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Opioid inhibition of synaptic transmission in the guinea-pig myenteric plexus

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1 Intracellular recordings were made from neurones in the myenteric plexus of the guinea-pig ileum. Presynaptic nerves were excited by a focal stimulating electrode on an interganglionic strand.

2 Fast excitatory postsynaptic potentials (e.p.s.ps) were depressed in amplitude by morphine and [Met^5]enkephalin in the concentration range of 1 nM–1 μM. Nicotinic depolarizations evoked by exogenously applied acetylcholine (ACh) were not affected by these opioids.

3 Hyperpolarization of the presynaptic fibres probably contributed to the depression of the fast e.p.s.p. because fast e.p.s.ps evoked by low stimulus voltages were more depressed than those evoked by high stimulus voltages and fast e.p.s.ps resulting from activation of a single presynaptic fibre were blocked in a non-graded manner.

4 Opioids depressed the slow e.p.s.p. in those neurones in which they did not change the resting membrane potential.

5 The slow e.p.s.p. was increased in amplitude in those neurones hyperpolarized by opioids. Depolarizations resulting from application of barium, substance P or ACh were also enhanced by opioids. Equivalent circuit models in which opioids increase, and substance P or ACh decrease, the same potassium conductance could account for this enhancement.

6 The actions of opioids were prevented or reversed by naloxone (1 nM–1 μM).

7 It is concluded that morphine and enkephalin inhibit the release of ACh and a non-cholinergic transmitter from fibres of the myenteric plexus, and that this may involve a hyperpolarization of presynaptic fibres. Additionally, opioids can interact postsynaptically with other substances which affect membrane potassium conductances.

Introduction

Narcotic analgesic drugs, such as morphine, have marked effects on gastrointestinal function which have long been exploited both therapeutically and experimentally. A widely used experimental preparation is the guinea-pig isolated ileum. Opioids inhibit the release of acetylcholine (ACh) from this preparation when the nerves within it are excited by passage of a brief electric current; opioids also inhibit the release of other transmitters similarly evoked, including substance P (see Kosterlitz & Waterfield, 1975; Schulz, 1978; Gintzler & Scalisi, 1982). In those kinds of experiments, a large number of neurones are activated synchronously and the transmitter so released is most often assayed by its effect on the longitudinal muscle layer. In the present experiments, the effects of opioids on transmitter release in the myenteric plexus were studied using the action of the transmitter on the membrane of a single myenteric neurone as the assay system in order to make inferences about the mechanism of the presynaptic inhibition.

About one-quarter of guinea-pig myenteric neurones appear to contain enkephalin and some others contain dynorphin (Furness & Costa, 1982; Furness *et al.*, 1983; Watson *et al.*, 1981) but little is known of the physiological role of these endogenous opioids. Opioids hyperpolarize a proportion of myenteric neurones by increasing their membrane potassium conductance (North & Tonini, 1977; North *et al.*, 1979) but effects on synaptic transmission within the

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myenteric plexus have not been studied in detail. Therefore, a further aim of the present experiments was to examine the effects of opioids, applied exogenously, on various types of synaptic potentials in the myenteric plexus. Knowledge of the effects of exogenous opioids was thought to be a prerequisite for understanding the role of endogenous opioids in the function of the enteric nervous system.

Methods

Intracellular recordings were made from myenteric plexus neurones of the guinea-pig ileum. The longitudinal muscle layer was dissected from the wall of the ileum, and an approximately rectangular piece was cut which had sides of 1–3 mm. Such a piece typically had five to twelve myenteric ganglia and the interconnecting strands of the plexus adherent to it. Ganglia from which intracellular recordings were made were immobilized by pinning to a silicone resin (Sylgard) which formed the base of the recording chamber. Ganglia were visualized with differential interference contrast optics at a final magnification of either 500 (Zeiss RA34 microscope) or 200 (Leitz inverted microscope). The preparations were superfused with a solution of the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11; the solution was gassed with 95% O_2 and 5% CO_2 and the temperature was 35–37°C. The methods of intracellular recording were essentially the same as those described by Nishi & North (1973); the amplifier (WP Instruments M701) had provision for intracellular current injection.

Synaptic potentials were evoked by focal stimulation of the ganglion surface or a strand of nerve fibres in the plexus leading to the ganglion; the glass stimulation electrode contained the solution described above and had a tip diameter of 10–20 μm. In some experiments, a bipolar tungsten electrode was used; this was insulated except for the tip. Single pulse stimuli (500 μs duration, typically 1–20 nA) evoked fast excitatory postsynaptic potentials (e.p.s.ps) in S-neurones (for definition of neuronal types see North, 1982). Many S-neurones also responded to a single pulse stimulus with a slow e.p.s.p. (Johnson *et al.*, 1980; North & Tokimasa, 1982). Repeated pulse stimuli (up to 10 Hz for 3 s) evoked slow e.p.s.ps in the majority of both S- and AH-neurones, and occasionally slow inhibitory postsynaptic potentials (i.p.s.ps). The frequency of occurrence, the time course, and the ionic mechanism of both depolarizing and hyperpolarizing synaptic potentials were similar to those previously described (Johnson *et al.*, 1980; 1981; Bornstein *et al.*, 1984). In confirmation of North & Tonini (1977), application of opioids caused a membrane hyperpolarization in about 50% of myenteric neurones. Synaptic potentials in the presence of the opioid were measured during the passage of sufficient depolarizing current across the soma membrane to restore the membrane potential to its control value before application of the opioid.

Drugs were applied to the ganglia in three ways. The first was by changing the superfusing solution to one which differed only in its content of the drug. ACh, substance P and γ-aminobutyric acid (GABA) were also applied by ionophoresis and by pressure ejection. For ionophoresis, a micropipette (tip diameter 1–2 μm) was filled with ACh (500 mM), substance P (10 mM) or GABA (500 mM) and its tip was positioned within 5 μm of the impaled neurone. Drugs were applied by passing brief (10–100 ms) pulses of outward current (5–100 nA). A retaining current of 3–5 nA was routinely applied in order to reduce leakage from the pipette (see Katayama *et al.*, 1979). For pressure ejection, a micropipette (tip diameter 5–10 μm) was filled with ACh (100 μM), substance P (10 μM) or GABA (10 mM) and its tip was placed 30–50 μm from the cell soma. A brief pulse (10–50 ms) of pressure (up to 100 kPa) was applied to eject the drug.

Drugs used were acetylcholine chloride (Sigma), atropine sulphate (Merck), α-chymotrypsin (Millipore), [D-Ala^2,D-Leu^5]enkephalin (DADLE) (Peninsula), GABA (Sigma), hexamethonium bromide (Sigma), [Met^5]enkephalin (Peninsula, Miles), morphine sulphate (Mallinckrodt), naloxone hydrochloride (gift of Endo Laboratories), normorphine hydrochloride (courtesy of Dr Jacobsen), oxotremorine sesquifumarate (Sigma) and substance P (Peninsula).

Results

Opioids depress the fast excitatory postsynaptic potential

The fast e.p.s.p. in myenteric neurones is due to the release of ACh acting on nicotinic receptors and its characteristics are similar to those observed in a variety of autonomic ganglia (Nishi & North, 1973). Superfusion with morphine (100 pM–3 μM), normorphine (1 nM–1 μM) and [Met^5]enkephalin (100 pM–1 μM) reduced the e.p.s.p. amplitude. The sensitivity to the opioids varied considerably from cell to cell. However, in a given neurone the degree of depression was concentration-dependent. For example, morphine (100 pM) reduced the fast e.p.s.p. amplitude by 16 ± 3% (n = 8) (these and other values are means ± s.e.mean). Morphine (1 μM) depressed the e.p.s.p. amplitude by 38 ± 5% (n = 33) of control (Figure 1). Similar effects were caused by [Met^5]enke-

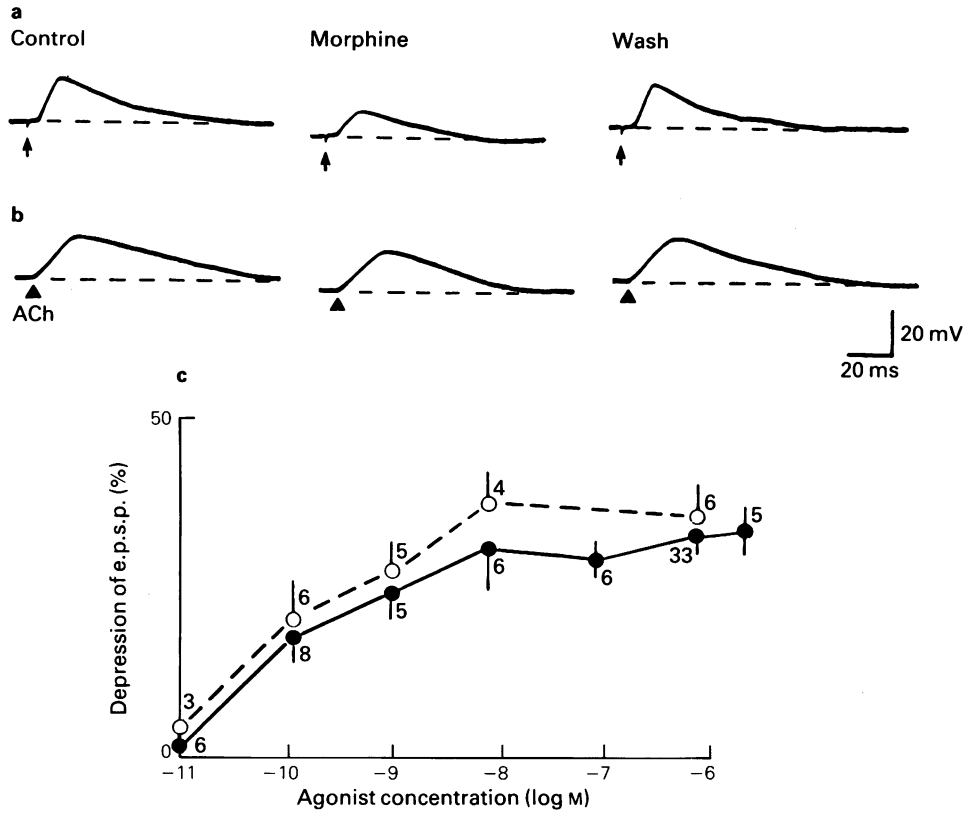

Figure 1 The depression of the fast excitatory postsynaptic potential (e.p.s.p.) by morphine. (a) Fast e.p.s.p. evoked by a single presynaptic pulse (arrow). During superfusion with morphine (10 nM) the amplitude of the fast e.p.s.p. was reduced and this effect reversed with 20 min wash. (b) Superfusion of morphine (10 nM) did not depress the nicotinic depolarization induced in the same neurone by ionophoretic application of ACh (▲; 10 nA for 10 ms) on the postsynaptic membrane. The membrane was hyperpolarized 5 mV by morphine. (c) Mean depression of the fast e.p.s.p. amplitude, expressed as percentage of control, induced by superfusion of morphine (○) and [Met^5]enkephalin (●). The vertical lines indicate s.e.mean for number of applications of agonists shown; the number of neurones is somewhat less because two or three concentrations were sometimes applied successively while recording from the same neurone.

phalin (Figure 1c). The mean maximal depression caused by morphine or [Met^5]enkephalin (at 1 μM) was about 35% (Figure 1c). This action of opioids was seen whether or not there was any membrane hyperpolarization of the neurone from which the recording was made.

The effect of opioids on the e.p.s.p. began within 10–30 s of the drug reaching the tissue bath and washed out within 10–20 min. In about one-third of the experiments, the washout of the opioid was associated with an increase in the e.p.s.p. amplitude over its control level: this enhancement of the e.p.s.p. sometimes reached the threshold for action potential initiation. This 'rebound' increase in the e.p.s.p. amplitude was also dependent upon the opioid concentration. The concentrations of opioids which caused maximal depression of the e.p.s.p (100 nM–5 μM) also caused a maximal 'rebound' increase on washout (maximum increase was 18 ± 4.5% (n = 28) of the control amplitude).

During prolonged (more than 5 min) exposure to opioid agonists, a fading of the opioid action was observed. In this case, the 'rebound' increase in the amplitude of the e.p.s.p. occurred even in the continued presence of the agonist (n = 7). This form of desensitization to the effect of the opioids was more marked when the agonist was applied for a second time – that is to say, the second application caused a smaller initial depression and a larger 'rebound' increase than the first application. Naloxone (3 nM–1 μM), which alone had no effect on the e.p.s.p. amplitude, prevented both the depression and the augmentation of the e.p.s.p. In these experiments, naloxone was applied for 2–5 min before, and then concomitant with, the application of the opioid agonist. Although no systematic study of the effective

naloxone concentrations was carried out, it was apparent that equimolar concentrations generally caused complete block of the agonist effects.

The possibility that the depression of the fast e.p.s.p. resulted from a change in the sensitivity of the postsynaptic membrane to the nicotinic action of ACh was tested. Ionophoretic application of ACh (10–20 nA, 5–20 ms) to the membrane of the impaled cell caused depolarizations which were similar in time course to the fast e.p.s.p. These ACh potentials were not affected by superfusion with opioids in concentrations which caused maximal depression of the e.p.s.p. (Figure 1b).

The depression of the fast e.p.s.p. by opioids could result from one or more of the following mechanisms: a reduction of the number of fibres excited which contributed to the e.p.s.p., a block of action potential propagation along the fibres, or a depression of the amount of ACh released by each action potential arriving at the release site apposing the impaled neurone. The first two possibilities were suggested by earlier experiments (North & Tonini, 1977; Morita & North, 1981) and also by the results of experiments employing pressure application of enkephalin. When [Met^5]enkephalin or [D-Ala^2,D-Leu^5]enkephalin was applied by pressure pulse, it was found in four of six neurones that a depression of the e.p.s.p. was observed only when the opioid was ejected in the vicinity of the fibres approaching the ganglion which contained the impaled cell, and not when applied over the soma of the cell itself; in two neurones, the pressure application of opioid at either position depressed the e.p.s.p. Experiments were therefore preformed in an effort to distinguish between these possible mechanisms of presynaptic action.

Dependence of opioid effect on strength of stimulation

The e.p.s.p. evoked by low stimulus strengths was depressed more by opioids than the larger e.p.s.p. resulting from a higher stimulus voltage. In the experiments illustrated in Figure 1c no account was taken of the strength of stimulation. However, in four neurones to which [Met^5]enkephalin (100 mM) was applied the depression of the e.p.s.p. which was observed was 75 ± 12% for low stimulus strengths and 33 ± 11% for high stimulus strengths. In these neurones, the control amplitude of the e.p.s.p. were 6.3 ± 1.3 mV (low stimulus strength) and 14.1 ± 2.6 mV (high stimulus strength). Superfusion with low concentrations of [Met^5]enkephalin

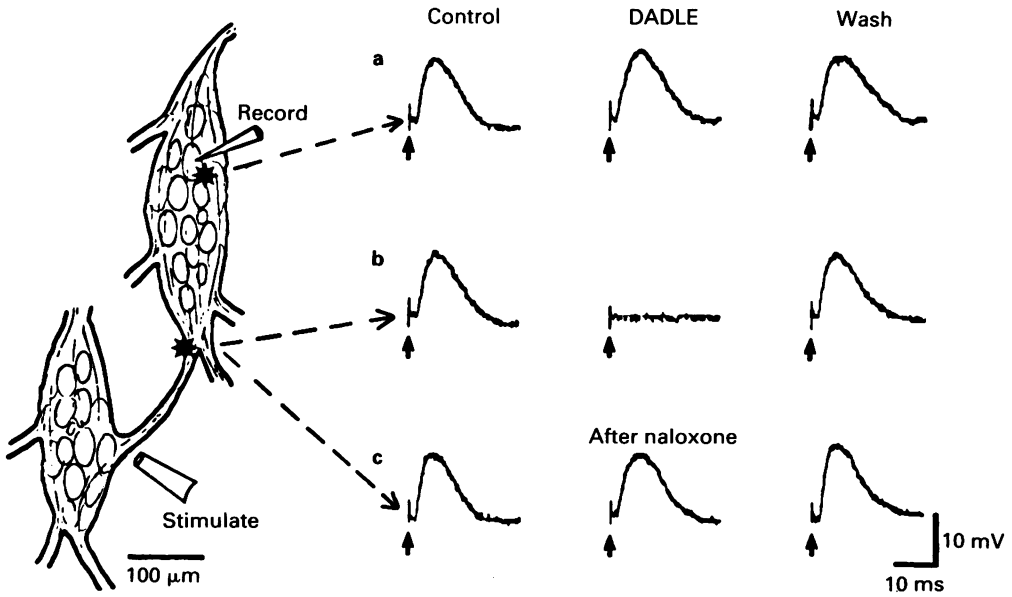

Figure 2 The effect of [D-Ala^2,D-Leu^5]enkephalin (DADLE) on single fibre excitatory postsynaptic potential (e.p.s.p.). Left, drawing of two connected myenteric ganglia to illustrate position of recording and stimulating electrodes, and positions of application of DADLE (asterisks). Right, electrophysiological records of single fibre e.p.s.p. evoked by a single low stimulus strength pulse to an interconnecting nerve entering the ganglion. DADLE was applied by pressure ejection (50 ms, 40 kPa; concentration in the pipette 10 μM) from the tip of a pipette positioned on the surface of the ganglion. (a) Ejection of DADLE close to the soma of the impaled neurone had no effect. (b) The same ejection over the fibres entering the ganglion completely abolished the fast e.p.s.p. (c) This effect was not seen during superfusion of naloxone (100 nM).

(1–10 mM) often almost completely abolished the e.p.s.p. evoked with a low stimulus strength while having little or no effect on the e.p.s.p. evoked by a high stimulus strength.

All-or-nothing depression of single fibre e.p.s.p. It was difficult to evoke e.p.s.ps which clearly resulted from excitation of single fibres. This was possible only when the focal stimulating electrode was moved to a distance of several hundred μm from the impaled cell, on the surface of another ganglion or on an interconnecting strand. In these cases (four experiments), progressively increasing the stimulus voltage resulted in an e.p.s.p. which appeared in an all-or-nothing manner and had a relatively fixed amplitude, suggesting that it arose from excitation of a single presynaptic fibre. Superfusion with morphine (100 mM) depressed this e.p.s.p.; recovery was complete within 5 min of discontinuing exposure to morphine. Pressure ejection of [D-Ala^2,D-Leu^5]enkephalin had a similar effect but only when the tip of the pipette containing the opioid was positioned over the fibres entering the ganglion. There was no depression when an equal amount of [D-Ala^2,D-Leu^5]enkephalin was ejected from the pipette placed directly over the impaled cell (Figure 2). Superfusion with naloxone (100 mM) prevented these opioid effects.

Run-down of e.p.s.p The amplitude of the fast e.p.s.p. declines when e.p.s.ps are evoked at frequencies exceeding 0.5 Hz (Nishi & North, 1973). The amplitude of ACh depolarizations elicited at similar frequencies does not decline, so the run-down has been attributed to a reduction in the amount of ACh released from the presynaptic nerves. Morphine (1 μM) depressed the fast e.p.s.p. but did not change the ratios of the amplitudes of the first to the successive e.p.s.ps in trains at 1, 2, 5 or 10 Hz. In other words, all e.p.s.ps in a train were depressed by the same proportionate amount.

Opioids depress or enhance the slow excitatory postsynaptic potential

The slow e.p.s.ps observed in the present study were similar to those previously described (Johnson *et al.*, 1980; 1981; Bornstein *et al.*, 1984). Single pulse stimuli evoked slow e.p.s.ps in about 30% of S cells and these were reversibly abolished by atropine. Single pulse stimuli evoked slow e.p.s.ps in only about 10% of AH cells and these potentials were atropine-insensitive. Stimuli comprising trains of pulses (typically 10 Hz for 1–3 s) evoked slow e.p.s.ps, in both S- and AH-neurones, which were atropine-insensitive. In those cells in which morphine did not change membrane potential ($n = 16$), the slow e.p.s.p. was usually depressed in a dose-dependent manner. In three of these

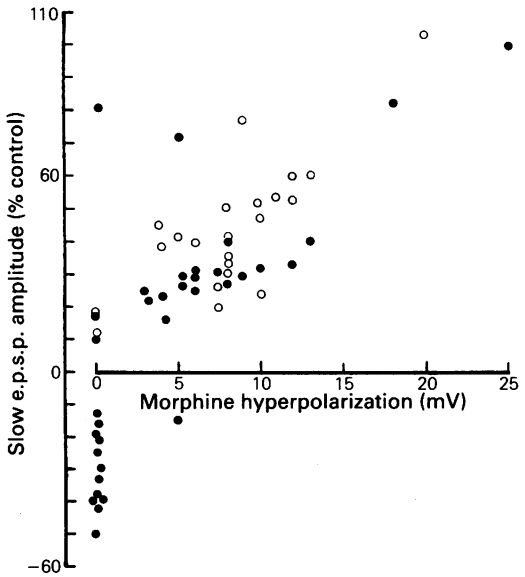

Figure 3 Relation between effect of morphine on membrane potential and effect on amplitude of slow depolarizations. Each point indicates the change, during morphine exposure, in the amplitude of the slow e.p.s.p. (filled circles) or in the slow muscarinic depolarization induced by ionophoretic application of ACh (open circles), expressed as percentage of their control amplitude. Abscissae are the hyperpolarizations caused by the superfusion of morphine (10–100 nM) in the same neurones. E.p.s.ps and ACh responses were all evoked after restoring the membrane potential to its control level during the superfusion of morphine. In the absence of any membrane hyperpolarization, morphine usually depressed the amplitude of the slow e.p.s.p.

neurones, the slow e.p.s.p. was increased in amplitude (by 10, 17 and 80%). Morphine hyperpolarized 19 neurones and in 18 of these cells the amplitude and duration of the slow e.p.s.p. was increased (Figure 3).

Neurones which were not hyperpolarized by opioids The slow e.p.s.p. evoked by a single pulse in the S-neurones (which was also shown to be blocked by atropine (300 nM)) was reversibly depressed in amplitude by superfusion with morphine (100 nM). Morphine and normorphine had a similar effect on the slow e.p.s.p. evoked by repeated pulse stimuli in both S- and AH-neurones. The depression of the slow e.p.s.p. was dependent upon the concentration of morphine applied (Figure 4). This inhibition occurred within 1–3 min of the morphine reaching the tissue bath and the effect reversed completely during 10 min of washing with drug-free solution. The depression of the slow e.p.s.p. was reversed or prevented by concomitant superfusion with naloxone (100 nM–1 μM)

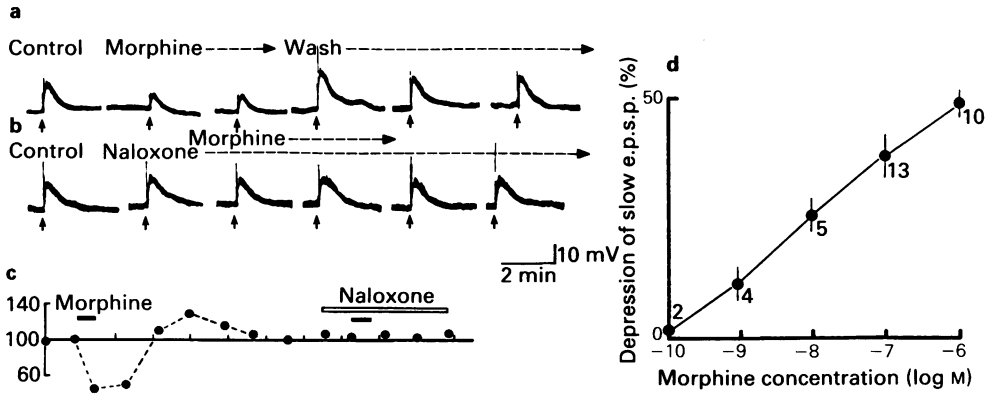

Figure 4 Depression of the slow excitatory postsynaptic potential (e.p.s.p.) by morphine. (a) Slow non-cholinergic e.p.s.ps evoked by repetitive presynaptic nerve stimulation (5 Hz, 2 s) in an S cell. Superfusion of morphine (1 μM) depressed the amplitude of the slow e.p.s.p. Immediately after discontinuing exposure to morphine, there was a rebound increase in amplitude of the slow e.p.s.p.; full recovery occurred after 20 min. (b) The effect of morphine was prevented by superfusion of naloxone (1 μM). Naloxone alone had no effect on the slow e.p.s.p. Same neurone (a) and (b); resting membrane potential − 60 mV. (c) Time course of the change of the slow e.p.s.p amplitude shown in (a) and (b). Ordinates are e.p.s.p. amplitudes expressed as a percentage of control. Abscissae are time points at 5 min intervals. Solid and open bars indicate periods of superfusion with morphine and naloxone respectively. (d) Concentration-dependence of depression of the slow e.p.s.p. by morphine. Each point shows the mean depression, with vertical lines representing s.e.mean, observed of the number of applications indicated (34 applications to 15 neurones; 12 S- and 3 AH-neurones).

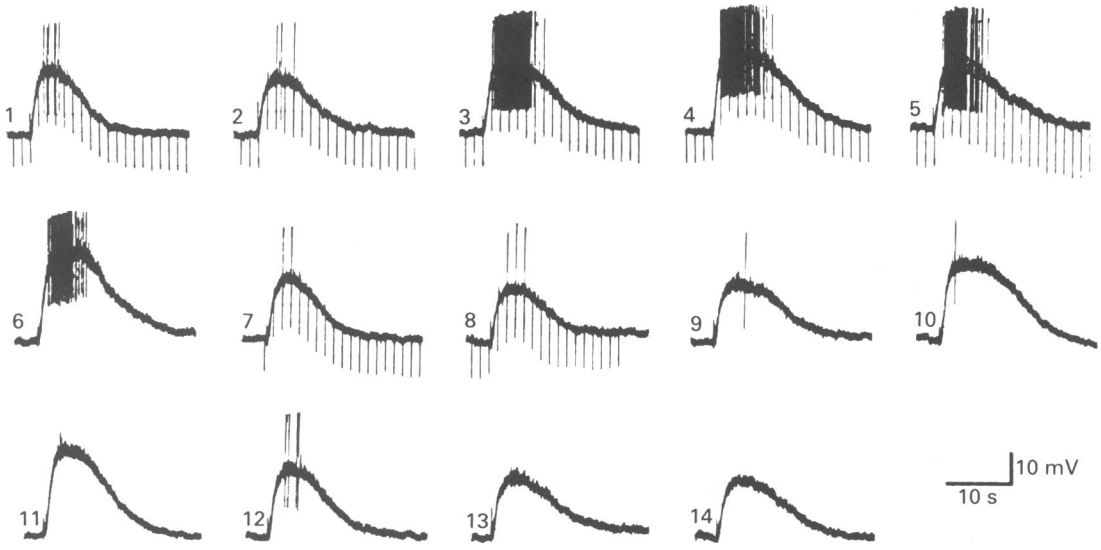

Figure 5 The potentiating effect of normorphine on the slow e.p.s.p. and the inhibitory effect of naloxone. Each slow non-cholinergic e.p.s.p. was evoked by a repetitive pulse stimulus (15 Hz for 500 ms). 1 and 2, controls; 3–6, during superfusion of normorphine (1 μM). Normorphine caused a membrane hyperpolarization (6 mV) and increased the amplitude and the duration of the slow e.p.s.p. (these were evoked after restoring the membrane potential to the resting level). 7–9, during superfusion with normorphine and naloxone (100 nM). 10 and 11, after removal of naloxone by washing out the slow e.p.s.p. increased. 12–14, during washout of normorphine. Full recovery occurred 8 min after washing out normorphine. Downward deflections on traces 1–5, 7 and 8 are electrotonic potentials evoked by fixed amplitude hyperpolarizing current pulses (200 pA). S-neurone, resting potential − 54 mV.

($n = 4$) (Figure 4). Depolarizations evoked by iontophoretic application of ACh, oxotremorine or substance P were not affected by opioids. In most neurones, the initial depression of the slow e.p.s.p. was followed by a rebound increase in amplitude when the morphine was washed out, as was observed for the fast e.p.s.p. The rebound increase in amplitude was prevented by concentrations of naloxone (100 nM) which also prevented the initial depression of the slow e.p.s.p. [Met^5]enkephalin caused a similar depression of the slow e.p.s.p.; the reduction in amplitude was $30 \pm 7.7\%$ ($n = 4$) by 100 nM, and $42 \pm 4.4\%$ ($n = 5$) by 1 μM. These effects were also prevented by naloxone (100 nM) ($n = 3$). Naloxone alone had no consistent effect on the e.p.s.p. amplitude.

Neurones which were hyperpolarized by opioids A single pulse stimulus to the presynaptic nerve evoked a slow e.p.s.p.; this was shown to be reversibly abolished by atropine (300 or 500 nM) in five neurones (4 S and 1 AH). Morphine (10 and 100 nM) hyperpolarized three of these cells (7.6 ± 3.7 mV) and had no effect on the membrane potential of the others. In the three cells which were hyperpolarized, the slow e.p.s.p. amplitude was increased by $58 \pm 6.9\%$; this enhancement caused action potentials to be generated by a slow e.p.s.p. which was previously too small to reach threshold for spike generation. These effects of morphine occurred within 10–30 s of the drug reaching the tissue and were reversed within 10–20 min when exposure to morphine was discontinued.

Presynaptic nerve stimuli comprising many pulses were more commonly used to evoke the slow e.p.s.p. In some cases, atropine was present but in most experiments it was not known whether the slow potential was cholinergic or due to other transmitters, such as substance P (Bornstein *et al.*, 1984). The presynaptic stimuli were typically 1–3 s in duration at 10–15 Hz. The enhancement of the slow e.p.s.p. was observed whether or not the membrane potential was restored to its value prior to morphine application before evoking the synaptic response. In a given cell, the enhancement was larger for higher morphine concentrations, but among many cells there was considerable variability in the amplitude of the effect. The largest hyperpolarizations which were observed (18 and 25 mV) were associated with increases in the slow e.p.s.p. amplitude (measured at the original membrane potential) of 82 and 100% respectively (Figure 3). There was a strong positive correlation between the increase in e.p.s.p. amplitude (measured at the resting membrane potential) and the magnitude of the morphine-induced hyperpolarization ($r = 0.85$, $P < 0.001$, $n = 22$) (Figure 4). This action of morphine started within 10–30 s of the morphine reaching the bath but it was noted in several cases that the slow e.p.s.p. was transiently depressed just before the membrane started to hyperpolarize in response to morphine. This may indicate a concomitant presynaptic action. [Met^5]-enkephalin had an action similar to that of morphine. The enhancement of the slow e.p.s.p. by morphine was not observed when morphine was applied at the same time as naloxone (100 nM and 1 μM; $n = 4$) (Figure 5).

In two cells, slow depolarizations occurred spontaneously and these had very similar time courses to the evoked responses. The amplitudes of these depolarizations were also increased by morphine, which hyperpolarized both cells.

Opioids enhance depolarizations evoked by acetylcholine, substance P and barium

Muscarinic depolarization ACh was applied to the postsynaptic membrane of 35 neurones by iontophoresis or pressure ejection. This evoked a fast (nicotinic) and a slow (muscarinic) depolarization in S-cells ($n = 16$) and a slow muscarinic depolarization in AH-cells ($n = 19$) (North & Tokimasa, 1982; Morita *et al.*, 1982a). Superfusion of morphine (10–100 nM) hyperpolarized 20 of these neurones (15 S, 5 AH). This was associated with an increase in the amplitude and duration of the muscarinic potential (Figures 3 and 6). This increase in amplitude of the muscarinic potential was observed whether or not the membrane potential was restored to its original level after the morphine application. The fall in membrane conductance produced by the muscarinic action of ACh was also increased by morphine; morphine did not change the reversal potential of the muscarinic response. In three neurones, the input resistance was measured (from the amplitudes of hyperpolarizing electrotonic potentials) at various times throughout the muscarinic depolarization. The input resistance rose and fell as a double exponential function of time (see North & Tokimasa, 1984), with a maximum value which was 37% greater than the control input resistance. During application of morphine (10 nM), which hyperpolarized these cells by 5–6 mV, the muscarinic depolarization was associated with a peak rise in input resistance to 52% of control. However, morphine did not change the time course with which the input resistance rose and later declined (time constants 800 ms and 5 s respectively).

Although the degree of enhancement of the muscarinic response varied from cell to cell, there was a high degree of correlation between the morphine-induced hyperpolarization and the increase in the muscarinic depolarization ($r = 0.79$, $P < 0.0025$, $n = 22$). For a given morphine concentration, the enhancement of the muscarinic depolarization was greater than the enhancement of the slow e.p.s.p. (compare open and filled circles in Figure 3). This was particularly obvious in those neurones in which both the muscarinic response to ACh and the slow e.p.s.p.

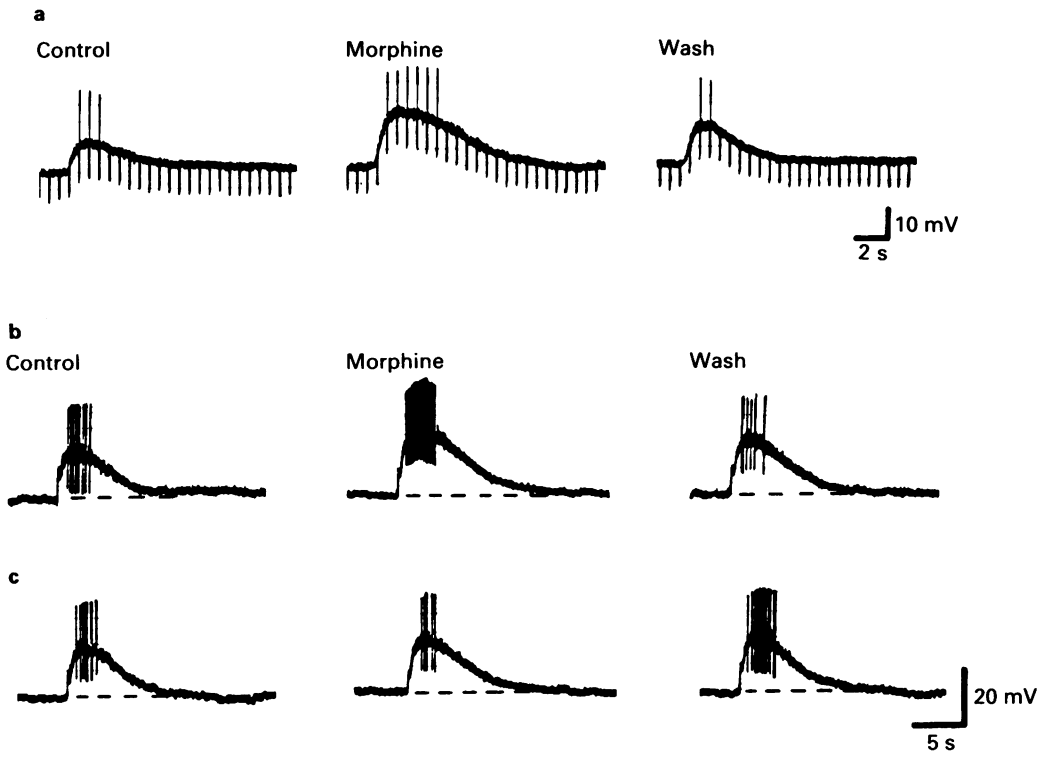

Figure 6 Potentiation of the slow muscarinic depolarization during morphine hyperpolarization. (a) AH-neurone; (control) ACh was applied by ionophoresis (50 nA for 80 ms). Superfusion of morphine (1 μM) caused a membrane hyperpolarization (10 mV) and a slight increase in membrane conductance and in the amplitude and duration of the slow muscarinic depolarization (evoked at resting potential, − 60 mV; current pulses to test input resistance were 200 pA). (b and c) Fast nicotinic and slow muscarinic depolarization induced by ionophoretic application of ACh (50 nA for 30 ms) onto an S cell. (The nicotinic component is visible at this time scale only as the rapidly rising initial part of the depolarization). (b) Superfusion of morphine (100 nM) caused a membrane hyperpolarization (8 mV) and increased the amplitude and duration of the slow muscarinic depolarization (the membrane potential was restored to the resting level). No change in the nicotinic depolarization occurred. (c) The effect of morphine (100 nM) was not observed when naloxone (100 nM) was present in the superfusing solution.

were enhanced in parallel during morphine superfusion. This may be because the slow e.p.s.p. is depressed by the presynaptic action of morphine in addition to being enhanced by the postsynaptic action. Thirteen cells were not hyperpolarized by morphine and in these cells there was no change in the amplitude or time course of the muscarinic depolarization. In two neurones, morphine (10 and 100 nM) caused small increases in the amplitude of the muscarinic depolarization (11 and 16%) without any accompanying change in membrane potential. The enhancement of the muscarinic depolarization was not seen when morphine was applied at the same time as naloxone (100 nM–1 μM, $n = 4$).

Substance P depolarization Ionophoretic or pressure application of substance P caused a membrane depolarization and conductance decrease similar in all respects to that described previously (Katayama *et al.*, 1979). Superfusion with morphine (100 nM–1 μM) hyperpolarized four cells (5.7 ± 0.8 mV) and did not affect one cell. In all five neurones, the amplitude of the substance P response was increased (by 32.6 ± 4.7%). Naloxone prevented the action of morphine in the one cell in which it was tested.

Barium depolarization Barium ions are known to mimic the muscarinic action of ACh in central (Krnjevic *et al.*, 1971) and peripheral neurones (Constanti *et al.*, 1981; North & Tokimasa, 1984). We compared the effect of barium applied by brief pressure pulse before and after morphine. Morphine (10 nM–100 nM) increased the amplitude of the barium depolarization and prolonged its time course. This effect occurred in three neurones which were hyperpolarized by morphine and in one which was unaffected by morphine.

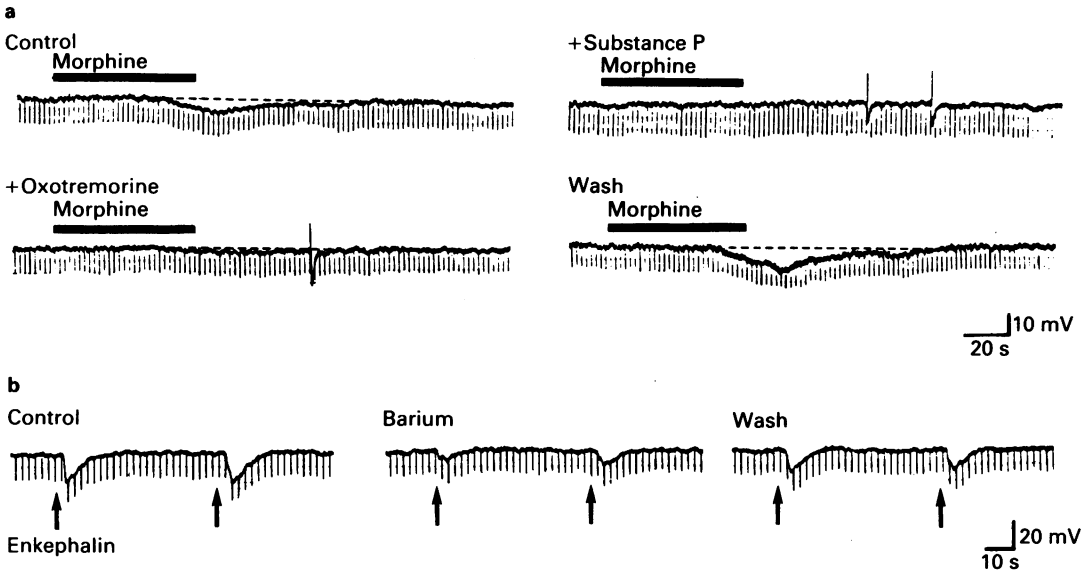

Figure 7 The effect of substance P, oxotremorine and barium on opioid-induced hyperpolarizations. (a) Superfusion of AH-neurone with morphine (1 μM) (solid bars above traces) caused a hyperpolarization associated with a conductance increase. During prior and concomitant superfusion with substance P (10 nM), or oxotremorine (1 nM), the morphine hyperpolarization was much reduced, but subsequently recovered after washout of the drugs. This concentration of oxotremorine did not change the membrane potential or conductance. Substance P caused a small depolarization (less than 3 mV), and the morphine was applied after the potential was restored to the control level by passing a small hyperpolarizing current (resting potential − 58 mV; current pulses were 200 pA). (b) [Met^5]enkephalin (arrows) was applied by pressure ejection (5 pulses of 70 kPa for 100 ms) from the tip of a pipette positioned 30 μm from the cell soma; this caused a hyperpolarization with a conductance increase. Superfusion of barium (30 μM) did not change the resting potential but depressed the response to [Met^5]enkephalin. The response recovered after washing out the barium. S-neurone; resting potential − 52 mV, current pulses were 200 pA.

γ-Aminobutyric acid depolarizations GABA depolarizes myenteric neurones by increasing their chloride conductance (Cherubini & North, 1984). Four neurones were hyperpolarized (6.7 ± 1.1 mV) by morphine 100 nM–1 μM). The depolarization and conductance increase caused by local ionophoresis of GABA onto these cells were not increased by morphine.

Muscarinic agonists, substance P and barium depress morphine-induced hyperpolarizations

Oxotremorine was used instead of ACh in these experiments in order to avoid any nicotinic component of the depolarization (Morita *et al.*, 1982a). Opioid hyperpolarizations were depressed by oxotremorine, substance P and barium, applied at concentrations which themselves caused little (up to 3 mV) or no effect on membrane potential or conductance (oxotremorine 3–10 nM; substance P 3–10 nM; barium 30 μM) (Figure 7). Similar observations were made whether the opioid hyperpolarization resulted from perfusion of morphine (100 nM–1 μM) ($n = 5$) or pressure application of [Met^5] enkephalin ($n = 2$).

Slow inhibitory postsynaptic potential (i.p.s.p.)

The slow i.p.s.p. was graded in amplitude with the number of pulses applied to the presynaptic nerves and it had a reversal potential similar to that of the afterhyperpolarization following the action potential. In these respects it was similar to the slow i.p.s.p. described by Johnson *et al.* (1980). The slow i.p.s.p. was not affected by chymotrypsin (200 μg ml^{-1}) or atropine (1 μM). Superfusion with morphine reversibly depressed the slow i.p.s.p. in a manner similar to that with which it depressed the slow e.p.s.p. It was difficult to examine the reversal of this morphine effect by naloxone, because it was observed that naloxone itself (100 nM and 1 μM) partially depressed the slow i.p.s.p. in five of the six cells to which it was applied, indicating perhaps that an endogenous opioid peptide contributes to the generation of the slow i.p.s.p. in some neurones.

Discussion

Opioid depression of the fast excitatory postsynaptic potential

A presynaptic inhibition of ACh release is indicated by two main findings. First, the e.p.s.p. was depressed even in those neurones in which opioids had no effect on the membrane potential or input resistance. Second, the depolarization evoked by ionophoresis of ACh was not depressed by opioids. This presynaptic inhibition of ACh release by opioids is similar to that described for the guinea-pig inferior mesenteric ganglion (Konishi *et al.*, 1979a; 1979b; 1981), cat ciliary ganglion (Katayama & Nishi, 1984), and frog neuromuscular junction (Frederickson & Pinsky, 1971; Bixby & Spitzer, 1983a). The presynaptic inhibition may occur for one or more of the following reasons: (1) fibres are hyperpolarized by opioids and are therefore no longer exicted by the same electrical stimulus; (2) a hyperpolarization (and/or conductance increase) of presynaptic fibres causes the action potential to block between the site of its initiation and its arrival at the synapse apposing the impaled cell; (3) less transmitter is released by the action potential invading the release site.

The focal stimulating electrode was positioned over the ganglion surface or an interconnecting strand of the plexus. The applied opioids superfused the entire tissue; therefore, it is possible that an opioid-induced hyperpolarization of the presynaptic fibres would simply prevent their excitation by a given electrical current. Since a substantial proportion of myenteric neurones, including their cell processes, are hyperpolarized by opioids (North & Tonini, 1977; Morita & North, 1981; North *et al.*, 1979), this hyperpolarization may contribute to the inhibition of ACh release when electrical field stimulation of the entire plexus is used (see Szerb, 1982). One of the present results provides evidence for this mechanism; this is the finding that the large amplitude e.p.s.ps were less depressed by opioids than the small amplitude e.p.s.ps. A hyperpolarization of fibres at the point of stimulation would prevent their excitation when low stimulus currents were used, but would be less effective when the stimulus current was supramaximal. On the other hand, the possibility cannot be excluded that high and low threshold fibres simply differ in their sensitivity to opioids.

As it was found previously that opioids can block the propagation of the action potential along the processes of myenteric neurones (Morita & North, 1981), is it possible that a similar action of presynaptic fibres could lead to depression of the e.p.s.p.? One observation in favour of this is the finding that an e.p.s.p. resulting from excitation of a single presynaptic fibre was depressed in an all-or-nothing manner when the opioid was applied locally over the path of the presynaptic fibre (Figure 2).

The final possible way in which the presynaptic inhibition may occur is a reduction in the amount of calcium entering the terminal each time it is invaded by an action potential. There is evidence that opioids reduce calcium entry through voltage-sensitive channels, either by a direct action on the calcium conductance (Mudge *et al.*, 1979; Bixby & Spitzer, 1983b; Werz & Macdonald, 1983) or by an indirect action secondary to an increased potassium conductance (North & Williams, 1983). The present experiments do not allow one to judge which of these mechanisms may operate; however, if the membrane properties of the neurone cell bodies can be taken to represent those of the nerve terminal release sites, then it may be important that morphine increases the potassium conductance of many myenteric neurones. In this context, it should be noted that morphine does not affect the calcium action potential recorded in the presence of tetrodotoxin (Cherubini *et al.*, 1984a; Cherubini & North, 1985). In brief, an increase in the potassium conductance of the entire membrane of the presynaptic cell could give rise to all of the present experimental findings; namely, a failure to excite the presynaptic cell, a failure of propagation in presynaptic fibres and a reduction in the amount of ACh released when the action potential reaches the synapse.

Presynaptic depression of the slow excitatory postsynaptic potential

The depression of the slow e.p.s.p. was qualitatively similar to that described by Konishi *et al.* (1979b) and Jiang *et al.* (1982). It seems likely that this depression is also presynaptic because depolarizations evoked by muscarinic agonists and substance P were not reduced in amplitude. It is clear that any or all of the factors discussed in the preceding section could contribute to this presynaptic inhibition; the present results do not allow one to discriminate between them.

Postsynaptic enhancement of the slow excitatory postsynaptic potential

When morphine hyperpolarized myenteric neurones, the responses to a variety of substances which reduce potassium conductance were enhanced. This may be because morphine opens the same potassium conductance as that closed by muscarinic agonists, substance P and barium. Such a conclusion was supported by calculating the interaction to be expected on the basis of a simple equivalent circuit model (see Appendix).

Concentrations of ACh and substance P, too low to cause detectable resistance changes, also decreased opioid hyperpolarizations. It has been found that the same low concentrations of ACh and substance P also

shorten or abolish the calcium-dependent after hyperpolarization of AH-neurones (Katayama *et al.*, 1979; North & Tokimasa, 1983). One interpretation is that opioids increase a potassium conductance in the membrane which can also be increased by the calcium entry of the action potential, and that substance P and ACh interfere with the process by which the intracellular calcium leads to the increase in potassium conductance (see North & Tokimasa, 1983). There are several similarities between the potassium conductance increased by opioids and that which is activated by a transient influx of calcium into AH-neurones. Both appear to be almost independent of membrane potential in the range − 60 to − 110 mV (Morita *et al.*, 1982b; Morita & North, 1982); both are sensitive to barium (see Figure 7) and to quinine (Cherubini *et al.*, 1984b). According to this interpretation, opioids and intracellular calcium are less able to increase this potassium conductance when low concentrations of ACh or substance P are applied to the cell. A component of the resting potassium conductance may be sensitive to the concentration of calcium in an intracellular compartment (Grafe *et al.*, 1980). ACh and substance P may reduce the potassium conductance by reducing the calcium concentration in this compartment, as has been argued previously for barium (North & Tokimasa, 1983; 1984).

In conclusion, opioids cause presynaptic inhibition of the release of ACh and the non-cholinergic transmitter which mediates the slow e.p.s.p., probably substance P (Bornstein *et al.*, 1984); considerable, but as yet indirect, evidence suggests that this may result from an increase in the membrane potassium conductance of the presynaptic fibres. Opioids also cause an increase in the potassium conductance of the cell bodies of myenteric neurones and this will have its own effect on synaptic transmission in the plexus. The fast e.p.s.p. will be increased in amplitude by membrane hyperpolarization and reduced in amplitude by the conductance increase, in so far as the synaptic current is resistive rather than capacitative (Edwards *et al.*, 1975). In the case of the slow e.p.s.p., the opioid-induced increase of potassium conductance of the postsynaptic membrane would tend to enhance the postsynaptic effectiveness of the transmitter which reaches it. This may occur because the slow synaptic transmitters and opioids both alter the same potassium conductance.

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APPENDIX

It is assumed that the membrane of a myenteric plexus neurone can be represented by a simple equivalent circuit in which all conductances are independent of membrane voltage. At the resting potential (E_r), there is an inward current through a conductance G_l, which is equal to the outward current through G_K. Taking values for the potassium equilibrium potential (E_K) of − 90 mV, equilibrium potential for the inward leak current (E_l) of + 40 mV, G_K 20 nS and G_l 6 nS, this current is 600 pA and the value for E_r is − 60 mV. This agrees reasonably well with experimental observations (see Morita *et al.*, 1982b for circuit diagram and choice of values).

When ACh is applied, a depolarization ΔV_A results from a reduction in G_K to a new value $y_A G_K$, where $y_A < 1$. Using the above values, application of Kirchoff's law to the equivalent circuit shows that $\Delta V_A = [30 - 130/(1 + 3.33y_A)]$. These values for the amplitude of the ACh depolarization appear on the top line of Table 1. When morphine is applied, G_K changes to $y_M G_K$ (where $y_M > 1$) and the resulting hyperpolarization (ΔV_M) is given by the analogous expression $\Delta V_M = [30 - 130/(1 + 3.33y_M)]$. Values for y_M and ΔV_M appear in the most left hand column of Table 1.

Table 1 The amplitude of the depolarization (mV) which would result from application of acetylcholine (ACh) assuming that ACh reduced the resting membrane potassium conductance to 90, 80, 70, 60, 50 or 30% of its resting value.

y_A	1	0.9	0.8	0.7	0.6	0.5	0.3
$y_M = 1$ ($\Delta V_M = 0$ mV)	0	2.5	5.5	9.0	13	19	35
$y_M = 1.5$ ($\Delta V_M = -8.3$ mV)	0	2.9	6.4	11	16	24	50
$y_M = 2$ ($\Delta V_M = -13$ mV)	0	3.1	6.8	12	18	26	57
$y_M = 3$ ($\Delta V_M = -18$ mV)	0	3.2	7.2	12	19	28	63

The top row ($y_M = 0$) gives the values in the absence of any morphine. The second to fourth rows give the values in the presence of the various conductance increases caused by morphine (y_M). The hyperpolarizations which would result from these conductance increases (before restoration of the membrane potential, see text) are also shown in mV (ΔV_M).

In the present study, the membrane potential was restored to its control value (E_r) during the application of morphine by passing a steady current (I) from a constant source. If this current is assumed to pass entirely through y_M G_K, then its amplitude will be $\Delta V_M y_M G_K$. Under this condition, application of ACh now causes the potassium conductance to change to $y_A y_M G_K$. The total membrane current through potassium channels will now be given by $(i + I)$ where $i = 130/[1/G_K y_A y_M) + (1/G_l)]$ and the amplitude of the resulting depolarization can be calculated from

$$\Delta V_A = 30 - [130/(1 + 3.33 y_A y_M) + (30/y_A) + 130/(y_A + 3.33 y_A y_M)]$$

Table 1 shows the expected ACh depolarizations which would result from a given conductance decrease in the present experimental conditions, in which the morphine first increased the conductance and the membrane potential was then restored to its control level by passing an external current. The effect of ACh is larger in the presence of morphine, and there is a positive correlation between the morphine hyperpolarization and the amplitude of the ACh depolarization (cf. Figure 3).

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