et al.*, 1984; Wetzel & Heller Brown, 1985). Secondly, low concentrations of choline were preferentially utilized by the high affinity mechanism for rapid and efficient transport of choline and its conversion into acetylcholine. This high affinity transport of choline has been demonstrated in intestinal neuronal elements (Pert & Snyder, 1974). The actions of hemicholinium-3 in reducing uptake of choline and, more importantly, the evoked release of [^{3}H]-ACh confirmed this mechanism was operating in human taenia. Thirdly, the depressant effect of tetrodotoxin (TTX) and calcium ion deprivation on ACh release from taenia is strong evidence that the released ACh is neuronal in origin.

The residual amount of tritiated material released by field stimulation, in the presence of TTX, may have originated from AH-neurones. Some action potentials from these neurones, which may be cholinergic, are

not completely blocked by TTX (North, 1982). More plausibly, perhaps, field stimulation may have caused direct depolarization of nerve terminals. The observation that TTX failed to reduce basal release of tritiated material appears to be at variance with the results of Duthie & Kirk (1978). They found that the continuous electrical and mechanical activity displayed by taenia muscle strips was changed to intermittent activity by TTX and proposed that this may be due to a reduction in cholinergic nerve activity. As previous investigations have not shown an inhibitory effect of TTX on taenia muscle strip tone (Crema *et al.*, 1968; Burleigh, 1977), a possible explanation for the observations of Duthie & Kirk might be the considerable variations in spontaneous activity which can occur within the same taenia muscle strip (Bucknell & Whitney, 1966). In the guinea-pig ileum TTX reduces basal (i.e. resting) release of ACh (Paton *et al.*, 1971; Cowie *et al.*, 1978; Vizi *et al.*, 1984). The use of physostigmine in these investigations may have led to sufficient accumulation of ACh at neural synapses to initiate action potentials in synapsing neurones (Sato *et al.*, 1973; Ehrenpreis *et al.*, 1976).

The effect of morphine on ACh output under resting and stimulated conditions was judged by its effect on release of tritiated material. Analysis of basal and stimulated release of tritiated material indicates that evoked release (i.e. stimulated minus basal) of tritiated material by field stimulation is almost entirely attributable to an increase in [^{3}H]-ACh release. As morphine could significantly depress stimulated but not basal release of tritiated material, it seems reasonable to suppose that morphine depresses [^{3}H]-ACh release during stimulation, but not during resting conditions. Reversal of the effect of morphine by naloxone is further evidence of an action on opioid receptors. Since naloxone did not significantly enhance stimulated release of tritiated material, it is likely that opioid drugs received during an operation do not subsequently affect the response of taenia to morphine.

The fractional release of ACh per pulse decreases with increasing frequency of stimulation (Paton, 1957; Cowie *et al.*, 1978). This decline may be related to enhanced activation of presynaptic inhibitory autoreceptors at higher frequencies of stimulation, there being a more rapid accumulation of ACh within the synapse at high frequencies (James & Cubeddu, 1984). When the negative feedback muscarinic mechanism of ACh release becomes operative, Met-enkephalin caused no inhibition of ACh release (Vizi *et al.*, 1984). Such observations would explain why the action of morphine diminishes as stimulus frequency increases.

North & Tonini (1977) proposed that the reduction of ACh release by morphine, results from hyperpolarization of the nerve cell membrane and its processes. Evidence for this mechanism was the reversal of the action of morphine observed when the stimulus current strength was increased. In the present study, increasing stimulus current strength by 50% prevented a significant reduction in release of tritiated material by morphine (1.32 μM).

Although morphine does not exert a direct effect on smooth muscle cells of the human distal gut it does reduce the activity of stimulated cholinergic neurones. If ACh output is reduced by an action on presynaptic opioid receptors (Vizi *et al.*, 1984) then a number of types of cholinergic nerve may be affected (Paton *et al.*, 1971). For example nerves extrinsic to the bowel (e.g. sacral pelvic nerves), extrinsic to the preparation (e.g. submucosal plexus neurones) or intrinsic nerves (i.e. either interneurones or the final motor neurones). If, however, the actions of the drug were on the cell body (North & Tonini, 1977), then only cholinergic neurones intrinsic to the preparation could be affected.

The observation that morphine did not affect the motor response of taenia to EFS is supported by previous investigations. Thus morphine did not antagonize responses of the distal gut to stimulation of non-adrenergic, non-cholinergic inhibitory nerves (Burleigh, 1983; Small & Yong, 1983; Laniyonu *et al.*, 1984). However, these inhibitory nerves receive excitatory inputs from cholinergic interneurones and from the efferent limb of the descending inhibitory nerve pathway that is involved in caudal propulsion of food (Costa & Furness, 1982). Reduction in ACh release from such interneurones would, therefore, reduce activity of the non-adrenergic, non-cholinergic inhibitory nerves, resulting in impairment of propulsion and an increase of smooth muscle tone. A similar mechanism of action has been proposed to explain the excitatory action of opiates in the rat colon (Gillan & Pollock, 1980).

We are grateful to Dr M.K.S. Hathorn for assistance with the computer programme for calculation of release of radioactive material, to S.E.R.C. and Janssen Pharmaceutical Ltd for financial support (C.A.S.E. studentship for S.J.T.) and to surgeons at The London, St. Bartholomew's and St. Marks hospitals for their co-operation in providing specimens of colon.

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(Received July 19, 1985.
Revised February 17, 1986.
Accepted February 27, 1986.)