Br. J. Pharmac. (1977), **61**, 541–549

THE MECHANISM OF ACTION OF NARCOTIC ANALGESICS IN THE GUINEA-PIG ILEUM

R.A. NORTH & M. TONINI[1]

Neurophysiology Laboratory, Department of Pharmacology, Loyola University Stritch School of Medicine, 2160 South First Avenue, Maywood, Illinois 60153, U.S.A.

[1] Present address: Istituto di Farmacologia, Piazza Botta 10, 27100 Pavia, Italy.

1 Intracellular recordings were made from neurones in the myenteric plexus of the guinea-pig ileum. Single myenteric ganglia were maintained *in vitro* and drugs were applied by adding them to the perfusing solution.

2 Narcotic analgesics hyperpolarized the membrane of a proportion of neurones in the myenteric plexus.

3 The membrane hyperpolarization was sometimes associated with a decrease in input resistance. These effects reduced the excitability of myenteric neurones.

4 The effects of narcotics occurred at low concentrations (10 nM to 1 μM), were stereospecific and were reversed by naloxone.

5 It is proposed that the morphine-sensitive neurones may be the cholinergic efferents to the muscle layers. By hyperpolarizing these neurones, morphine may prevent their excitation by electric field stimulation. This may explain why narcotic analgesics reduce the output of acetylcholine and the contractile response of this preparation when it is excited by field stimulation.

Introduction

Morphine inhibits the peristaltic reflex of the guinea-pig isolated ileum by reducing the output of acetylcholine from the nerves in the myenteric (Auerbach's) plexus which innervate the muscle layers (Schaumann, 1956; 1957; Paton, 1957). Localization of the action of morphine to the myenteric plexus neurones was shown by Paton & Zar (1968) by using a preparation which contained only the myenteric plexus and the longitudinal muscle (Rang, 1964).

The guinea-pig isolated ileum and the myenteric plexus-longitudinal muscle preparation are now widely used as model systems in the investigation of narcotic analgesics (see review by Kosterlitz & Waterfield, 1975). Both the acetylcholine output from these preparations and the contractile response of the longitudinal muscle layer to electrical stimulation of its nerves are greatly depressed by narcotic analgesics. These actions of narcotics correlate well with their analgesic potencies (Kosterlitz & Waterfield, 1975), are reversed by specific narcotic antagonists such as naloxone (Kosterlitz & Watt, 1968) and are stereospecific (Creese & Snyder, 1975).

The primary action of morphine on the myenteric plexus was generally believed to be a direct interference with the process by which the action potential released acetylcholine from the neurones which innervate the longitudinal muscle. In support of this were the findings that the effect of morphine was not substantially altered by hexamethonium (Gyang & Kosterlitz, 1966; Greenberg, Kosterlitz & Waterfield, 1970; Waterfield & Kosterlitz, 1974), and that the excitatory postsynaptic potentials (e.p.s.ps) recorded from the myenteric ganglion cells were unaffected by morphine (North & Nishi, 1974; North & Henderson, 1975).

Recently, we found that low concentrations of narcotic analgesics hyperpolarize a proportion of neurones in the myenteric plexus (North & Tonini,

1976). This seems likely to be the basis of the inhibition by morphine of the firing recorded from myenteric neurones with extracellular electrodes (Sato, Takayanagi & Takagi, 1973; Dingledine, Goldstein & Kendig, 1974; Dingledine & Goldstein, 1975; 1976); indeed, Dingledine & Goldstein (1975) postulated that a membrane hyperpolarization might be the primary action of morphine. The purpose of the present experiments was to test the hypothesis that the membrane hyperpolarization might also be the basis for the inhibition by morphine of the acetylcholine output from the myenteric plexus. Preliminary accounts of parts of this work have been published (North, 1976; North & Tonini, 1976).

Methods

Single myenteric ganglia from the ilea of adult guinea-pigs were immobilized *in vitro* in the manner previously described (Nishi & North, 1973). The ganglia were maintained at 37°C by perfusion with a pre-warmed Krebs solution of the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, gassed with 5% CO_2 and 95% O_2. The flow rate of the Krebs solution was 1–3 ml/min and the bath volume 1–2 ml. Drugs were applied to the tissue by changing the perfusion solution to one which differed only in its content of the drug. Steady state bath concentrations would be unlikely to occur in less than 2 min from the time of arrival of a new solution at the tissue.

The ganglia were viewed with differential interference contrast (Zeiss-Nomarski) optics at a magnification of 500. The somata of individual cells were clearly visualized and impaled with glass microelectrodes. The microelectrodes were filled with 3 M KCl and had tip resistances between 60 and 120 MΩ. Intracellular potentials were amplified, displayed and photographed in the conventional manner. In some experiments the membrane potential was displayed on a potentiometer pen recorder (Varian G-14A-1).

Values for resting membrane potential were estimated by sudden withdrawal of the microelectrode from the cell. Values for input resistance were determined by passing a known current through the recording microelectrode by means of an active bridge circuit (WP Instruments, M701). Resistance measurements were accepted only if (a) the electrode showed resistive linearity over the range of currents used, (b) electrode resistance did not appear to change during cell impalement (i.e. there were no obvious time-independent transients at the start and end of the current pulse) and (c) the electrode resistance was the same after withdrawal from the cell as it was prior to insertion. Satisfactory resistance measurements were achieved in less than half of all the cells impaled.

A glass microelectrode (tip diameter 10 to 30 μm) filled with Krebs solution or 4M NaCl was used for extracellular focal stimulation. The tip of this electrode was placed on the surface of the ganglion at a distance of up to 100 μm from the impaled cell. A brief (200 μs to 1 ms) cathodal current pulse was used to excite neuronal elements within the ganglion. If the response of the neurone to focal stimulation was an e.p.s.p., it was classed as Type 1 (Nishi & North, 1973; Hirst, Holman & Spence, 1974). Neurones were also excited by passing a depolarizing current pulse through the recording microelectrode. If the action potential was followed by a slow after-hyperpolarization the neurone was classed as Type 2 (Nishi & North, 1973; Hirst, Holman & Spence, 1974). Both Type 1 and Type 2 cells sometimes responded to focal stimulation with an 'antidromic' action potential caused by stimulation of one of its cellular processes; that is, the action potential so produced invaded the soma of the cell. Cells which gave no action potential on direct intracellular depolarization, no response to focal stimulation, no anode break excitation at the time of impalement and which had high (more than 65 mV) resting membrane potentials were classed as Type 3 (glial) cells.

Drugs used were: dextrorphan tartrate (Roche), levorphanol tartrate (Roche), (±)-methadone (Lilly), morphine sulphate (Mallinckrodt) naloxone (Endo laboratories) and normorphine (base).$2H_2O$ (Dr E.L. May). Concentrations given in the text refer to the compounds listed above.

Results

The present results are based on applications of normorphine to 217 myenteric neurones from which satisfactory intracellular recordings were made. Only those neurones are included from which a stable resting membrane potential was recorded for a period of at least 15 minutes. In many cells, intracellular recordings were maintained for several hours. All the effects of drugs which are described were reversible on washing with a drug-free Krebs solution. The effects observed differed among the three cell types of the plexus.

Type 1 cells

Membrane potential and resistance. About 60% of all the Type 1 cells tested with normorphine (10 nM to 1 μM) responded with a membrane hyperpolarization. The noise level of the intracellular recordings was typically 200 μV to 1 mV and many cells showed small spontaneous fluctuations in potential of 2 to 3 mV; for these reasons, potential changes of less than 2 mV could not be attributed reliably to effects of the drugs. The hyperpolarizing response began within a few seconds of the normorphine solution reaching the tissue (Figure 1a). The response reached its maximum

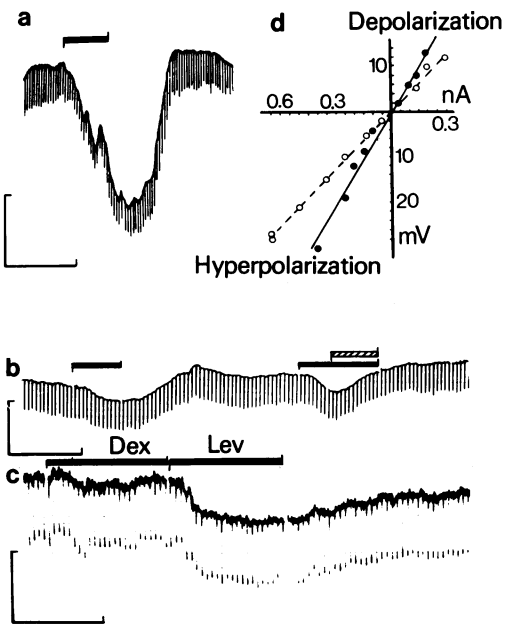

Figure 1 Hyperpolarization of four different myenteric neurones by narcotics. (a)–(c) Intracellular recordings of membrane potential displayed on a pen recorder. Constant current pulses (150 pA) were passed through the recording electrode at intervals of 10 seconds. Pulse duration (400 ms) was sufficient to charge fully the input capacitance of the pen recorder. During the periods indicated by the bars, the solution which perfused the tissue was changed to one which contained a drug. There was a delay of 30 s before a new solution reached the tissue. (a) Solid bar indicates application of normorphine (1 μM). The hyperpolarization observed in this neurone was the largest which has been observed. Calibrations: horizontal 5 min, vertical 20 mV. (b) Solid bar indicates normorphine (300 nM); hatched bar indicates naloxone (30 nM). The degree of hyperpolarization produced by normorphine was approximately the same in both applications, but it was reversed by naloxone. In this cell there was no detectable change in input resistance. Calibrations: horizontal 3 min, vertical 10 mV. (c) Solid bars indicate dextrorphan (Dex, 2 μM) and levorphanol (Lev, 200 nM). The dextrorphan had only a slight effect on membrane potential but a ten-fold lower concentration of levorphanol caused a long-lasting hyperpolarization of about 7 mV. No change in resistance was detected. Calibrations: horizontal 3 min, vertical 10 mV. (d) Voltage–current relation of a myenteric neurone before (solid line and ●) and after (broken line and ○) exposure to normorphine (1 μM). The ordinate scale indicates the steady state potential displacement caused by passing known currents (abscissae) across the cell membrane. The normorphine caused a fall in input resistance from 61 to 41 mΩ. This was associated with a hyperpolarization of 10 mV (not indicated in the figure).

amplitude within 1 to 2 min, which was the approximate time for a single change in the Krebs solution which covered the tissue (1–2 ml). With low concentrations of normorphine (less than 100 nM), the hyperpolarization persisted for as long as the perfusing solution contained the drug (up to 5 minutes). With higher concentrations of normorphine (300 nM to 1 μM), the hyperpolarization often declined progressively after a period of 2 to 3 minutes. In some cells the hyperpolarization completely disappeared despite the continued perfusion with normorphine (1 μM).

The amplitude of the membrane hyperpolarization was related to the concentration of normorphine applied. Four neurones responded to 10 nM normorphine (e.g. Figure 2a), the effects ranging from 2 to 8 mV; the responses to 30 and 100 nM normorphine ranged up to 21 mV and the largest hyperpolarizations (25 and 40 mV) were observed with 1 μM normorphine. Concentrations higher than 1 μM were not tested. Because of a large variability in the amplitude of the response shown by different cells to the same concentration of normorphine, it was difficult to correlate this amplitude with the resting membrane potential of the neurone. However, those neurones in which normorphine caused large (more than 20 mV) hyperpolarizations generally had low (less than 40 mV) resting potentials as determined by sudden withdrawal of the microelectrode. Less often, relatively large effects were observed in cells which appeared to be well polarized on micro-electrode withdrawal (e.g. Figure 2b). The difficulty in correlating the size of the response with the resting membrane potential is compounded by the fact that the high resistance micro-electrodes employed are likely to have high, and perhaps quite variable, tip potentials.

The membrane hyperpolarization was accompanied by a fall in neurone input resistance in most of the neurones in which satisfactory resistance measurements were obtained (see Methods section). The time course of the resistance change was similar to that of the potential change (Figure 1a). The resistance seldom fell to less than 75% of its control value, although in a few neurones more substantial falls in resistance were observed (e.g. Figure 4).

In some experiments a single neurone was tested repeatedly with normorphine. In these cases, the degree of hyperpolarization usually increased as the concentration of normorphine was increased (Figure 2a) during the first two or three exposures. However, the quantitative interpretation of these experiments was made difficult by the finding that the response eventually became smaller and smaller during the repeated application of the same or increasing concentrations of normorphine (Figure 2a). This progressive loss in responsiveness to normorphine varied considerably from cell to cell. It may represent real 'desensitization' or it may be a

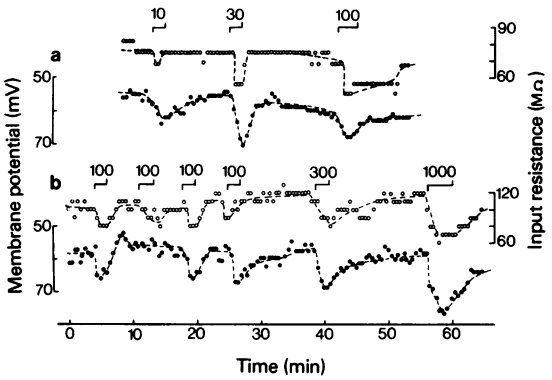

Figure 2 The effect of normorphine on membrane potential and input resistance of two Type 1 myenteric neurones. (a) and (b) represent two experiments on different myenteric neurones. In both (a) and (b) (●) indicates membrane potential and (○) indicates neurone input resistance. During the periods indicated by the horizontal bars, the perfusing solution covering the tissue contained normorphine in the concentration indicated (nM). The relatively low rate of flow of the perfusing solution make it unlikely that the steady state concentrations indicated would be achieved in less than 2 min (see Methods). (a) The hyperpolarization of the membrane at first increased when the concentration of normorphine was increased. Application of an even higher concentration of normorphine produced a smaller hyperpolarization even though a substantial change in resistance still occurred. Such an effect was often observed. (b) This cell was exposed four times to the same concentration of normorphine. The second exposure produced no discernible effects on membrane potential. The cause of such variability in effects is uncertain. Subsequently, the hyperpolarization was increased by increased concentrations of normorphine.

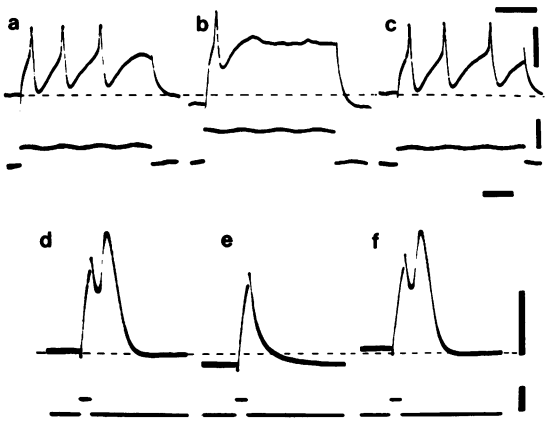

Figure 3 Effect of normorphine on excitation by direct intracellular passage of current. Upper traces, membrane potential. Lower traces, transmembrane current. (a) Control. The cell fired repetitively in response to a depolarizing current pulse. (b) Three minutes after changing to a solution containing normorphine (1 μM). The membrane became hyperpolarized by 12 mV; this, and the fall in resistance, necessitated the use of a higher current to bring the membrane to threshold. The cell fired only one spike in response to the same duration of depolarizing current. (c) After 5 min of washing with drug-free Krebs solution. The membrane potential and resistance have reverted to their control values and the cell fires repetitively. Spikes are retouched in (a)–(c). Calibrations (a)–(c): horizontal 10 ms, vertical (voltage) 40 mV (current) 500 pA. (d) Control. An action potential was elicited by passing a brief (1 ms) but strong depolarizing pulse through the recording micro-electrode. (e) After 2 min exposure to normorphine (100 nM). The hyperpolarization is sufficient to prevent the same current pulse from depolarizing the membrane to threshold. A slight increase in either current strength or duration was sufficient to restore the action potential (not illustrated). (f) After 3 min wash with drug-free Krebs solution. Calibrations: horizontal 2 ms, vertical (voltage) 40 mV (current) 2 nA. The experiments in (a)–(c) and (d)–(f) are from two different neurones.

reflection of a changing condition of the neurone during prolonged recordings. The loss in response was much less often observed when the intracellular electrodes were filled with potassium citrate or potassium acetate (North, unpublished observations), thus raising the possibility that the response declines due to a rise in intracellular chloride ion concentration.

Excitability. Neurones were excited by passing a depolarizing current through the recording micro-electrode so as to bring the membrane to threshold. The results of such an experiment are shown in Figure 3a–c. In the control circumstances, Type 1 cells responded to a depolarizing current by firing repetitively (Nishi & North, 1973). In the presence of normorphine, the same current was usually insufficient to bring the membrane to threshold because of the hyperpolarization and fall in resistance. In some experiments, the membrane still reached threshold but in the presence of normorphine the neurone fired only a single spike (Figure 3b). These effects were readily reversible.

Similar experiments were performed using a short duration (500 μs to 1 ms) current pulse with which to depolarize the soma membrane (Figure 3d–f). This pulse duration is similar to that used to excite the myenteric neurones by field stimulation in studies of the contractile response of the longitudinal muscle. The current strength was adjusted so that the cell gave an action potential in every trial, and then increased by 10%. This simulated the arrangement for supramaximal field stimulation. The hyperpolarization and

fall in resistance produced by normorphine were sufficient to prevent the same current from depolarizing the cell to threshold (Figure 3e). During the action of normorphine, the membrane could be depolarized to threshold by increasing either the amplitude or the duration of the current pulse. This finding suggests that an important action of normorphine in the experiments with field stimulation might be to hyperpolarize the neuronal membrane sufficiently to prevent its excitation by a stimulus intensity which was formerly supramaximal.

Neurones were also excited by close focal stimulation of a cellular process—the action potential then propagated to the soma. The results of two such experiments are shown in Figure 4. In these experiments, the strength of the focal stimulation was adjusted so that it was well above the threshold level. Each stimulus (500 μs duration) was followed by an 'antidromic' action potential in the soma. Normorphine hyperpolarized the soma membrane sufficiently to prevent invasion of the soma by the propagating action potential. This blockade is not likely to be due entirely to hyperpolarization at the point of stimulation for two reasons. First, increasing the strength of stimulation was ineffective in restoring the soma spike. Second, in some cells (e.g. Figure 4e–h) the membrane hyperpolarization produced by normorphine was sufficient to fractionate the soma spike but not to block it completely. This fractionation of the 'antidromic' spike is readily observed in myenteric neurones; the small all-or-nothing component is the potential change in the soma produced by current flow from the activated region of the proximal part of the process or the tapering somatic cone (see Nishi & North, 1973).

Excitatory synaptic potentials. In confirmation of earlier reports (North & Nishi, 1974; North & Henderson, 1975), we found no consistent effects of normorphine on the amplitude or time course of the e.p.s.p. Those cells which were hyperpolarized by normorphine sometimes showed an appropriate increase in e.p.s.p. amplitude. Other neurones which were hyperpolarized showed no change in e.p.s.p. amplitude, perhaps because of the concomitant reduction in input resistance. Miniature e.p.s.ps could not be reliably identified; therefore, the action of morphine was not examined.

Specificity of narcotic effects. The hyperpolarization by normorphine of the myenteric neurones, and the conductance change when it was observed, could be reversed by changing the perfusing solution to one which contained both normorphine and naloxone. In several experiments a concentration of naloxone ten times lower than that of normorphine was sufficient to reverse the effects of the normorphine (Figures 1b and 4d). The naloxone reversal of the membrane hyperpolarization usually occurred with a more rapid time

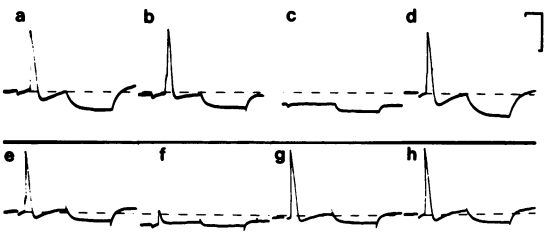

Figure 4 Effect of normorphine on the responses of myenteric neurones to 'antidromic' excitation (for meaning, see Methods). In each of the eight panels, a single focal stimulus to the surface of the ganglion was followed by passing a hyperpolarizing current pulse through the recording micro-electrode. (a)–(d) and (e)–(h) are from two different neurones. In each experiment, (a)–(d) and (e)–(h), the focal stimulus and hyperpolarizing current were constant throughout. (a) The stimulus is followed by an 'antidromic' action potential. (b) Two minutes after changing to a solution containing normorphine (1 μM). The hyperpolarization has accentuated the inflexion on the rising phase of the action potential and the membrane resistance is reduced. (c) Three and a half minutes after changing to the normorphine solution. The 'antidromic' action potential is completely blocked and the membrane resistance is much reduced. (d) Six minutes after changing to a solution which contained both normorphine (1 μM) and naloxone (100 nM). (e) Control. (f) Two and a half minutes after changing to a solution containing normorphine (100 nM). The 'antidromic' action potential was fractionated but not completely blocked by the membrane hyperpolarization (and fall in resistance). (g) Four minutes after changing to a solution containing normorphine (100 nM) and naloxone (100 nM). (h) Six minutes after washing with drug-free Krebs solution. Spikes are retouched. Calibrations apply to all traces: horizontal 10 ms, vertical 50 mV. The hyperpolarizing current pulse was 500 pA throughout.

course than the reversal by washing with drug-free Krebs solution. Naloxone alone (up to 1 μM) had no effect on the resting membrane potential or resistance.

A membrane hyperpolarization qualitatively similar to that produced by normorphine was also observed with methadone (1 μM), morphine (1 μM) and levorphanol (200 nM). Those cells which were hyperpolarized by levorphanol (200 nM) were not affected by dextrorphan (2 μM) (Figure 1c).

Type 2 cells

Only about 10% of Type 2 cells were affected by normorphine. Responses were seldom observed with concentrations lower than 1 μM. The cells which were affected showed an immediate hyperpolarization of 2–10 mV. This hyperpolarization almost always

declined after 1–2 min despite the continued presence of the normorphine. Second applications of normorphine to sensitive Type 2 cells usually produced no effect. The neurones which were hyperpolarized sometimes showed a slight fall in input resistance; those which showed no change in membrane potential also showed no change in input resistance. The lack of effect in these cells is, therefore, not likely to be due solely to the fact that the Type 2 cells generally have higher resting membrane potentials than the Type 1 cells (Nishi & North, 1973).

Type 3 cells

Normorphine (300 nM to 1 μM) was applied to eight Type 3 cells and in no case was any change in potential or resistance observed.

Discussion

The present results indicate that the majority (60%) of Type 1 myenteric neurones are hyperpolarized by narcotic analgesics. Only 10% of Type 2 and no Type 3 cells were affected. The concentrations of narcotics used were comparable to those required to inhibit the output of acetylcholine from the myenteric plexus (Paton, 1957; Cox & Weinstock, 1966; Fennessy, Heimans & Rand, 1969; Waterfield & Kosterlitz, 1974) or to inhibit the nerve-mediated contractile response of the longitudinal muscle (Paton & Zar, 1968; Cowie, 1968; Kosterlitz, Lord & Watt, 1973). The hyperpolarization is considered to have been mediated by opiate receptors in view of the findings that (a) the effective concentrations of agonists are low, (b) the effect is reversed by even lower concentrations of antagonists, and (c) only the (−)-isomer of optical enantiomers is effective.

The site of action of morphine

Previous experiments have indicated that the primary site of action of morphine is the cholinergic neurone which innervates the muscle layers. The inhibition by morphine of the acetylcholine output is not prevented by hexamethonium (Greenberg *et al.*, 1970; Waterfield & Kosterlitz, 1974). The present finding is that the majority of Type 1 cells respond to normorphine. This is compatible with an interpretation that the morphine-sensitive Type 1 cells are the cholinergic efferents, whilst the morphine-insensitive Type 1 cells might be the non-adrenergic inhibitory neurones. This is lent support by the findings that morphine does not affect the amplitude of the inhibitory junction potential recorded from the muscle layers of the guinea-pig ileum (North & Henderson, unpublished observations). There is considerable indirect evidence that the Type 2 cells in the myenteric plexus are afferent cells (Nishi & North, 1973; Hirst *et al.*, 1974; Hirst & McKirdy, 1974; North & Nishi, 1974). If this were the case, it is perhaps surprising that a small proportion of them are sensitive to morphine, even though the concentration required to elicit a hyperpolarization was greater than that required for Type 1 cells. In this respect, it is interesting that Gyang, Kosterlitz & Lees (1964) showed that morphine depresses not only the peristaltic reflex proper of the isolated ileum, but also has a slight inhibitory effect on the non-synaptic graded reflex response of the longitudinal muscle. It is possible that a proportion of the Type 2 cells release acetylcholine which acts directly upon the longitudinal muscle to mediate the graded reflex.

Our supposition that the morphine-sensitive cells are the cholinergic efferents to the muscle is at variance with the interpretation by Diab, Dinerstein, Watanabe & Roth (1976) of their autoradiographic experiments. They found the [^{3}H]-morphine bound to a population of small cells in the myenteric plexus. Diab *et al.* (1976) referred to these cells as satellite cells but presented no ultrastructural evidence for such an identification. There are two reasons why the cells which they found to bind [^{3}H]-morphine might be neurones rather than glial cells. First, in the present experiments normorphine had no effect on the membrane potential of cells identified electrophysiologically as glial cells (Type 3 cells, Nishi & North, 1974). Second, Diab *et al.* (1976) demonstrated that the cells which bound [^{3}H]-morphine were also surrounded by a dense network of nerve fibres which showed fluorescence typical of noradrenaline. The electron-microscopic study by Gabella (1972) showed clearly that neurones and not glial cells received synapses from fibres containing the small granular vesicles characteristic of adrenergic fibres.

In many neurones the hyperpolarization by normorphine was accompanied by a fall in input resistance. This is not likely to be due to anomalous rectification because the voltage–current relationship for the Type 1 cells is usually linear for hyperpolarizations up to 20 to 30 mV (Figure 1d; Nishi & North, 1973; Hirst *et al.*, 1974). The fall in resistance therefore indicates that normorphine increases membrane conductance to one or more ion. Those cells which showed relatively large conductance increases might be affected by morphine predominantly on their somata, whilst those neurones which showed smaller changes or no change in input resistance might be affected at a distance from the soma on their cellular processes.

Interpretation of studies of acetylcholine output

The aim of the present experiments was to correlate the findings with intracellular electrodes with the observations that morphine inhibits the output of acetylcholine from the nerves of the myenteric plexus

and thereby depresses the contractile response of the muscle. The membrane hyperpolarization in the presence of morphine might prevent some neurones from being excited, and this will be especially likely to occur if the stimulus were depolarizing the individual neurones only just to their threshold level. With submaximal electrical field stimulation of the whole plexus, a certain proportion of all the neurones excited must be just reaching threshold for spike initiation. The small hyperpolarization produced by a low concentration of morphine will be sufficient to prevent the excitation of those neurones for which the stimulus only just reached threshold depolarization. That is, the preparation would be highly sensitive to the depressant effect of morphine. With supramaximal electrical field stimulation of the whole plexus, a larger hyperpolarization would be required before *any* neurones will fail to reach their firing threshold. That is to say, a higher concentration of morphine would be required; the preparation would be less sensitive.

Those workers who have compared the effects of submaximal and supramaximal stimulation on the same preparation have found that a lower concentration of morphine is effective when submaximal stimulation is used. Cox & Weinstock (1966) reported that the ED_{50} for morphine was approximately 40 nM with submaximal stimulation and 80 nM with maximal stimulation. Cowie (1968) found ED_{50}s of 60 and 190 nM for submaximal and supramaximal stimulation respectively. These findings for the inhibition of the twitch of the whole ileum have been replicated on the myenteric plexus-longitudinal muscle preparation; Cowie (1968) found ED_{50}s of 100 nM and 500 nM depending on whether she used submaximal or supramaximal stimulation.

In the present experiments, we found that the membrane hyperpolarization (and in some cases the fall in resistance) produced by normorphine was sufficient to prevent a depolarizing current pulse from exciting the neurone. This affect could be overcome by increasing the amplitude of the current pulse or, when the pulse duration was small compared to the cell membrane time constant, by increasing the duration of the current pulse. Therefore we might expect that the effect of morphine in depressing the acetylcholine output or in inhibiting the contractile response of the muscle could also be overcome simply by increasing the stimulus strength. Such a reversal of the morphine inhibition by increasing the stimulus strength has previously been demonstrated using submaximal stimulation (Ehrenpreis, Light & Schonbuch, 1972). When submaximal stimulation is used, it is possible that the increase in stimulus strength is simply recruiting a previously unexcited population of neurones which are not affected by morphine. However, when supramaximal stimulation is used all the neurones which innervate the longitudinal muscle are being excited, and any reversal of a morphine inhibition by increasing the strength of stimulation must reflect a renewed excitation of neurones prevented from firing by morphine. If morphine acts directly on the process by which acetylcholine is released by the action potential, it is difficult to see how any part of its action could be overcome merely by increasing the stimulus strength.

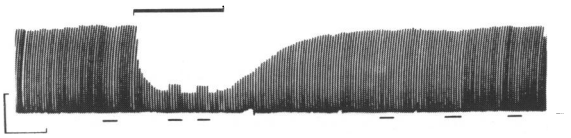

Figure 5 The contractile response of the myenteric plexus-longitudinal muscle preparation of the guinea-pig. The isolated preparation was suspended vertically in a 1.4 ml tissue bath at 37°C. The contractions in response to electric field stimulation were recorded isometrically. Stimulation pulses (70 V, 35 mA; 500 μs duration) were monophasic and delivered at a frequency of 0.1 Hz. During the periods indicated by the lines beneath the trace, the voltage was increased to 100 V (50 mA). During the period indicated by the horizontal bar above the trace, normorphine (300 nM) was present in the tissue bath. Before the application of normorphine, and on three occasions during the wash-out, increasing the stimulus strength to 100 V caused no further increase in amplitude of the contractile response. However, when the twitch was depressed by normorphine, such an increase produced a clear augmentation of the response. The control response is clearly not the maximum response of which the tissue is capable because increasing the frequency of stimulation greatly increased the response (not shown). Calibrations: horizontal 3 min, vertical 2 grams.

In unpublished experiments (North & Karras), we have confirmed that the depression of the contractile response by normorphine can be overcome by increasing the stimulus current, even though the stimulus current was already supramaximal in the absence of normorphine (see also Figure 5). In practice, a large reversal of the normorphine inhibition is impossible to demonstrate because any large increase in stimulus strength causes a reduction in the contractile response in either the control or the morphine-treated preparation; this may represent a concomitant excitation of non-adrenergic inhibitory nerves; or even sympathetic nerve terminals, as the stimulus strength is increased.

The present findings therefore suggest that morphine inhibits the release of acetylcholine from the field-stimulated myenteric plexus not by a primary action on the mechanism of release but by preventing a proportion of myenteric neurones from reaching threshold for excitation. On the other hand, that part of the *spontaneous* release of acetylcholine which is due to ongoing neuronal activity (that is, which is sensitive to tetrodotoxin) is also inhibited by morphine (Paton,

1957; Paton & Zar, 1968). In this case, a hyperpolarization of the somata might inhibit the ongoing activity in the neurones. However, an additional action at the site of release cannot be excluded by the present experiments. A hyperpolarization occurring on the cellular processes might prevent nerve terminal invasion (Van Essen, 1973) or the conductance change itself might account for presynaptic inhibition (Slawsky & Getting, 1976). Such mechanisms of action might be more relevant to the actions of morphine in the central nervous system, where the artificial circumstances of field stimulation do not pertain.

This work was supported by USPHS NIH Grant NSO6772. We thank Dr May and Endo Laboratories for gifts of drugs.

References

COWIE, A.L. (1968). An investigation of the factors which modify activity in the guinea-pig isolated ileum with particular reference to the release of acetylcholine. *Ph.D. Thesis:* University of Aberdeen.

COX, B. & WEINSTOCK, M. (1966). The effect of analgesic drugs on the release of acetylcholine from electrically stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **27**, 81–92.

CREESE, I. & SNYDER, S.H. (1975). Receptor binding and pharmacological activity of opiates in the guinea-pig intestine. *J. Pharmac. exp. Ther.*, **194**, 205–219.

DIAB, I.M., DINERSTEIN, R.J., WATANABE, M. & ROTH, L.J. (1976). ^{3}H-Morphine localization in myenteric plexus. *Science, N.Y.*, **193**, 689–691.

DINGLEDINE, R. & GOLDSTEIN, A. (1975). Single neuron studies of opiate action in the guinea-pig myenteric plexus. *Life Sci.*, **17**, 57–62.

DINGLEDINE, R. & GOLDSTEIN, A. (1976). Effect of synaptic transmission blockade on morphine action in the guinea-pig myenteric plexus. *J. Pharmac. exp. Ther.*, **196**, 97–106.

DINGLEDINE, R., GOLDSTEIN, A. & KENDIG, J. (1974). Effects of narcotic opiates and serotonin on the electrical behaviour of neurons in the guinea-pig myenteric plexus. *Life Sci.*, **14**, 2299–2309.

EHRENPREIS, S., LIGHT, I. & SCHONBUCH, G.H. (1972). Use of electrically stimulated guinea-pig ileum to study potent analgesics. In *Drug Addiction: Experimental Pharmacology*. ed. Singh, J.M., Miller, L.H. & Lal, H. pp. 319–342. New York: Futura.

FENNESSY, M.R., HEIMANS, R.L.H. & RAND, M.J. (1969). Comparison of effect of morphine-like analgesics on transmurally stimulated guinea-pig ileum. *Br. J. Pharmac.*, **37**, 436–449.

GABELLA, G. (1972). Fine structure of the myenteric plexus in the guinea-pig. *J. Anat.*, **111**, 69–97.

GREENBERG, R., KOSTERLITZ, H.W. & WATERFIELD, A.A. (1970). The effects of hexamethonium, morphine and adrenaline on the output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the ileum. *Br. J. Pharmac.*, **40**, 553P.

GYANG, E.A. & KOSTERLITZ, H.W. (1966). Agonist and antagonist actions of morphine-like drugs on the guinea-pig isolated ileum. *Br. J. Pharmac. Chemother.*, **27**, 514–527.

GYANG, E.A., KOSTERLITZ, H.W. & LEES, G.M. (1964). The inhibition of autonomic neuro-effector transmission by morphine-like drugs and its use as a screening test for narcotic analgesic drugs. *Naumyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **248**, 231–246.

HIRST, G.D.S., HOLMAN, M.E. & SPENCE, I. (1974). Two types of neurone in the myenteric plexus of the guinea-pig duodenum. *J. Physiol., Lond.*, **236**, 303–326.

HIRST, G.D.S. & McKIRDY, H.C. (1974). A nervous mechanism for descending inhibition in the guinea-pig small intestine. *J. Physiol., Lond.*, **238**, 129–143.

KOSTERLITZ, H.W., LORD, J.A.H. & WATT, A.J. (1973). Morphine receptor in the myenteric plexus of the guinea-pig ileum. In *Agonist and Antagonist Actions of Narcotic Analgesic Drugs*. ed. Kosterlitz, H.W., Collier, H.O.J. & Villareal, J.E. pp. 45–61. Baltimore: University Park.

KOSTERLITZ, H.W. & WATERFIELD, A.A. (1975). *In vitro* models in the structure-activity relationships of narcotic analgesics. *A. Rev. Pharmac.*, **15**, 29–47.

KOSTERLITZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonist and antagonists, with particular reference to N-allylnoroxy-morphine (naloxone). *Br. J. Pharmac. Chemother.*, **3**, 266–276.

NISHI, S. & NORTH, R.A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. *J. Physiol., Lond.*, **231**, 471–491.

NORTH, R.A. (1976). Effects of morphine on myenteric neurones. *Neuropharmac.*, **15**, 719–721.

NORTH, R.A. & HENDERSON, G. (1975). Action of morphine on guinea-pig myenteric plexus and mouse vas deferens studied by intracellular recording. *Life Sci.*, **17**, 63–66.

NORTH, R.A. & NISHI, S. (1974). Properties of the ganglion cells in the myenteric plexus of the guinea-pig ileum determined by intracellular recording. *Proc. IV Intern. Symp. Gastrointestinal Motility*. pp. 667–676. Vancouver: Mitchell.

NORTH, R.A. & TONINI, M. (1976). Hyperpolarization by morphine of myenteric neurones. In *Opiates and Endogenous Opioid Peptides*. ed. Kosterlitz, H.W. pp. 205–212. Amsterdam: North Holland.

PATON, W.D.M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **12**, 119–127.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of the acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J. Physiol., Lond.*, **194**, 13–34.

RANG, H.P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmac. Chemother.*, **22**, 356–365.

SATO, T. TAKAYANAGI, I. & TAKAGI, K. (1973). Pharmacological properties of electrical activities obtained from neurons in Auerbach's plexus. *Japan. J. Pharmac.*, **23**, 665–671.

SCHAUMANN, W. (1956). Influence of atropine and

morphine on the liberation of acetylcholine from the guinea-pig's intestine. *Nature, Lond.*, **178**, 1121–1122.

SCHAUMANN, W. (1957). Inhibition by morphine of the release of acetylcholine from the intestine of the guinea-pig. *Br. J. Pharmac. Chemother.*, **12**, 115–118.

SLAWSKY, M.T. & GETTING, P.A. (1976). A mechanism for presynaptic inhibition in Triton diomedia. *Neurosci. Abtrs.*, **2**, 356.

VAN ESSEN, D.C. (1973). The contribution of membrane hyperpolarization to adaptation and conduction block in sensory neurones of the leech. *J. Physiol., Lond.*, **230**, 509–534.

WATERFIELD, A.A. & KOSTERLITZ, H.W. (1974). Release of acetylcholine from the myenteric plexus of the guinea-pig ileum. *Proc. IV Intern. Symp. Gastrointestinal Motility*. pp. 659–666. Vancouver: Mitchell.

(Received April 14, 1977.
Revised June 3, 1977.)