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METHYL XANTHINES, ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND THE SPINAL TRANSMISSION OF NOCICEPTIVE INFORMATION

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1 In spinal cats anaesthetized with either α-chloralose or sodium pentobarbitone, a study was made of the effects of adenosine 3',5'-cyclic monophosphate (cyclic AMP), mono- and di-butyryl cyclic AMP and the methyl xanthines, theophylline and isobutyl methyl xanthine (IBMX), on the responses of neurones of laminae I, IV and V to noxious and innocuous skin stimuli. The compounds were administered from micropipettes positioned in the substantia gelatinosa. IBMX was also given intravenously.

2 When administered in the substantia gelatinosa, neither cyclic AMP, its butyryl derivatives, nor the methyl xanthines had any effect on the excitation of neurones of spinal laminae IV and V by noxious heating of the skin or deflection of hairs. When the nociceptive responses of cells had been reduced by electrophoretic morphine, methyl xanthines and cyclic AMP failed to modify the effects of morphine on these deeper neurones. Electrophoretically administered naloxone reversed the effect of morphine.

3 Intravenously administered IBMX (1 to 2 mg/kg) produced large transient increases in the firing rate of both C fibres and the excitation of dorsal horn neurones by noxious heating of the skin. These increases coincided with decreases in the mean systemic blood pressure, and probably resulted from increased temperatures being attained in the dermis by each noxious stimulus. When dorsal horn neurones were activated by electrical stimulation of the tibial nerve by a stimulus adequate to excite C fibres, intravenous IBMX produced a small or no increase in the number of spikes per stimulus.

4 These results in the spinal cord do not support the hypothesis that the inhibition of synthesis of cyclic AMP is relevant to the analgesic action of morphine in mammals.

Introduction

Several lines of evidence have suggested that opiates and endorphins produce their effects on the central nervous system by inhibiting the synthesis of adenosine 3',5'-cyclic monophosphate (cyclic AMP). Collier & Roy (1974) found that opiates inhibited prostaglandin-stimulated synthesis of cyclic AMP in rat brain homogenates, and that this was blocked by opiate antagonists such as naloxone. Both opiates (Traber, Fischer, Latzin & Hamprecht, 1975) and endorphins (Goldstein, Cox, Klee & Nirenberg, 1977) reduced prostaglandin-stimulated synthesis of cyclic AMP by cultured neuroblastoma × glioma hybrid cells. Administration of relatively large doses of methyl xanthines to rats produced signs which were likened to those of morphine withdrawal in dependent animals (Collier, Francis, Henderson & Schneider, 1974; Francis, Roy & Collier, 1975). It was suggested that inhibition of phosphodiesterase by methyl xanthines was responsible for these results, and, by analogy, elevated levels of cyclic AMP in neurones were considered to be responsible for the signs of opiate withdrawal. Ho, Loh & Way (1973) found that morphine-induced analgesia in mice was reversed by cyclic AMP administered intracerebroventricularly, and even when given intravenously in doses of 10 mg/kg. The xanthines, caffeine and theophylline, have been found to lower the vocalisation threshold for presumed painful electrical stimulation of the tail of the rat (Paalzow & Paalzow, 1973).

Not all reports have supported the proposed relationship between opiates and adenylcyclase. Katz & Catravas (1977) observed no antagonism by morphine of prostaglandin-stimulated increases in cyclic AMP levels in a rat brain mince. Sawynok & Jhamandas (1976) found that cyclic AMP acted similarly to morphine in inhibiting electrically evoked contractions of the guinea-pig ileum. Theophylline antagonized the actions of both morphine and cyclic AMP. Jhamandas, Sawynok & Sutak (1978) found that methyl xanthines antagonized the reduction by morphine of

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acetylcholine release from the rat cerebral cortex *in vivo*, but attributed this to an action on bound calcium and not to inhibition of phosphodiesterase.

The spinal cord is a suitable site to test possible interactions between morphine and cyclic AMP by neurophysiological techniques. When administered from micropipettes positioned in the substantia gelatinosa, both morphine (Duggan, Hall & Headley, 1976; 1977a) and methionine enkephalin (Duggan, Hall & Headley, 1977b) reduced the excitation of neurones of spinal laminae IV and V by noxious cutaneous stimuli. This action of morphine was antagonized by naloxone administered either at the same site as morphine, or intravenously. It was common in these experiments for naloxone, when administered after morphine, to increase the responses of neurones to cutaneous noxious stimuli to levels well above those present prior to administration of morphine.

If cyclic AMP is involved both in the depression of nociceptive responses of spinal neurones by morphine, and in the enhancement of these responses by naloxone administered after morphine, it might be expected that (a) administration of methylxanthines either systemically or in the substantia gelatinosa would enhance the nociceptive responses of dorsal horn neurones and (b) administration of these compounds at the same sites as morphine would reverse the actions of morphine.

When planning these experiments it was considered improbable that administering cyclic AMP derivatives in the extracellular space would raise intracellular levels sufficiently either to mimic the effects of enhanced intracellular synthesis or to overcome the effects of possible inhibition of synthesis. Effects have been observed however, when cyclic AMP and its butyryl derivatives have been administered extracellularly near neurones of the cerebellar and cerebral cortices (Siggins, Hoffer & Bloom, 1971; Lake, Jordan & Phillis, 1973; Stone, Taylor & Bloom, 1975) although it was not known if these resulted from activation of neurone receptors or from elevated intracellular levels of these substances. The finding that intravenous cyclic AMP reversed the analgesic effects of morphine on rats, in the hot plate test (Hoh *et al.*, 1973), also suggested that extracellular administration of this substance should be attempted. These doubts about the possible effectiveness of electrophoretically administered compounds do not apply to systemically administered methyl xanthines and therefore the majority of experiments were performed using this latter form of administration.

Methods

Experiments were performed on 22 cats anaesthetized either with α-chloralose (50 to 70 mg/kg) or sodium pentobarbitone (35 mg/kg). The lumbar spinal cord was exposed and divided at the thoraco-lumbar junction. All animals were artificially ventilated, end tidal CO_2 levels being maintained at approximately 4%. Dorsal horn neurones were activated by cutaneous noxious and non-noxious stimuli. The noxious stimulus was heating of an area of skin (approximately 3 mm^2) with a focussed light beam. A thermocouple on the heated area measured skin surface temperature and also provided feedback to a circuit controlling the filament current of the heating lamp. This controlling circuit is essential when drugs are administered which alter skin circulation (Duggan, Headley, Griersmith & Maher, 1978), but, as will be discussed later, problems still arose when methyl xanthines were given intravenously. The non-noxious stimulus was deflection of hairs adjacent to the area of heated skin by a moving air jet.

Extracellular recordings were obtained from neurones of spinal laminae I, IV and V using the 4 M NaCl-filled centre barrels of 5 barrel micropipettes. The outer barrrels contained acid fast green (saturated solution in 2 M NaCl) for the marking of cell positions, and excitant (Na, D,L-homocysteate 0.2 M) and depressant (γ-aminobutyric acid 0.5 M) amino acids.

Cell firing rates were counted using a window discriminator and a ratemeter, and were displayed continuously on a pen recorder. To ensure accurate counting, action potentials, the upper and lower levels of the discriminator window and the derived pulses counted by the ratemeter were all displayed on the same oscilloscope. The number of action potentials evoked by each cutaneous stimulus was determined by a gated electronic counter. A correction for spontaneous firing was made with each of these counts.

In experiments studying the effects of compounds on opiate receptors of the substantia gelatinosa, two micropipette assemblies were used, one a seven barrel with its tip positioned in this lamina, the other a five barrel of the type described above but inclined at 18 so as to pass a defined distance below the tip of the seven barrel assembly. This technique has been described in detail in previous publications (Duggan *et al.*, 1977a, b). The solutions contained within the seven barrel micropipettes were: sodium adenosine 3',5'-cyclic monophosphate 0.25 M; sodium N^6 monobutyryl adenosine 3',5'-cyclic monophosphate 0.25 M; sodium N^6, O^2 dibutyryl adenosine 3',5'-cyclic monophosphate 0.25 M. Both 3-isobutyl-1-methyl xanthine (IBMX) and theophylline were dissolved in 0.1 M NaOH to a final pH of 8 to 8.4 and concentration of 0.1 M. Because of reported variation in the release of cyclic AMP and its derivatives from micropipettes (Bloom, 1975), more than one barrel of each seven barrel assembly was filled with a given solution. The large ejecting currents used were attained by ejecting

simultaneously from several barrels. Micropipettes were used within 24 h of filling.

For the recording of action potentials from C fibres of a peripheral nerve the tibial nerve was exposed in the thigh and immersed in a pool of liquid paraffin (BP). The nerve trunk was cut and the peripherally intact end was laid over a small, firmly supported platform. The outer sheath enclosing the nerve fascicles was carefully split and completely removed for a short distance. With the aid of forceps and a fine tungsten needle, fascicles were separated and fine strands of fibres teased apart. Each strand was laid over a single wire of a pair of silver hook electrodes, the other wire contacting muscle. Action potentials in C fibres were then sought by applying noxious heat to several digital pads. Conduction velocities of these fibres were determined by electrical stimulation of the skin adjacent to the area heated by means of subdermal bipolar electrodes. Square wave pulses of 0.5 ms duration were used for such stimulation.

Results

Drugs ejected electrophoretically in the substantia gelatinosa

When ejected alone or in combination, neither cyclic AMP, its butyryl derivatives, nor the xanthines had any significant effect on the excitation of neurones of spinal laminae IV and V by noxious and non-noxious cutaneous stimuli.

When ejected with currents of from 80 to 300 nA for periods of 6 to 31 min, cyclic AMP had no effect on the firing of 12 neurones. With 6, a methyl xanthine (theophylline 3 neurones, IBMX 3 neurones) was also ejected together with cyclic AMP but no effect was observed.

Monobutyryl cyclic AMP (ejecting current up to 200 nA for 30 min) was tested on 9 neurones. When ejected alone (2 neurones) or in combination with IBMX (7 neurones) or IBMX and cyclic AMP (2 neurones) no effect on the firing of deeper neurones was observed. Dibutyryl cyclic AMP was tested on 4 neurones. With 3 no effect was observed. With one there occurred a just detectable increase in spontaneous firing and that in response to noxious skin stimulation.

The methyl xanthines were ejected alone and in combination with cyclic AMP or its butyryl derivatives. Neither theophylline (6 neurones, ejecting currents of 50 to 100 nA for up to 35 minutes) nor IBMX (9 neurones, ejecting currents of 100 to 200 nA for up to 60 minutes) affected the responses of deeper neurones to cutaneous stimuli. To ensure that these substances were being administered near opiate receptors, with 12 neurones morphine was also ejected in the substantia gelatinosa. When the nociceptive responses of 8 of these cells had been selectively reduced by morphine, a xanthine and cyclic AMP were also ejected in the substantia gelatinosa. In no instance was the effect of morphine in any way modified by these substances. Such a result is illustrated in Figure 1. With this neurone, IBMX ejected for 20 min and monobutyryl cyclic AMP ejected for 13 min had no effect on the depression of nociceptive responses by morphine. Naloxone, administered at the same site, had a significant effect within 5 min and fully reversed the action of morphine within 10 min of the start of ejection.

Intravenously administered isobutyl methylxanthine (IBMX)

IBMX was administered intravenously to 14 animals while the activation of dorsal horn neurones by noxious heating of the skin was studied. These experiments were complicated by the circulatory effects of the drug, and evidence was obtained that these were sufficiently great to produce artefactual changes in the firing of peripheral nociceptors despite control of the skin surface temperature attained by each noxious stimulus.

In 4 experiments IBMX (1 to 2 mg/kg) was injected rapidly, over 10 to 20 s, in 1 to 2 ml of 165 mM saline. The drug produced a large (mean 140%) increase in nociceptive responses of the cells studied with little or no effect on excitation by deflection of hairs. However, this increase in excitation was transitory, lasting 4 to 10 min, and always coincided with a large decrease in mean systemic blood pressure.

In a further 8 experiments, IBMX (1 to 2 mg/kg) was injected relatively slowly over a period of 3 to 4 min resulting in a lesser but still large change in blood pressure (see Figure 2c). A large but transitory increase in excitation by noxious heat still occurred (Figure 2a). After this initial phase, the blood pressure remained depressed (Figure 2c) for periods in excess of 1 h, and the only remaining effect on the firing of dorsal horn neurones was a small increase in spontaneous firing.

If the circulation through the skin is reduced, the rate of transfer of heat away from the dermal layers will also be reduced. Thus, when radiant heat is applied to the skin as a stimulus, higher temperatures may be attained in these deeper layers even though the surface is maintained at a constant temperature. The circuit used to control the heating lamp in the present experiments has been shown to compensate for certain circulatory changes (Duggan *et al.*, 1978), but the possibility remained that the increased firing of central neurones following IBMX injection resulted from increased activation of nociceptors. This was investigated by studying (a) action potentials in C fibres

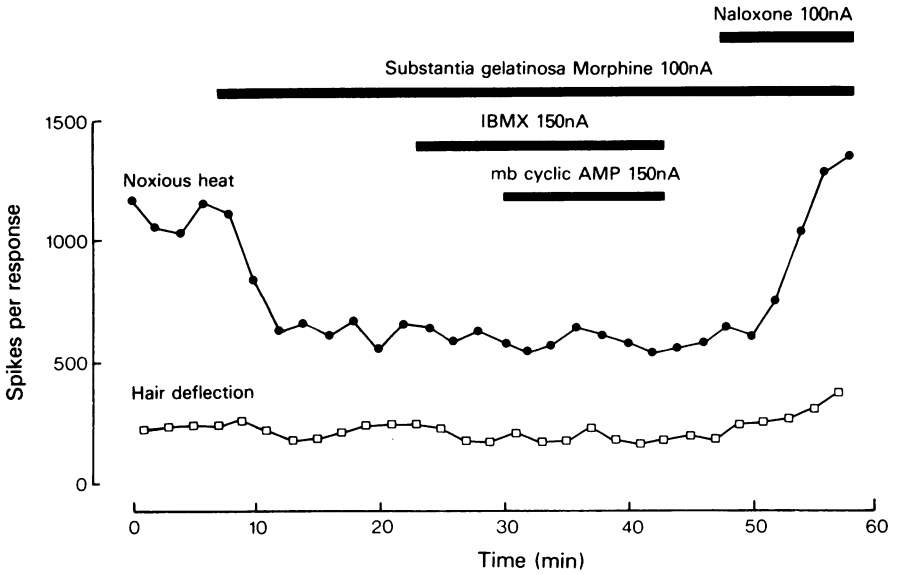

Figure 1 Failure of electrophoretic isobutyl methyl xanthine (IBMX) and monobutyryl cyclic AMP (mb cyclic AMP) to affect the action of morphine administered in the substantia gelatinosa. Ordinate scale: the numbers of action potentials recorded from a lamina III spinal neurone in response to alternate noxious (heating of the skin of the fourth digital pad of the left hind limb to 56 C) and non-noxious (deflection of hairs adjacent to the heated area) stimuli. Morphine ejected with a current of 100 nA at a distance of 130 μm dorsal to the tip of the recording electrode selectively reduced excitation by the noxious stimulus. Neither IBMX (150 nA) nor mb cyclic AMP (150 nA) ejected at the same site as morphine had any effect. Naloxone (100 nA) ejected at this site completely reversed the action of morphine. Abscissa scale: time (min).

of peripheral nerves before and after the administration of IBMX; (b) by activating dorsal horn neurones by noxious heating of the skin and by electrical stimulation of peripheral nerve; (c) by administration of IBMX by a slow infusion pump.

Figure 3 illustrates the type of result obtained when C fibres within the tibial nerve were studied. This particular fibre conducted with a velocity of 1.6 m/s, as determined from electrical stimulation of the skin. When the skin of the fourth left digital pad was heated to 55°C this fibre fired with a gradually increasing rate. Immediately following the injection of IBMX, 1 mg/kg, the response to noxious heat was increased. The record of firing in response to the second heat stimulus after IBMX shows moreover that the maximum firing rate was attained much earlier than before the administration of this drug. The records of skin surface temperature show that IBMX produced a slowing in the decline of skin surface temperature following each noxious stimulus. All of these observations are consistent with an increased rate of heating of the deeper layers of the skin as a consequence of a reduction in skin circulation, and it is probably unnecessary to postulate a direct effect of IBMX on nociceptors as explanation of the increased firing rate of the C fibre.

When dorsal horn neurones were activated by electrical stimulation of the tibial nerve by a stimulus adequate to excite C fibres, the response to this stimulus was either minimally or not increased when compared with the large increase in excitation resulting from noxious heating of the skin. Such a result is illustrated in Figure 2. This neurone responded to electrical stimulation of the tibial nerve ($\times 80$ threshold for activation of the cell) with early and late groups of spikes (Figure 2b). The latency of the latter indicates a conduction velocity of 0.9 m/s, assuming no interposed synapses. The mean number of later spikes per nerve stimulus was 12.4 ± 0.43 (s.e. mean) in the control period (H_1) and 13.7 ± 0.54 following the administration of IBMX (H_2). This 10% increase, although significant, is small when compared with the concurrent increase in excitation by noxious heat of 100% (1850 spikes before, 3606 just after IBMX, Figure 2a).

In two experiments IBMX (1.4 and 1.2 mg/kg) was administered by a slow infusion pump over periods of 11 and 12 min, thus minimizing circulatory effects. No increases were observed in the excitation of dorsal horn neurones by cutaneous noxious heat.

Collectively these results indicate that the large increases in excitation by noxious heat following intra-

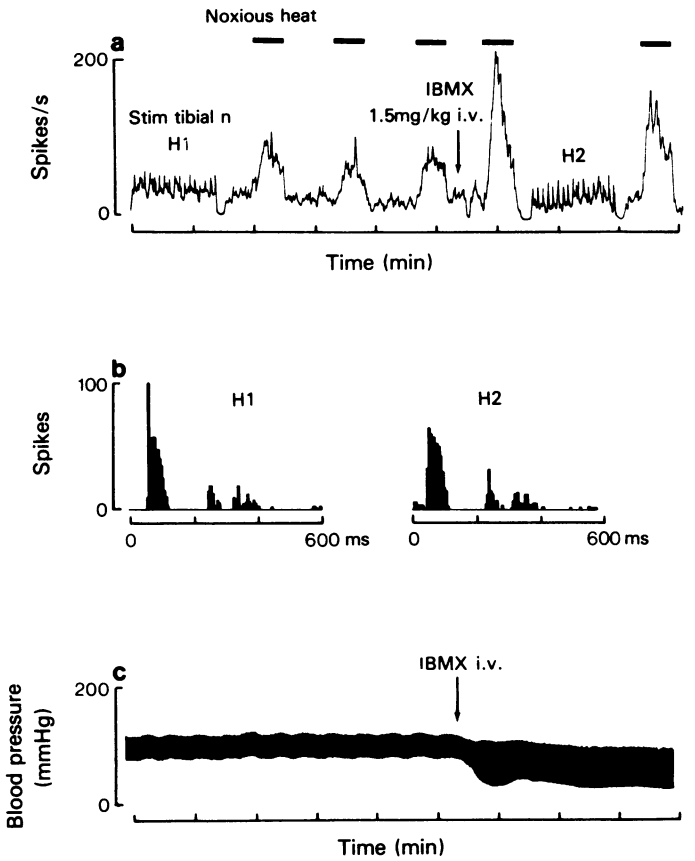

Figure 2 Increased firing of a dorsal horn neurone to noxious heating of the skin following intravenous isobutyl methyl xanthine (IBMX), without a parallel increase in excitation by electrical stimulation of C fibres. (a) The firing rate of a lamina IV dorsal horn neurone. At the periods marked H_1 and H_2, the ipsilateral tibial nerve was stimulated with a stimulus strength 80 times the threshold for activation of the cell, and post stimulus time histograms were compiled. The solid bars indicate the times of heating of the skin of the third digital pad of the ipsilateral hind limb to 53 C. (b) Post stimulus histograms compiled at H_1 and H_2. The stimulus was delivered at the start of each sweep, 16 responses were summed, bin width 5 ms. (c) The effect of intravenous IBMX on blood pressure.

venous IBMX were most probably produced by an increase in the firing of peripheral nociceptors, and that the only central effect of IBMX was to produce a relatively small and long lasting increase in the spontaneous firing of dorsal horn neurones.

Discussion

The present results provide no support for the hypothesis that the inhibition of the synthesis of cyclic AMP observed in brain homogenates and cultured cells is relevant to the analgesic action of morphine in mammals. It is possible that the opiate receptors of the spinal cord differ from those of other parts of the central nervous system but there is now good evidence that the spinal actions of morphine do contribute to the analgesic action of this alkaloid (reviewed by Yaksh & Rudy, 1978).

The negative results obtained from electrophoretic administration of cyclic AMP derivatives in the substantia gelatinosa do not exclude a role for cyclic AMP in the spinal transmission of nociceptive information. As outlined in the introduction, even if relatively large amounts were administered extracellularly, it is uncertain whether intracellular levels could be raised adequately to overcome possible inhibition of a synthesizing enzyme or to mimic enhanced synthesis.

These reservations about the administration of sub-

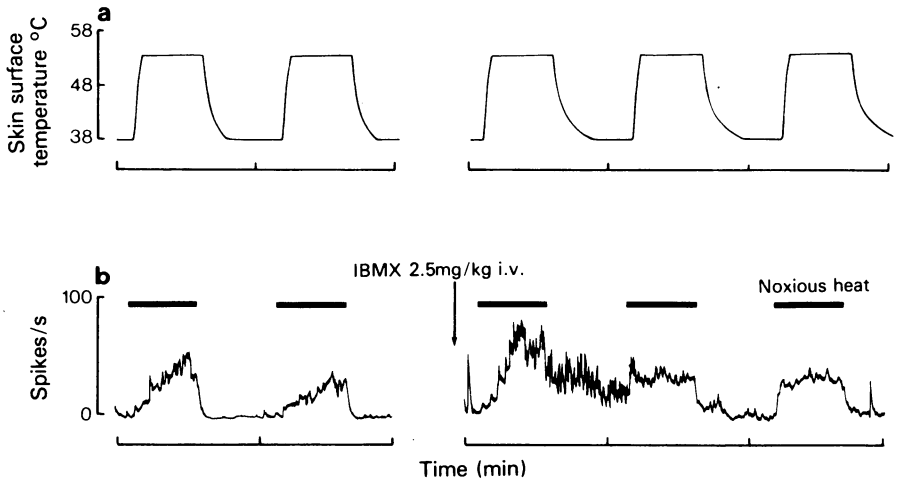

Figure 3 Increased firing of nociceptors to noxious heating of the skin following intravenous administration of isobutyl methyl xanthine (IBMX). (a) Records of skin surface temperature measured with a thermocouple. The rises in temperature were produced by a focussed light beam. (b) Pen recordings of the firing rate of impulses in a C fibre within the tibial nerve, conduction velocity 1.6 m/s. The two records are concurrent.

stances electrophoretically do not apply to intravenous IBMX. The doses given intravenously were the maximum that could be given under the conditions used, and these did not enhance the spinal transmission of nociceptive information. The small increases in spontaneous firing of cells which IBMX produced may be relevant to the syndrome described in the rat by Collier *et al.*, (1974), but in the present experiments it was clear that intravenous IBMX did not produce large increases in the excitation of spinal neurones by cutaneous noxious stimuli, a situation which is readily produced when naloxone is administered after morphine (Duggan *et al.*, 1977a). This result gives no support, at least in the spinal cord, to the proposal that the central actions of morphine result from an inhibition of adenyl cyclase.

Both this and a previous publication (Duggan *et al.*, 1978) highlight the problems associated with the use of radiant heat as a noxious stimulus to the skin. Accurately controlling the skin surface temperature does compensate for certain changes in skin circulation (Duggan *et al.*, 1978), but it was clear in the present experiments that the apparatus used was insufficient to prevent changes in the firing of nociceptors when IBMX was administered intravenously. It is possible that artefacts of this type occur in commonly used tests of analgesia such as tail flick of the rat (D'Amour & Smith, 1941). Indeed, before central theories of action are proposed for any compound which antagonizes the action of morphine in such a test, the absence of a change in the firing of peripheral nociceptors needs to be demonstrated.

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