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INHIBITION OF NEURONAL FIRING BY OPIATES: EVIDENCE AGAINST THE INVOLVEMENT OF CYCLIC NUCLEOTIDES

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1 Extracellular recordings were made *in vitro* from single neurones of the myenteric plexus of the guinea-pig ileum.

2 Neuronal firing was inhibited by morphine and normorphine (10 nM to 1 μM). Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (100 μM to 1 mM) also inhibited the firing of the majority of the neurones. Prostaglandin E_2 usually caused a short-lasting excitation of myenteric neurones and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was usually without effect on firing rate.

3 The inhibition of neuronal firing by normorphine was unaffected by prior and/or concurrent administration of cyclic AMP, dibutyryl cyclic adenosine 3′,5′-monophosphate, prostaglandin E_2 or 3-isobutyl-1-methylxanthine. As these four treatments might be expected to elevate intracellular levels of cyclic AMP, the results lend no support to the notion that a reduction in intracellular cyclic AMP is essential to the inhibition of firing produced by morphine.

Introduction

It has been proposed that some of the acute effects of opiates such as analgesia may be mediated by a direct effect on intracellular cyclic nucleotide metabolism. Originally this hypothesis was based on rather indirect evidence such as the antagonism of morphine antinociception by administration of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) or dibutyryl cyclic AMP (db cyclic AMP); similar effects were observed with theophylline and prostaglandin, which could owe their effects to an elevation of cyclic AMP levels (Ho, Loh & Way, 1973; Ferri, Santagostino, Braga & Galatulas, 1974). Stronger and more direct evidence for an effect of opiates on adenyl cyclase has come from work on brain homogenates and cultured neuroblastoma × glioma hybrid cells. In the first case, it was shown that morphine and its surrogates inhibit the stimulation by E prostaglandins of cyclic AMP formation in rat brain homogenates (Collier & Roy, 1974; Collier, Francis & Roy, 1976; Wilkening, Mishra & Makman, 1976). In the second case, opiates inhibit the adenylate cyclase of the cultured cells, whether this is basal activity or the enhancement of this activity which is produced by prostaglandin E (PGE) (Sharma, Klee & Nirenberg, 1975; Traber, Fischer, Latzin & Hamprecht, 1975). These actions of opiates are stereospecific and are reversed by relevant concentrations of naloxone. However, there is no substantial evidence that this particular action of opiates is an essential intermediate step in the production of analgesia or any other of their manifold acute effects. There is considerable evidence, on the other hand, which implicates the adenylate cyclase system in a causal manner in the changes which occur following long term exposure to narcotic analgesia (Collier, Francis, McDonald-Gibson, Roy & Saaed, 1975; Francis, Roy & Collier, 1975; Collier *et al.*, 1976; Sharma, Klee & Nirenberg, 1977).

Narcotic analgesics inhibit neuronal firing at several sites in the central nervous system (see reviews by Bradley, Gayton & Lambert, 1978; Zieglgänsberger & Fry, 1978). Narcotics also inhibit neuronal firing in the myenteric plexus of the guinea-pig ileum (Sato, Takayanagi & Takagi, 1973; 1974; Dingledine, Goldstein & Kendig, 1974; Dingledine & Goldstein, 1975; 1976; Ehrenpreis, Sato, Takayanagi, Comaty & Takagi, 1976; North & Williams, 1977). This action is mediated by the opiate receptors known to exist in this tissue (Kosterlitz & Watt, 1968; Creese & Snyder, 1975) and it has been shown to be a direct action of opiates on myenteric neurones which is independent of any trans-synaptic influences (Dingledine & Goldstein, 1976; North & Williams, 1977). The opiate receptors in the myenteric plexus appear to be pharmacologically identical to the μ-receptors of the central nervous system (Creese & Snyder, 1975; Kosterlitz & Waterfield, 1975; Lord, Waterfield, Hughes & Kosterlitz, 1977).

The myenteric plexus allows electrophysiological measurement of an immediate effect of occupation of the opiate receptor on a morphine-sensitive neurone,

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and is therefore a good site at which to investigate the involvement of cyclic nucleotides in the mediation of this effect. Being an *in vitro* technique, it has the further advantage that opiates and other substances can be added in precisely known concentrations. It has not been shown that opiates do inhibit adenylate cyclase in the neurones of the myenteric plexus; the present experiments indicate that if they do, then this is probably not an intermediate step in the inhibition of neuronal firing which they produce. Morphine was found to be equally effective in inhibiting neuronal firing in three conditions under which cyclic AMP levels would be expected to be markedly elevated: first, administration of cyclic AMP or db cyclic AMP; second, exposure to PGE_2; and third, phosphodiesterase inhibition with 3-isobutyl-1-methylxanthine (IBMX).

Methods

Extracellular recordings were made from single myenteric neurones with glass suction electrodes. The full details of the recording technique have been described (North & Williams, 1977). Neuronal activity is readily distinguished from smooth muscle activity by its shorter spike duration and its sensitivity to tetrodotoxin. The action potentials from single neurones were of constant configuration throughout the experiments; the potential changes were amplified (time constant 1 s), gated with a window discriminator and counted. Spike frequencies were plotted in histogram form with a bin width of 10 s.

The preparation was perfused with a Krebs solution of the following composition (mmol/l): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11.5; gassed with 95% O_2 and 5% CO_2. This solution was preheated and pumped over the tissue at a constant rate (1 to 2 ml/min) so that the temperature at the recording site was 37°C. Drugs were applied to the tissue by changing the perfusing solution to one which differed only in its content of the drug. Because of delays in the pump and heater, there was a 'dead time' of 40 to 80 s between turning a tap and the time of arrival of a new solution at the tissue. Figures in this paper indicate the time at which the tap was turned. The bath volume was 1 to 2 ml.

Drugs used were normorphine hydrochloride (Dr E.L. May), morphine sulphate (Mallinkcrodt), naloxone hydrochloride (Endo), theophylline (1,3-dimethylxanthine) (Sigma), 3-isobutyl-1-methylxanthine (Aldrich), adenosine 3',5'-cyclic monophosphoric acid (Sigma), prostaglandin E_2 (Dr S. Ehrenpreis), N^6, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (sodium salt) (Sigma). Concentrations in this paper are those of the compounds listed above. None of these substances significantly changed the pH of the Krebs solution in the concentrations used.

Results

The present results are based on recordings from 130 neurones in ganglia removed from 39 guinea-pigs.

Morphine

Morphine and normorphine (10 nM to 1 μM) inhibited neuronal firing in 86% of neurones tested ($n = 106$). No significant differences were noted between the effects of morphine and normorphine and those reported by Dingledine & Goldstein (1976) and North & Williams (1977).

Cyclic nucleotides

Cyclic AMP caused an inhibition of neuronal firing in 80% of neurones tested whilst 20% were not affected. The inhibition of firing was rapid in onset and decline, lasted throughout the period of application (2 to 10 min) and was related to the concentration of cyclic AMP (5 μM to 1 mM). Prior exposure of the tissue to cyclic AMP (5 μM to 1 mM) for at least 6 min did not affect the inhibition of firing caused by morphine; nor did cyclic AMP reverse the inhibition of firing by morphine.

The effects of db cyclic AMP were more variable than those of cyclic AMP. Eighteen % of neurones were excited, 25% of neurones were inhibited (Figure 1)

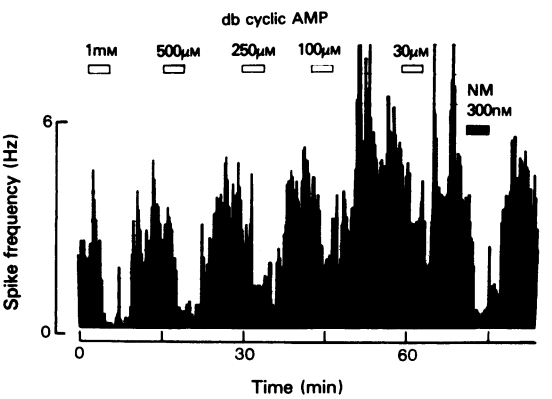

Figure 1 Inhibition of neuronal firing by dibutyryl cyclic adenosine 3',5'-monophosphate (db cyclic AMP) and normorphine (NM). During the periods indicated by the open and filled bars, the solution which perfused the tissue contained db cyclic AMP and normorphine (300 nM) respectively. The firing of this neurone was inhibited in a dose-dependent manner by db cyclic AMP. It was also sensitive to morphine.

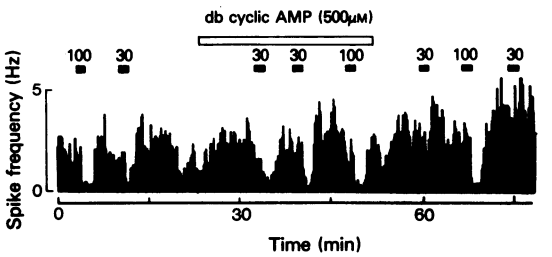

Figure 2 Lack of effect of dibutyryl cyclic adenosine 3',5'-monophosphate (db cyclic AMP) on the inhibition of firing caused by normorphine in a single myenteric neurone. During the periods indicated by the open and filled bars, the solution which perfused the tissue contained db cyclic AMP (500 μM) and normorphine (concentration indicated in nM) respectively. The neuronal firing rate was depressed by normorphine before, during and after the exposure to db cyclic AMP. The sensitivity to normorphine was not reduced during concurrent application of db cyclic AMP.

and 57% were unaffected ($n = 28$). No particular tendency was noted for higher concentrations to cause excitation and lower concentrations to cause inhibition, or *vice-versa*. The onset and decline of the inhibition caused by db cyclic AMP were similar to those caused by cyclic AMP. The inhibitory effect was clearly dose-related (Figure 1), and when inhibition occurred the effect could be induced repeatedly by repeated applications of db cyclic AMP at intervals of 6 min.

Prior exposure to db cyclic AMP did not alter the sensitivity of the neurones to inhibition by morphine. Normorphine and morphine still caused a powerful inhibition of neuronal firing in the presence of db cyclic AMP (500 μM) (Figure 2). Indeed, many neurones appeared to be slightly more sensitive to the inhibitory effect of morphine in the presence of db cyclic AMP (e.g. Figure 2).

Prostaglandin E_2

PGE_2 (10 to 30 nM) increased the firing rate of 80% of the myenteric neurones tested ($n = 26$); it was difficult to perform experiments with concentrations greater than 30 nM because muscle contractions occurred which sometimes dislodged the recording electrode. The increase in firing caused by PGE_2 did not last throughout the period of exposure (5 to 15 min) (Figure 3a). The ability of PGE_2 to cause an excitation was not dependent upon the release of acetylcholine because marked excitation occurred even in the presence of hexamethonium (300 μM) and hyoscine (1 μM).

Exposure of the tissue to PGE_2 (10 to 30 nM) did not prevent the inhibition of firing caused by morphine (300 nM to 1 μM) (Figure 3a). PGE_2 (10 to 30 nM) did not reverse the inhibition of firing caused by normorphine (300 nM) (Figure 3b).

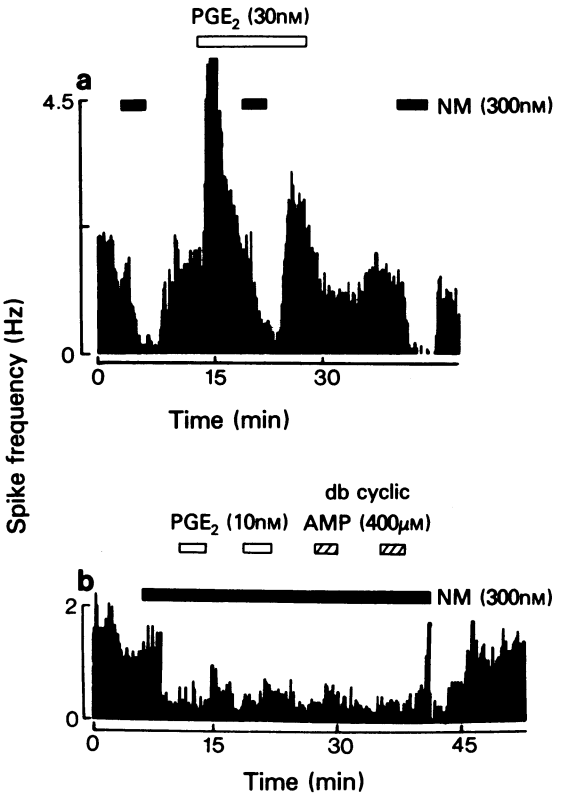

Figure 3 Interactions between prostaglandin E_2 and normorphine on single myenteric neurones. During the periods indicated by the open, cross-hatched and filled bars, the solution which perfused the tissue contained prostaglandin E_2 (PGE_2), dibutyryl cyclic adenosine 3',5'-monophosphate (db cyclic AMP) and normorphine (NM) respectively. (a) Neuronal firing was inhibited by normorphine. PGE_2 caused an excitation of the neurone but this did not persist throughout the exposure. Normorphine was equally effective in inhibiting neuronal firing in the presence of PGE_2. (b) Normorphine inhibited the firing of this neurone, and the inhibition remained throughout the 30 min period of application. PGE_2 did not excite the cell during the inhibition by normorphine; db cyclic AMP was also without effect.

Isobutylmethylxanthine

IBMX (6 to 50 μM) did not affect the firing rate of the majority of myenteric neurones; 6 of the 28 neurones tested were excited. In these neurones, the excitation was slight (1.2 to 2 × the control firing rate),

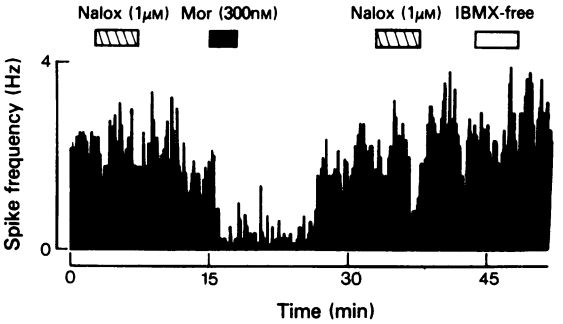

Figure 4 Lack of effect of isobutylmethylxanthine (IBMX) on the inhibition of neuronal firing by morphine. Prior to this experiment, the isolated ileum had been incubated in a Krebs solution containing IBMX (50 μM) for 24 h; the IBMX was present in the perfusing solution throughout the period of recording with the exception of the period indicated by the open bar. During the periods indicated by the cross-hatched and filled bars, the solution which perfused the tissue contained not only IBMX but also either naloxone (1 μM) or morphine (300 nM) respectively. Naloxone was quite without effect and morphine was as effective as usual in causing an inhibition of firing. Wash out of the IBMX was also without effect on the firing rate. Note the long duration of action of morphine compared to normorphine (Figures 1 to 3).

was rapid in onset and passed off during the period of exposure.

The inhibitory effect of morphine on the firing of neurones pretreated with IBMX was not distinguishable from its effect on the same neurones in the absence of IBMX ($n = 12$). Morphine inhibited the neuronal firing rate even after continuous exposure of the myenteric neurones to IBMX (50 μM) for 24 h (Figure 4). The IC_{50} for inhibition of cyclic AMP phosphodiesterase in rat brain homogenates (Collier, Butt, Francis, Roy & Schneider, 1978) and isolated fat cells (Beavo, Rogers, Croggord, Hardman, Sutherland & Newman, 1970) is approximately 5 μM. It therefore seems unlikely that an insufficiently high concentration of IBMX was employed.

Discussion

The principal aim of the present experiments was to test the hypothesis that inhibition of adenylate cyclase is an intermediate step between the occupation of the opiate receptor by an agonist and the inhibition of neuronal firing which this produces.

Narcotic analgesics have two actions in the myenteric plexus of the guinea-pig ileum, namely the inhibition of neuronal firing which is the subject of the present study, and the inhibition of acetylcholine release following electrical field stimulation of the plexus.

Inhibition of neuronal firing

The cyclic nucleotides did not reverse or prevent the inhibitory action of morphine on neuronal firing. Indeed, a substantial proportion of neurones were inhibited by cyclic AMP or db cyclic AMP alone, an effect similar to that reported at other sites (Siggins, Hoffer & Bloom, 1969; Segal & Bloom, 1974; Kostopoulous, Limacher & Phillis, 1975; Stone, Taylor & Bloom, 1975). Bloom (1975) has already commented that the fact that both narcotic analgesics and cyclic nucleotides almost invariably inhibit the firing of central neurones poses difficulties for theories which propose that a reduction in intracellular cyclic AMP levels leads to an inhibition of firing. The present experiments in which cyclic nucleotides were applied directly to myenteric neurones in known concentrations suggest that inhibition of adenylate cyclase is not likely to be an intermediate step between occupation of the opiate receptor and the inhibition of neuronal firing.

The results of the experiments with PGE_2 and IBMX are compatible with this interpretation: in neither case was there any effect on the inhibitory action of morphine. PGE_2 (10 to 30 nM) alone often had a transient effect on neuronal firing; higher concentrations caused a marked excitation of neurones and a direct excitation of the longitudinal muscle layer. On the other hand, the concentration of PGE_2 needed to cause a 2.7 fold increase in cyclic AMP formation in rat brain homogenates was about 90 μM (Collier & Roy, 1974), and the concentration required to stimulate adenylate cyclase in cultured neuroblastoma cells was about 10 μM (Traber *et al.*, 1975). The possibility remains that a 1000 fold increase in the concentration of PGE_2 might have revealed an antagonism of the action of morphine on neuronal firing. Similar criticisms probably do not apply to the experiments with IBMX. In this case a concentration was used which is ten times that required to inhibit by 50% the activity of cyclic AMP phosphodiesterase in rat brain homogenates (Collier *et al.*, 1978). Therefore the finding that morphine is equally effective in inhibiting neuronal firing in the presence of IBMX constitutes evidence against the involvement of cyclic AMP in this action of morphine.

The administration of naloxone to rats which have previously been given large and repeated doses of morphine produces a typical syndrome of withdrawal signs. A closely similar syndrome (quasimorphine withdrawal syndrome, QMWS) can be precipitated by equal doses of naloxone in rats which have never received morphine, but which have been pretreated with one of several compounds which are known to

inhibit cyclic AMP phosphodiesterase. Some correlation exists between the propensity of these compounds to induce the QMWS and to inhibit phosphodiesterase *in vitro* (Collier *et al.*, 1978). The present experiments with naloxone were done in order to see whether a similar phenomenon could be induced in single neurones. Even long term (24 h) exposure of myenteric neurones to IBMX was without significant effect on their firing rate, nor did naloxone affect neurones pretreated with IBMX. On the other hand, it is known that clear manifestations of tolerance and dependence can be induced in myenteric neurones either *in vivo* (North & Zieglgänsberger, 1978) or by 24 h incubation *in vitro* (North & Karras, 1978).

Inhibition of acetylcholine release

Adenosine and related nucleotides depress the nerve-mediated contractile response of the longitudinal muscle of guinea-pig ileum by inhibiting the release of acetylcholine (Takagi & Takayanagi, 1972; Ginztler & Musacchio, 1975; Sawynok & Jhamandas, 1976; Hayashi, Mori, Yamada & Kunimoto, 1978). However, the mechanism of their action appears to be distinct from that of morphine (Hayashi *et al.*, 1978). Prostaglandins were first reported to antagonize the inhibitory effects of morphine on the contractile response (Ehrenpreis, Greenberg & Belman, 1973) but it now seems that this action is relatively non-specific as prostaglandins also reverse the depression of the twitch caused by tetrodotoxin that is to say, they 'sensitize' the muscle to acetylcholine (Schulz & Cartwright, 1976). Caffeine, IBMX (Hammond, Schneider & Collier, 1976) and theophylline (Sawynok & Jhamandas, 1976) reverse the inhibition of the contractile response by morphine, whilst Gintzler & Musacchio (1975) reported that theophylline and other phosophodiesterase inhibitors either had no effect or potentiated the effect of morphine. Interpretation of such experiments is fraught with difficulties; the methylxanthines have several actions other than phosphodiesterase inhibition (see Sawynok & Jhamandas, 1976), and the measurement of the contractile response of the muscle affords many possibilities for interactions between agents occurring at different sites and by different mechanisms. There is evidence that a membrane hyperpolarization and/or conductance increase might underlie not only the inhibition of firing of myenteric neurones by narcotics but also the inhibition of transmitter release (North & Tonini, 1977; North, Katayama & Williams, 1978). If this is the case, then the present findings would support the interpretation of Hayashi *et al.* (1978) that cyclic nucleotides are probably not involved in the reduction of transmitter release by opiates; however, more direct emperiments are required to test this point.

Opiates have two temporally distinct types of action. Their manifold acute effects seem likely to have their basis in the inhibition of neuronal firing or transmitter release which has been demonstrated at numerous sites in the central and peripheral nervous system. The subacute effects, namely the initiation of changes leading to the development of tolerance and dependence, might of course have their basis in the inhibition of adenylate cyclase (Sharma *et al.*, 1975; 1977). It will therefore be of interest to examine whether the cyclic nucleotides have any effect on the development of tolerance and dependence in myenteric neurones.

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