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A STUDY OF THE ROLE OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE IN THE DEPRESSION BY OPIATES AND OPIOID PEPTIDES OF EXCITATORY JUNCTION POTENTIALS IN THE MOUSE VAS DEFERENS

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1 Excitatory junction potentials (e.j.ps) were recorded with intracellular microelectrodes from smooth muscle cells of the mouse isolated vas deferens. The amplitude of the e.j.p. was used as a measure of transmitter release evoked by applying single pulse stimuli to the intramural nerves.

2 Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) and dibutyryl cyclic AMP (db cyclic AMP, up to 1 mM) depressed the amplitude of e.j.ps, probably by interacting with extracellular sites on the nerve terminals, similar to those responsive to adenosine.

3 The phosphodiesterase inhibitors, 1-methyl-3-isobutyl xanthine (IBMX) and 1-ethyl-4-hydrazino-1*H*-pyrazolo(3,4-b)pyridine-5-carboxylic acid, ethylester, hydrochloride (SQ20,006) increased e.j.p. amplitude; this increase was much greater when the phosphodiesterase inhibitor was applied together with db cyclic AMP.

4 Neither the cyclic nucleotides nor the phosphodiesterase inhibitors altered the resting membrane potential of smooth muscle cells.

5 The amplitude of the e.j.p. was depressed by normorphine, D-Ala^2-Met^5-enkephalinamide (DAEA) and D-Ala^2-D-Leu^5-enkephalin (DADL) with respective EC_{50}s of 560 nM, 49 nM and 510 pM.

6 There was no change in the EC_{50} for normorphine in the presence of cyclic AMP (1 mM) or in the presence of a combination of IBMX (50 μM) and db cyclic AMP (500 μM). Similarly, the depression of the e.j.p. by DAEA or DADL was not affected by the combination IBMX (500 μM) and db cyclic AMP (250 μM).

7 These findings provide evidence against the hypothesis that a reduction in cyclic AMP levels in nerve terminals is an essential step in the inhibition by opiates and opioid peptides of transmitter release.

Introduction

It has been proposed that an important acute effect of opiates on neurones is inhibition of adenylate cyclase. This idea is based on three kinds of evidence. First, morphine antinociception in rodents is reduced by prior administration of adenosine-3′,5′-cyclic monophosphoric acid (cyclic AMP), its dibutyryl derivative (db cyclic AMP) or the phosphodiesterase inhibitor theophylline (Ho, Loh & Way, 1973). Second, opiates inhibit the prostaglandin-induced rise in cyclic AMP levels in rat brain homogenates (Collier & Roy, 1974). Third, opiates directly inhibit adenylate cyclase activity in neuroblastoma × glioma hybrid cells (Sharma, Klee & Nirenberg, 1975; Traber, Fischer, Latzin & Hamprecht, 1975).

Opiates inhibit the release of neurotransmitters at several peripheral and central sites and, although the mechanism of this action is not yet fully understood, there is a good correlation between this effect of opiates and their activity as analgesics in man (*inter alia* Kosterlitz & Waterfield, 1975; Henderson, Hughes & Kosterlitz, 1978). We wished to test the hypothesis that a decrease in nerve terminal levels of cyclic AMP was a necessary intermediate step in the inhibition of neurotransmitter release by opiates.

The mouse isolated vas deferens was used for two reasons. First, the depression by opiates of noradrenaline release is stereospecific and reversed by naloxone (Henderson, Hughes & Kosterlitz, 1972; Henderson

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& Hughes, 1976). Second, by measuring the amplitude of the excitatory junction potential (e.j.p.) with an intracellular electrode in the smooth muscle cells it is possible to estimate the amount of transmitter released by a single stimulus to the presynaptic nerves. Thus, the e.j.p. amplitude is depressed in a dose-dependent manner by opiates (Henderson & North, 1976). This presynaptic action of opiates was examined in the presence of drugs which might elevate the cyclic AMP concentrations in the nerve terminals: cyclic AMP, db cyclic AMP and the phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine (IBMX). If opiates depress transmitter release by decreasing the intraterminal concentration of cyclic AMP, then the elevation of cyclic AMP levels produced by these substances might reduce or prevent this action of opiates.

Methods

Intracellular recording

Mice (CF-1 strain) were killed by a blow to the head and the vasa deferentia dissected out of the abdominal cavity. A single vas deferens was pinned into a perspex bath and perfused at 1.5 ml/min with Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, KH_2PO_4 0.93, $CaCl_2$ 2.54, $MgSO_4.7H_2O$ 1.19, glucose 11 and $NaHCO_3$ 25. This solution was gassed with 95% O_2 and 5% CO_2 and heated so that the temperature at the recording site was 35 to 37°C. Drugs were applied by changing the perfusing solution. E.j.ps were recorded from single smooth muscle cells as previously described (Henderson & North, 1976; North & Vitek, 1980). E.j.ps were evoked by single rectangular pulses (500 μs in duration) which were repeated every 30 s. These stimuli were applied through two platinum ring electrodes placed around the muscle on either side of the site of recording. The stimulus voltage was adjusted so that the e.j.p. amplitude was between 10 and 25 mV before an opiate drug was tested.

Contractions of vas deferens

A vas deferens was suspended in an organ bath (volume 1.7 ml) through which Krebs solution continuously flowed at 1.5 to 2.5 ml/min. Flow was stopped for the injection of drugs. A linear motion transducer was used to record the changes in length of the vas deferens evoked by electrical field stimulation (0.1 Hz, 1 ms, supramaximal voltage) or by injection of noradrenaline or potassium chloride. Noradrenaline solutions were made daily and contained abscorbic acid (100 μM) and EGTA (50 μM).

Drugs

The drugs used were: adenosine 3',5'-cyclic monophosphoric acid (Sigma), D-Ala^2-D-Leu^5-enkephalin (Biosearch), D-Ala^2-Met^5-enkephalinamide (Peninsula), N^6,O^2-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (sodium salt) (Sigma), 1-ethyl-4-hydrazino-1*H*-pyrazolo(3,4-b)pyridine-5-carboxylic acid, ethyl ester, hydrochloride (SQ20,006; Squibb), 1-methyl-3-isobutyl xanthine (Sigma), (±)-noradrenaline hydrochloride (dl-arterenol; Sigma), and normorphine sulphamate (Dr A. E. Jacobson). Molar concentrations refer to the final bath concentration of these substances.

Results

The following results are based on recordings from 163 cells (82 mice).

Cyclic nucleotides

Both cyclic AMP and db cyclic AMP (up to 1 mM) depressed the amplitude of the e.j.p. without causing any change in the resting membrane potential of the smooth muscle cells. This effect, like that of adenosine (see North & Vitek, 1980) was rapid in onset and decline (2 to 5 min) and its magnitude was larger with higher concentrations of nucleotide. The depression of the e.j.p. caused by db cyclic AMP (1 mM) was 22.5 ± 3.7% (mean ± s.e. mean, $n = 8$), and by cyclic AMP (1 mM) was 52.0 ± 3.9% ($n = 9$). These effects of cyclic nucleotides were reproducible on the same preparation, in that repeated applications caused equivalent depressions of the e.j.p.

Phosphodiesterase inhibitors

Both IBMX and SQ20,006 (up to 1 mM) increased the amplitude of the e.j.p., although neither altered the resting membrane potential of the smooth muscle cells. This increase was relatively slow in onset, reaching its maximum after 8 to 12 min of drug perfusion, and subsequently was slow to reverse upon washing (15 to 30 min). The increase in e.j.p. amplitude was related to the concentration of phosphodiesterase inhibitor applied, but a second application of a given concentration of either drug to the same preparation was less effective than the initial application. Thus, IBMX (50 μM) increased e.j.p. amplitude by 58 ± 8.7% ($n = 6$) during its first application, but was significantly less effective (12 ± 6.3%, $n = 5$) when applied a second time. In the case of IBMX (500 μM), the increase in e.j.p. amplitude during the first application was 167 ± 20% ($n = 7$), and 82 ± 9.2% ($n = 6$) during the second application.

Phosphodiesterase inhibitors and db cyclic AMP

Although db cyclic AMP alone depressed e.j.p. amplitude (see above), when the db cyclic AMP was applied together with IBMX, the e.j.p. was increased to a greater extent than with IBMX alone. Similarly, although second applications of IBMX (50 μM) to a preparation increased the e.j.p. amplitude only slightly (see above), second applications of IBMX (50 μM) together with db cyclic AMP (500 μM) markedly increased the e.j.p. amplitude (by 103 ± 16%, $n = 5$). The effect of the non-xanthine phosphodiesterase inhibitor, SQ20,006, was also enhanced by concomitant application of db cyclic AMP. SQ20,006 (500 μM) and db cyclic AMP (250 μM) together increased e.j.p. amplitude by 392 ± 43% ($n = 4$).

The absence of any change in the resting potential of the smooth muscle cells suggests that the facilitatory action of IBMX and SQ20,006 on neuro-effector transmission is caused by presynaptic enhancement of transmitter release. The possibility was considered that these agents changed the sensitivity of the smooth muscle cells to noradrenaline. This possibility is unlikely because the contraction of the vas deferens evoked by noradrenaline (10 μM) was completely abolished by a combination of IBMX (500 μM) and db cyclic AMP (250 μM). The responses to nerve stimulation and high potassium (50 mM) solutions were also abolished by this drug combination. This implies a direct action on excitation-contraction coupling similar to that observed at other sympathetic end-organs (Cubeddu, Barnes & Weiner, 1974; Stjarne, Bartfai & Alberts, 1979).

Effect of normorphine

Normorphine depressed the amplitude of evoked e.j.ps; this effect was rapid in onset (peak effect reached in 3 to 6 min), washed readily and was dependent on the concentration applied. A dose-response curve for this action of normorphine was constructed by pooling data from many cells and preparations (Figure 1). The EC_{50} for normorphine was 560 nM, which is similar to the value found in a previous study (Henderson & North, 1976).

The effect of normorphine on e.j.p. amplitude was also measured in the presence of cyclic AMP (1 mM). This concentration of cyclic AMP depressed e.j.p. amplitude by about 50%; stimulus voltage was initially adjusted so that the e.j.p. amplitude was between 10 and 25 mV at the time of application of normorphine. The preparation was perfused with cyclic AMP (1 mM) for 5 to 30 min before testing normorphine. There was no change in the ability of normorphine to depress e.j.p. amplitude in the presence of this cyclic nucleotide (Figure 1). Normorphine was also tested after the preparation had been perfused

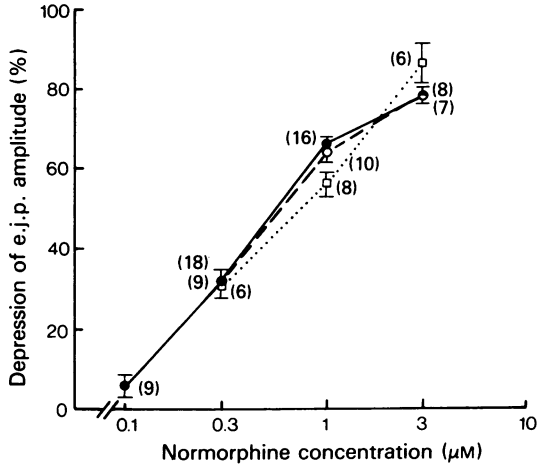

Figure 1 Normorphine dose-response curves for depression of the amplitude of the evoked e.j.p. in the mouse isolated vas deferens: (●) control; (□) in cyclic AMP (1 mM); (○) in IBMX (50 μM) + db cyclic AMP (500 μM). Bars represent s.e. mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration. Differences between control values and values after incubation in either cyclic AMP or IBMX + db cyclic AMP were tested by one-way analysis of variance at 3 concentrations (300 nM, 1 μM and 3 μM). No significant change from control values were found for any of the concentrations tested ($P > 0.05$).

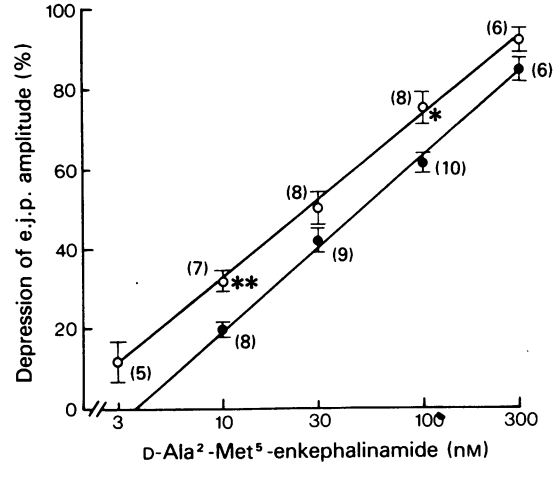

Figure 2 Dose-response curves for depression of evoked e.j.p. amplitude in the mouse isolated vas deferens by D-Ala²-Met⁵-enkephalinamide (DAEA): (●) control; (○) in IBMX (500 μM) + db cyclic AMP (250 μM). Bars represent s.e. mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration. Differences between control values and values after incubation in IBMX + db cyclic AMP were tested by Student's t test (** $P < 0.01$; * $P < 0.05$).

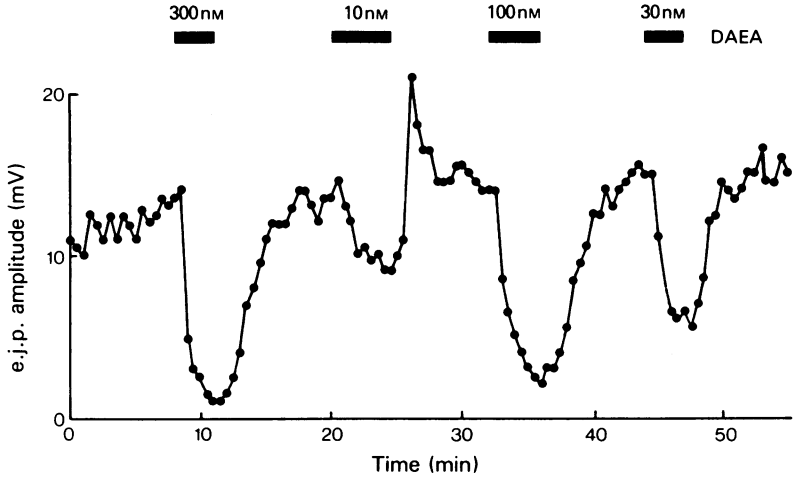

Figure 3 Continuous record of the amplitude of the evoked e.j.p. in a vas deferens which had already been exposed to IBMX (500 μM) and db cyclic AMP (250 μM) for 12 min at time zero. The preparation continued to be perfused with these compounds throughout the recording period. The perfusing Krebs solution also contained D-Ala^2-Met^5-enkephalinamide (DAEA) in the concentrations indicated (nM) during the times denoted by the solid bars.

with a combination of IBMX (50 μM) and db cyclic AMP (500 μM) for at least 12 min. The dose-response curve for normorphine in the presence of both IBMX and db cyclic AMP was almost identical to the dose-response curve in the absence of these drugs (Figure 1).

Effect of D-Ala^2-Met^5-enkephalinamide

D-Ala^2-Met^5-enkephalinamide (DAEA) is an enkephalin analogue which is resistant to enzymatic inactivation (Pert, Bowie, Fong & Chang, 1976). DAEA (3 to 300 nM) also depressed the amplitude of the evoked e.j.p. in the mouse vas deferens. Such an action had been previously reported for Met^5-enkephalin (Waterfield, Smokcum, Hughes, Kosterlitz & Henderson, 1977). The inhibitory effect of DAEA was more rapid in onset and decline than that of normorphine. The depression of the e.j.p. by DAEA was dose-dependent with an EC_{50} of 49 nM (Figure 2).

The effect of DAEA was tested in the presence of both IBMX (500 μM) and db cyclic AMP (250 μM). This combination increased the amplitude of the e.j.p. by 2 to 3.2 fold (182 ± 21%, $n = 5$). Figure 3 shows a continuous recording from a cell which was perfused with IBMX (500 μM) and db cyclic AMP (250 μM). The inhibitory effect of DAEA (10 nM to 300 nM) was not reduced by this combination. Actually, the potency of DAEA was slightly increased in the presence of IBMX and db cyclic AMP, as is evident from the composite dose-response curves (Figure 2). The magnitude of the depression of the e.j.p. by DAEA in the presence of IBMX and db cyclic AMP was significantly different from the magnitude of the effect in the absence of these drugs at two concentrations (10 nM: $P < 0.01$; 100 nM: $P < 0.05$, Student's t test).

Effect of D-Ala^2-D-Leu^5-enkephalin (DADL)

Neuroblastoma cells in tissue culture possess opiate receptors only of the δ-type (Chang, Cooper, Hazum & Cuatrecasas, 1979). Because DADL is a potent and, in low concentrations, selective agonist at the δ-receptor (Chang & Cuatrecasas, 1979) and because the mouse vas deferens possesses a preponderance of δ-receptors (Lord, Waterfield, Hughes & Kosterlitz, 1977), it was of interest to examine the interaction between elevated cyclic AMP levels and this enkephalin analogue on the evoked e.j.p. DADL was extremely potent in reducing the amplitude of the e.j.p. (EC_{50} = 510 pM), but its effect was not altered by the concomitant presence of both IBMX (500 μM) and db cyclic AMP (250 μM) (Figure 4).

Discussion

Effects of cyclic nucleotides and phosphodiesterase inhibitors on transmitter release

Cyclic AMP and db cyclic AMP both depressed the amplitude of the e.j.p. without changing the resting

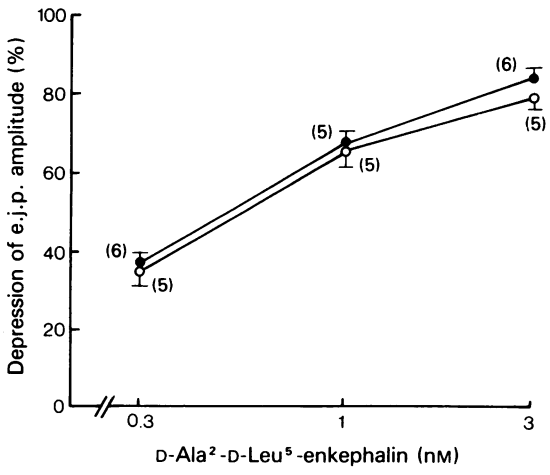

Figure 4 Dose-response curves for depression of e.j.p. amplitude by D-Ala²-D-Leu⁵-enkephalin: (●) control; (○) in IBMX (500 μM) + db cyclic AMP (250 μM). Bars represent s.e. mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration. There were no differences between control values and values after incubation in IBMX + db cyclic AMP (Student's t test, $P < 0.05$).

membrane potential of the smooth muscle cells. This is similar to the effect of adenosine (North & Vitek, 1980). Results from the latter study and the present one indicate that adenosine is the most potent and db cyclic AMP the least potent of the three nucleotides tested. The simplest explanation of this inhibitory effect of the nucleotides is that they interact with an extracellularly located purine 'receptor' similar to that purported to inhibit transmitter release in the rat vas deferens (Clanachan, Johns & Paton, 1977), the guinea-pig ileum (Hayashi, Mori, Yamada & Kunitomo, 1978) and the somatic neuromuscular junction (Ginsborg & Hirst, 1972).

The finding that phosphodiesterase inhibitors, both xanthine and non-xanthine, increased the amplitude of the e.j.p. without changing the resting membrane potential constitutes strong evidence that these agents facilitate transmitter release. This action is closely similar to that reported for phosphodiesterase inhibitors at other sympathetic neuro-effector junctions (cat spleen: Cubbedu *et al.*, 1974; guinea-pig vas deferens: Stjärne *et al.*, 1979). The evidence that this facilitation is a consequence of accumulation of cyclic AMP at an intracellular site is largely indirect, based on extrapolation of known actions of similar or higher concentrations of these phosphodiesterase inhibitors at other sites (Beavo, Rogers, Crofford, Hardman, Sutherland, Newman, 1970; Hamprecht & Schultz, 1973; Schofield & McPherson, 1974). The finding that db cyclic AMP itself had little effect on transmitter release except when phosphodiesterase was inhibited is also compatible with the suggestion that the increase in e.j.p. amplitude does actually result from the accumulation of cyclic AMP within the nerve terminal.

Effects of normorphine in the presence of cyclic nucleotides and IBMX

The purpose of these experiments was to test the hypothesis that a reduction in nerve terminal cyclic AMP levels is an essential intermediate step between occupation of the opiate receptor by normorphine and the resulting inhibition of transmitter release. The sensitivity of transmitter release to the inhibition by normorphine was not altered in circumstances in which either (a) release was reduced by the presence of cyclic AMP or (b) release was enhanced by a mixture of db cyclic AMP and IBMX. The first experiment suggests that morphine and cyclic AMP are unlikely to have a common extracellular site of action. The second finding indicates that conditions in which intraneuronal cyclic AMP may be increased, as evidenced by the facilitation of transmitter release (see above), do not reduce the ability of normorphine to inhibit transmitter release. This constitutes evidence against the hypothesis that a reduction in cyclic AMP levels need occur for normorphine to depress transmitter release.

Effects of opioid peptides in the presence of IBMX

The depression of the e.j.p. amplitude by normorphine was mimicked by the two opioid peptides, DAEA and DADL, both of which were substantially more potent than normorphine. Current evidence suggests that DAEA acts primarily on μ-receptors (Shaw, Turnbull, Dutta, Gormley, Hayward & Stacey, 1978). Its ability to inhibit transmitter release was unaltered by the combination of IBMX and db cyclic AMP. However, the vas deferens of the mouse contains predominantly receptors of the δ-type (Lord *et al.*, 1977). Furthermore, the direct evidence that opiates and opioid peptides inhibit adenylate cyclase comes from neuroblastoma × glioma cells which also contain almost exclusively δ-receptors (Chang *et al.*, 1979). The possibility existed that only activation of δ-receptors is linked to inhibition of adenylate cyclase, and that the predominantly μ-receptor-mediated effects of normorphine are not so mediated. Our finding that the EC_{50} for DADL was unchanged in the presence of db cyclic AMP and IBMX argue against this possibility.

Conclusions

The two acute actions of opiates on intact neuronal

systems which appear to be most common are inhibition of transmitter release and inhibition of neuronal firing (see North, 1979). The present results make it unlikely that the first of these depends directly on the ability of opiates to inhibit adenylate cyclase, at least in the mouse vas deferens. Earlier work has also provided evidence that the direct inhibition of neuronal firing by opiates either *in vitro* (Karras & North, 1979) or *in vivo* (Duggan & Griersmith, 1979) is also insensitive to the presence of cyclic nucleotides or phosphodiesterase inhibitors.

In addition to these acute effects, opiates also initiate changes which, during prolonged exposure, lead to tolerance and dependence. In neuroblastoma × glioma hybrid cells, the initial inhibition of adenylate cyclase caused by opiate agonists passes off during prolonged exposure (Sharma *et al.*, 1975) and it is possible that this might also occur in intact mammalian neurones. This hypothesis, that cyclic AMP involvement distinguishes the acute from the long term actions of opiate drugs (Karras & North, 1978) has also been considered by Collier (1980). Further experiments in which tolerance and dependence are induced *in vitro* (North & Karras, 1979; Crain, Crain, Finnigan & Simon, 1979) may help to substantiate or refute it.

References

BEAVO, J.A., ROGERS, N.L., CROFFORD, O.B., HARDMAN, J.G., SUTHERLAND, E.W. & NEWMAN, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3'-5'-monophosphate phosphodiesterase activity. *Mol. Pharmac.*, **6**, 597–603.

CHANG, K.-J., COOPER, B.R., HAZUM, E. & CUATRECASAS, P. (1979). Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmac.*, **16**, 91–104.

CHANG, K.-J. & CUATRECASAS, P. (1979). Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. biol. Chem.*, **254**, 2610–2618.

CLANACHAN, A.S., JOHNS, A. & PATON, D.M. (1977). Presynaptic inhibitory actions of adenine nucleotides and adenosine on neurotransmission in the rat vas deferens. *Neurosci.*, **2**, 597–602.

COLLIER, H.O.J. (1980). Cellular site of opiate dependence. *Nature*, **283**, 625–628.

COLLIER, H.O.J. & ROY, A.C. (1974). Morphine-like drugs inhibit the stimulation by E prostaglandins of cyclic AMP formation by rat brain homogenates. *Nature*, **248**, 24–27.

CRAIN, S.M., CRAIN, B., FINNIGAN, T. & SIMON, E.J. (1979). Development of tolerance to opiates and opioid peptides in organotypic cultures of mouse spinal cord. *Life Sci.*, **25**, 1797–1802.

CUBEDDU, L., BARNES, E. & WEINER, N. (1974). Release of norepinephrine and dopamine-β-hydroxylase by nerve stimulation. II. Effects of papaverine. *J. Pharmac. exp. Ther.*, **191**, 444–457.

DUGGAN, A. W. & GRIERSMITH, B.T. (1979). Methylxanthines, adenosine 3'-5'-cyclic monophosphate and the spinal transmission of nociceptive information. *Br. J. Pharmac.*, **67**, 51–57.

GINSBORG, B.L. & HIRST, G.D.S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *J. Physiol.*, **224**, 629–645.

HAMPRECHT, B. & SCHULTZ, J. (1973). Stimulation by prostaglandin E_1 of adenosine 3'-5'-cyclic monophosphate formation in neuroblastoma cells in the presence of phosphodiesterase inhibitors. *FEBS Lett.*, **34**, 85–89.

HAYASHI, E., MORI, M., YAMADA, S. & KUNITOMO, M. (1978). Effects of purine compounds on cholinergic nerves. Specificity of adenosine and related compounds on acetylcholine release in electrically stimulated guinea pig ileum. *Eur. J. Pharmac.*, **48**, 297–307.

HENDERSON, G. & HUGHES, J. (1976). The effects of morphine on the release of noradrenaline from the mouse vas deferens. *Br. J. Pharmac.*, **57**, 551–557.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1972). A new example of a morphine-sensitive neuro-effector junction: adrenergic transmission in the mouse vas deferens. *Br. J. Pharmac.*, **46**, 764–766.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1978). Modification of catecholamine release by narcotic analgesics and opioid peptides. In *The Release of Catecholamines from Adrenergic Neurons.* ed. Paton, D.M. pp. 217–228. New York: Pergamon Press.

HENDERSON, G. & NORTH, R.A. (1976). Depression by morphine of excitatory junction potentials in the vas deferens of the mouse. *Br. J. Pharmac.*, **57**, 341–346.

HO, I.K., LOH, H.H. & WAY, E.L. (1973). Cyclic adenosine monophosphate antagonism of morphine analgesia. *J. Pharmac. exp. Ther.*, **185**, 336–346.

KARRAS, P.J. & NORTH, R.A. (1979). Inhibition of neuronal firing by opiates: evidence against the involvement of cyclic nucleotides. *Br. J. Pharmac.*, **65**, 647–652.

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

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature*, **267**, 495–499.

NORTH, R.A. (1979). Opiates, opioid peptides and single neurones. *Life Sci.*, **24**, 1527–1546.

NORTH, R.A. & KARRAS, P.J. (1978). Tolerance and dependence *in vitro*. In *Characteristics and Functions of Opioids.* ed. Van Ree, J.M. & Terenius, L. pp. 25–36. Amsterdam: Elsevier.

NORTH, R.A. & VITEK, L.V. (1980). The effect of chronic morphine treatment on excitatory junction potentials in the mouse vas deferens. *Br. J. Pharmac.*, **68**, 399–405.

PERT, C.B., BOWIE, D.L., FONG, B.T.W. & CHANG, J.-K. (1976). Synthetic analogues of met-enkephalin which resist enzymatic destruction. In *Opiates and Endogenous Opioid Peptides*. ed. Kosterlitz, H.W. pp. 79–86. Amsterdam: Elsevier.

SCHOFIELD, J.G. & MCPHERSON, M. (1974). Increase in pituitary adenosine 3′-5′-cyclic monophosphate content and potentiation of growth-hormone release from heifer anterior pituitary slices incubated in the presence of 3-isobutyl-1-methylxanthine. *Biochem. J.*, **142**, 295–300.

SHARMA, S.K., KLEE, W.A. & NIRENBERG, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. natn. Acad. Sci., U.S.A.*, **72**, 3092–3096.

SHAW, J.S., TURNBULL, M.J., DUTTA, A.S., GORMLEY, J.J., HAYWARD, C.F. & STACEY, G.J. (1978). A structure-activity study with enkephalin analogues: further evidence for multiple opiate receptor sites. In *Characteristics and Functions of opioids*. ed. Van Ree, J.M. & Terenius, L. pp. 185–196. Amsterdam: Elsevier.

STJARNE, L., BARTFAI, T. & ALBERTS, P. (1979). The influence of 8-br 3′-5′-cyclic nucleotide analogs and of inhibitors of 3′-5′-cyclic nucleotide phosphodiesterase on noradrenaline secretion and neuromuscular transmission in guinea-pig vas deferens. *Naunyn-Schmiedebergs Arch. Pharmac.*, **308**, 99–106.

TRABER, J., FISCHER, K., LATZIN, S. & HAMPRECHT, B. (1975). Morphine antagonizes action of prostaglandin in neuroblastoma and neuroblastoma × glioma hybrid cells. *Nature*, **253**, 120–122.

WATERFIELD, A.A., SMOKCUM, R.W.J., HUGHES, J., KOSTERLITZ, H.W. & HENDERSON, G. (1977). *In vitro* pharmacology of the opioid peptides, enkephalins and endorphins. *Eur. J. Pharmac.*, **43**, 107–116.

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