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INTERACTIONS BETWEEN MORPHINE AND THE OPIOID-LIKE PEPTIDES IN THE RAT VAS DEFERENS

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1 Morphine, methadone, levorphanol, pethidine, etonitazene and related morphine-like alkaloids produced an increase in the electrically-evoked muscular contraction of the rat vas deferens. In contrast, the enkephalins and β-endorphin caused inhibition of the twitching.

2 The concentration of β-endorphin required to inhibit by 50% the muscular twitch was about 50 to 100 times less than that of the enkephalins.

3 Pretreatment of the vasa with morphine antagonized the inhibition of the neuromuscular transmission caused by either β-endorphin or enkephalin.

4 Conversely, pretreatment with β-endorphin sensitized the vasa to the increase in twitch tension caused by morphine.

5 Morphine did not alter the sensitivity to exogenously administered noradrenaline, dopamine or potassium.

Introduction

The idea of multiple opiate receptors is of great interest in the understanding of the pharmacology of the recently discovered opioid-like peptides. Support for this hypothesis derives both from *in vivo* and *in vitro* studies using morphine and structurally related analogues and the endogenous opiate peptides. Gilbert & Martin (1976) and Martin, Eades, Thompson, Huppler & Gilbert (1976), on the basis of the results of neuropharmacological studies in dogs, concluded that three kinds of opiate receptors exist. Similarly, Smith (1977) and McGillard & Takemori (1978) produced evidence for different opiate receptors for the antinociceptive, respiratory and lenticular effects of morphine. Binding studies of radioactive opiates and enkephalins to brain synaptic membranes (Law & Loh, 1978; Chang, Cooper, Hazum & Cuatrecasas, 1979), or to the neuroblastoma glioma cell line (Chang & Cuatrecasas, 1979; Chang *et al.*, 1979) suggest that the morphine binding site is not identical to that of the endogenous opiate ligands. *In vitro*, the marked difference in potency of a series of opiates and endorphins in the myenteric plexus-longitudinal muscle of the guinea-pig ileum preparation or the mouse vas deferens has been interpreted as indicating heterogeneity of opiate receptors (Lord, Waterfield, Hughes & Kosterlitz, 1976; 1977).

Previous investigations from our laboratory demonstrated that in the rat vas deferens, the opioid-like peptides produced inhibition of neuromuscular transmission while morphine increased the electrically evoked twitch (Miranda, Huidobro & Huidobro-Toro, 1979). The purpose of the present paper was to investigate further the effect of opiates and endorphins in the rat vas deferens. Of special interest was the possibility of interactions between these two types of opiates. The present results demonstrate that pretreatment of the rat vas deferens with endorphins sensitized the tissue to the effect of morphine, while morphine pretreatment antagonized the response of the endorphins. These results suggest the existence of multiple opiate receptors in the rat vas deferens.

Methods

Vas deferens preparation

Vas deferentia of Sprague Dawley rats (200 to 250 g from the Catholic University vivarium) were dissected and mounted in a 30 ml double jacketed bath, maintained at 37°C, in Tyrode solution of the following composition (mM): NaCl 137, KCl 2.70, $MgCl_2$ 0.49,

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$CaCl_2$ 1.35, NaH_2PO_4 0.36, $NaHCO_3$ 24 and glucose 11. The tissues were connected to a force-displacement transducer as detailed by Miranda *et al.* (1979). The preparations were equilibrated for 1 h with 0.7 to 1.0 g tension before starting drug applications or electrical stimulation. Transmural stimulation was set at supramaximal voltage (70 to 80 V), 0.1 Hz and 2 ms duration. Pulses were delivered from a Grass S4 Stimulator.

Opiates were dissolved in saline (0.9% w/v NaCl solution) and applied in a volume of 0.1 to 0.3 ml. Concentrations are expressed in terms of the final molar concentration of the bases or the peptides.

Quantification of drug effects

Opiates were added to the bath containing the tissues and washed after the effect reached a maximum (6 min for the alkaloids, or about 2 min after application of the peptides). The opiate effects were calculated as the percentage increase in or inhibition of the control twitch tension. Dose-response curves for the effect of each opiate were obtained; concentrations of drugs causing a 50% increase (ED_{50}) or a 50% inhibition of the twitch (ID_{50}), were calculated by interpolation from these plots, as detailed by Miranda *et al.* (1979).

Interactions between opiate alkaloids and opiate-like peptides

Different sets of vasa were primed with either 1.1, 3.4 or 11.0 $\times 10^{-6}$ M morphine to increase the twitch tension. Six minutes after the application of morphine or *N*-cyclopropylmethylnorazidodihydroisomorphine (CAM) (i.e. when the response was maximal), doses of β-endorphin or D-ala^2-met-enkephalinamide were applied to obtain dose-response curves. As controls, dose-response curves for each peptide were obtained in the absence of morphine. Each vas deferens was used to study the effect of one priming dose of morphine on the responses to either peptide.

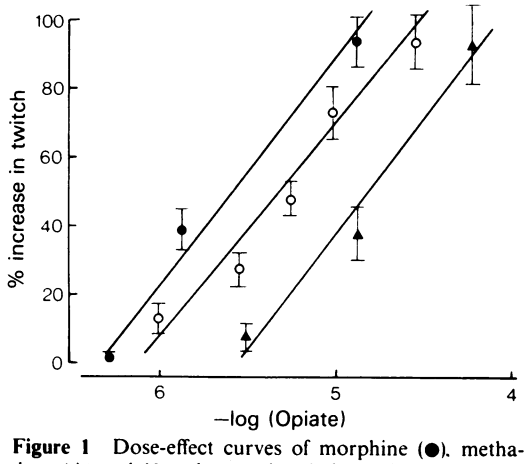

Figure 1 Dose-effect curves of morphine (●), methadone (▲) and *N*-cyclopropylmethylnorazidodihydroisomorphine (○) on the electrically-induced twitch of the rat vas deferens. The mean increase in the twitch tension caused by the opiate alkaloids was plotted against −log of the concentration of the opiates. Bars indicate s.e. mean of each set of determinations. The concentration causing a 50% increase in the twitch tension was extrapolated from graphs such as these.

In the second set of experiments, vasa deferentia were first exposed to 9 $\times 10^{-7}$ M β-endorphin; this dose produced a 80 to 90% reduction in the twitch tension that lasted, without recovery, for at least 6 min. Three minutes after the addition of β-endorphin, the preparations were treated with different doses of opiate alkaloids to increase the twitch height. Dose-effect curves for morphine, CAM, and naloxone were obtained, and the ED_{50} of the opiates was determined. Note that the tissue was not washed at the time of the application of the alkaloids. Results were compared to those obtained with the opiate-alkaloids in the absence of β-endorphin pretreatment.

Effect of morphine on the responses of noradrenaline, dopamine or potassium

To study whether morphine altered the responses of exogenously applied noradrenaline, dopamine or potassium, dose-effect curves to these agonists were obtained in vasa maintained in Tyrode containing 1.5 or 10 $\times 10^{-6}$ M morphine. The dose of agonist to produce half-maximal contraction (ED_{50}) was compared between control (no morphine added) and the morphine-treated group.

Statistical analysis

Student's *t* test was used to compare the results of paired experiments. The significance level was set at a *P* value less than 0.05.

Drugs

The following drugs were used: morphine, methadone, pethidine and nalorphine hydrochloride (Merck Chemical Co., Darmstad, Germany). Etonitazene hydrochloride was a gift from Ciba (Basel). Levorphanol tartrate and dextrorphan base were generous gifts from Hoffmann-La Roche (Nutley, N.J., U.S.A.). Naloxone, naltrexone hydrochloride and oxymorphone hydrochloride (Endo Labs, Garden City, N.Y., U.S.A.). *N*-methyl morphine was supplied by Dr E. L. May from N.I.H. (Washington, D.C., U.S.A.). Azidomorphine bitartrate and *N*-cyclopropylmethylnorazidodihydroisomorphine (CAM) were obtained from Professor J. Knoll (Budapest, Hungary). Methionine-enkephalin (met-enk), leucine-enkephalin (leu-enk),

and D-ala²-methionine enkephalinamide (D-ala²-met-enkephalinamide) (Bachem Chemicals, Marina del Rey, CA, U.S.A.). β-Endorphin was a kind gift from Professor C.H. Li (San Francisco, CA, U.S.A.). Inorganic salts and glucose to prepare the Tyrode solution were purchsed from Merck Chemical Co. (Darmstad, Germany).

Results

Effect of morphine and morphine surrogates on the electrically-induced twitch of the rat vas deferens

Morphine produced a dose-dependent increase in the electrically-induced twitch height, without affecting base-line tension (Figure 1). The concentration of morphine to cause a 50% increase in the twitch tension (ED_{50}) was found to be about 5×10^{-6} M. The effect of morphine was shared by other morphine-like alkaloids such as methadone, pethidine, azidomorphine. In general, the alkaloids produced at maximal doses an increase in the twitch tension of about 80 to 120% (Figure 1). Entonitazene, a synthetic analgesic, was the most potent drug to potentiate the neuromuscular twitch. It increased the twitch at doses of about 10^{-6} M by approximately 200%, and at maximal doses it potentiated the twitch 3 to 4 fold. CAM, a derivate of norazidomorphine, was as active as morphine and about twice as potent as azidomorphine, its parent compound. *N*-methyl-morphine or naloxone did not show agonist effects in doses up to 100 times that of morphine. Nalorphine exhibited properties of a weak agonist since it did not produce maximal effects; it increased the twitch response, at maximal doses, by 50 to 60%. The ED_{50} of the alkaloids studied is shown in Table 1; their dose-response curves were approximately parallel to that of morphine (Figure 1).

The effect of morphine appeared to be mediated via stereoselective opiate receptors as indicated by the fact that levorphanol was more active than its isomer, dextrorphan (Table 1).

Effect of enkephalins and β-endorphin on the electrically-induced twitch of the rat vas deferens

In contrast to the increase in twitch tension produced by the application of opiate alkaloids, the opiate-like peptides produced a dose-dependent inhibition of the muscular twitch (Figures 2 and 3). β-Endorphin was the most active peptide, being approximately 100 times more potent than the natural or synthetic enkephalins; its ID_{50} was about 6.3×10^{-7} M (Table 1). The dose-effect curves of the different peptides were parallel to that of β-endorphin, suggesting that probably all these compounds interact at a common site in the vas deferens.

Table 1 Effects of morphine, morphine surrogates and the opioid-like peptides on the rat vas deferens preparation

	Concentration of opiate to increase the twitch by 50% Mean ± s.e. mean × 10^{-6} M	*n*
Morphine	4.5 ± 1.17	8
Methadone	10.0 ± 1.87	4
Pethidine	8.4 ± 2.30	4
Etonitazene	0.6 ± 0.09	8
Azidomorphine	13.4 ± 0.23	6
CAM	7.1 ± 1.39	12
Nalorphine	11.4 ± 2.9	4
Naloxone	>400	8
N-methyl morphine	>450	4
Levorphanol	7.0 ± 1.5	4
Dextrorphan	30.0 ± 2.85	4
	Concentration of peptide to inhibit twitch by 50% Mean ± s.e. mean × 10^{-5} M	*n*
Methionine-enkephalin	8.5 ± 0.97	12
Leucine-enkephalin	6.8 ± 2.79	4
D-Ala²-methionine enkephalinamide	3.5 ± 0.72	8
β-Endorphin	0.06 ± 0.002	8

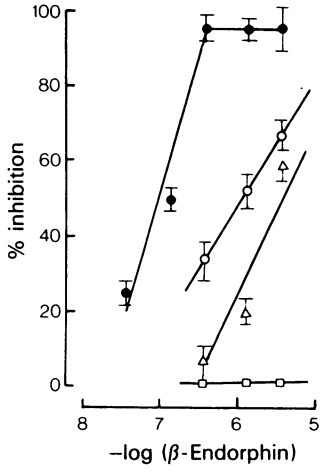

Figure 2 Morphine antagonism of the inhibitory response of β-endorphin. Dose-response curves for the inhibition of the electrically-induced twitch caused by β-endorphin were performed in the absence (control, ●, $n = 8$) or in the presence of various concentrations of morphine: (○) 1.1×10^{-6} M, $n = 7$; (△) 3.45×10^{-6} M, $n = 8$; (□) 11.0×10^{-6} M, $n = 4$. Morphine was applied first to increase the twitch tension, and 6 min later, without washing the preparation, different doses of β-endorphin were added to the bath in order to obtain a dose-response curve. Ordinate scale denotes the percentage inhibition of the twitch response caused by β-endorphin. Symbols indicate the mean value, bars the s.e. mean.

Interactions between morphine and the opiate-like peptides

It was of interest to look for possible interactions between the effects produced by the two types of opiates. Pretreatment with morphine antagonized in a dose-related fashion the inhibition of the twitch response caused by β-endorphin (Figure 2) or D-ala^2-met-enkephalinamide (Figure 3). The antagonism by low doses of morphine was apparently competitive as shown by the parallel displacement of the peptide's dose-effect curve to the right. However, pretreatment with 10^{-5} M morphine completely prevented the inhibitory effect of β-endorphin, suggesting that the mechanism of the interaction is more complicated in nature (Figure 2). Likewise, preparations primed with 4.7×10^{-6} M CAM showed complete blockade of the response of up to 10^{-5} M β-endorphin (Figure 4). Lower concentrations of CAM produced a gradual decrease in the effect of a challenge dose of 9×10^{-7} M β-endorphin (Figure 4). The antagonism of the inhibitory effect of the peptides caused by morphine or CAM pretreatment was completely reversible, since the inhibitory effect of β-endorphin on the muscular twitch was completely restored after washing.

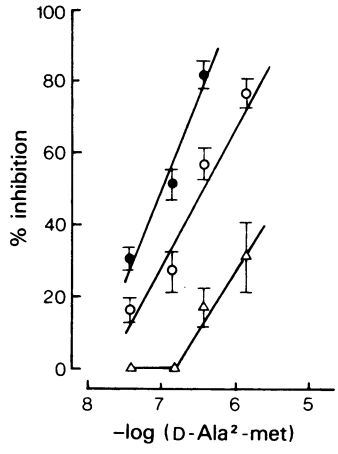

Figure 3 Antagonism of the inhibitory effect of D-alanine^2-methionine enkephalinamide (D-ala^2-met) by morphine. Dose-response curves for the inhibitory effect of D-ala^2-met-enkephalinamide were obtained in rat vas deferens incubated in the absence (control). ●, $n = 4$), and in the presence of 1.1×10^{-6} M (○, $n = 4$) or 3.45×10^{-6} M (△, $n = 4$) morphine. Experimental design is as described for Figure 2. Symbols represent the mean inhibitory value of the opiate-peptide, bars the s.e. mean.

Interactions between β-endorphin and morphine-like alkaloids

In the second series of experiments, vasa deferentia were primed with 9×10^{-7} M β-endorphin, and subsequently challenged with opiate alkaloids. The concentration of naloxone to produce a 50% reversal of the peptide inhibitory response was approximately 7×10^{-8} M (Figure 5). CAM and morphine also 'reversed' in a graded fashion the twitch previously inhibited by β-endorphin. However, the potency of morphine and CAM in increasing the neuromuscular twitching was markedly augmented following β-endorphin pretreatment. Figure 5 shows that the dose of CAM causing a 50% increase in the twitching was about 7×10^{-8} M, and that of morphine was about 7×10^{-7} M. These values are about 10 times lower than those shown in Table 1.

Effects of morphine on the contractile responses to noradrenaline, dopamine or potassium

The addition of 1.6 or 10×10^{-5} M morphine to the Tyrode solution did not significantly modify the contractile response to noradrenaline, dopamine or potassium. As can be seen in Table 2, the ED_{50} of the agonists was not significantly changed in the presence of morphine.

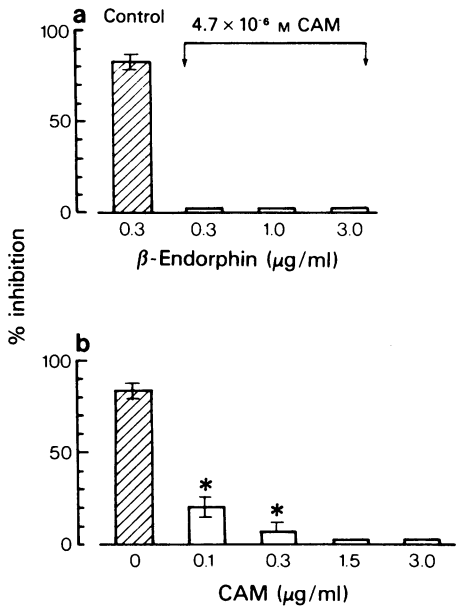

Figure 4 Effect of *N*-cyclopropylmethylnoraziododihydroisomorphine (CAM) on the inhibitory effect of β-endorphin. A challenge dose of 9.0×10^{-7} M β-endorphin was used to inhibit by about 85% the electrically-induced twitch of the rat vas deferens. This effect is represented in the hatched column of (a) (control). After application of 4.7×10^{-6} M CAM, 9×10^{-7} M β-endorphin was completely ineffective. A dose as high as 9×10^{-6} M β-endorphin was completely antagonized by CAM (a). Smaller doses of CAM (b), caused a graded reduction of the response to a challenge dose of 9.0×10^{-7} M β-endorphin (hatched column in b). Columns indicate the mean of 4 experiments; bars, the s.e. mean. $*P < 0.05$ in relation to the control, when no CAM was added.

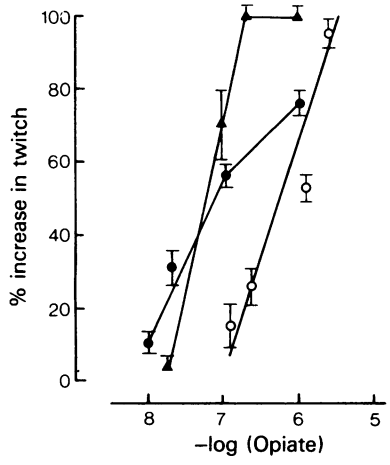

Figure 5 Reversal of the β-endorphin-induced inhibition of the twitch height by naloxone, morphine and *N*-cyclopropylmethylnorazidodihydroisomorphine (CAM). A challenge dose of 9.0×10^{-7} M β-endorphin was used to produce a prolonged inhibition of the electrical twitch (see Figure 4); 3 min after the application of the peptide, increasing doses of naloxone (●, $n = 8$), morphine (○, $n = 8$) or CAM (▲, $n = 4$) were applied to produce an increase in the twitch height. A separate preparation was used to obtain a dose-response curve for each opiate-like alkaloid. Symbols indicate the mean, bars the s.e. mean. Note that in the case of both CAM and morphine, the dose to cause a 50% increase in the twitch height was markedly potentiated as compared to the results presented in Table 1, where no peptide pretreatment was performed.

Discussion

The present results confirm and extend previous work indicating that the opioid-like peptides produce a de-

Table 2 Effect of morphine on the contractile responses to noradrenaline (NA), dopamine (DA), and potassium (K^+)

	ED_{50}		
	NA ($\times 10^{-6}$ M)	*DA* ($\times 10^{-6}$ M)	K^+ ($\times 10^{-2}$ M)
Control	3.71 ± 0.22 (6)	7.35 × 0.14 (8)	—
$+1.6 \times 10^{-6}$ M Morphine	3.81 ± 0.20 (6)	7.26 ± 0.14 (8)	—
Control	4.14 ± 0.31 (8)	6.90 ± 0.09 (8)	4.61 ± 0.12 (8)
$+1.0 \times 10^{-5}$ M Morphine	4.85 ± 0.16 (8)	7.11 ± 0.12 (8)	4.22 ± 0.10 (8)

Values are mean ± s.e. mean.
Number in parentheses refer to the different preparations used to obtain these results. Morphine did not significantly modify the ED_{50} of the agonists. *P* values were not significant ($P > 0.10$).

crease in the electrically-evoked twitch of the rat vas deferens (Lemaire, Magnan & Regoli, 1978; Miranda *et al.*, 1979; Schulz, Faase, Wuster & Herz, 1979). Curiously, morphine or its derivatives do not depress neuromuscular transmission or cause only a minor inhibition of the twitch (Hughes, Kosterlitz & Leslie, 1975; Lemaire *et al.*, 1978). In a recent communication we showed that morphine produced a potentiation of the neuromuscular twitch in the rat vas deferens. However, this effect was not blocked by naloxone (Miranda *et al.*, 1979). We have now shown that the morphine effect is common to a variety of opiate-like alkaloids. Etonitazene was the most active, in agreement with its high potency as an antinociceptive agent (Eddy as cited by Casy, 1971). CAM, the *N*-cyclopropylmethyl derivative of norazidomorphine had all the properties of an opiate agonist in full agreement with the studies of Knoll, Furst & Markeit (1977).

An immediate question that arose from these studies was that of interactions between the two types of opiates. Pretreatment with morphine antagonized the inhibitory effect of either β-endorphin or D-ala^2-met-enkephalinamide. One simple explanation would be that of a physiological antagonism between the effects of each opiate. However, this explanation does not reconcile two findings: first 11×10^{-6} M morphine or 4.7×10^{-6} M CAM completely prevented the inhibitory effect of β-endorphin. Secondly, preparations pretreated with β-endorphin and challenged with morphine or CAM, showed a marked increase in the ability of the alkaloids to increase the twitch compared to vasa not primed with the peptide. The reversal produced by morphine of the inhibition caused by β-endorphin appeared qualitatively similar to that of naloxone. Morphine could, then, be thought of as a pharmacological opiate antagonist, sharing properties like naloxone in this tissue. This possibility appears unlikely, firstly because morphine potentiated the neuromuscular twitch but naloxone did not (Table 1). Secondly, naloxone displaced the leucine-enkephalin dose-effect curve to the right in a parallel fashion (Miranda *et al.*, 1979), while morphine caused only an initial parallel displacement, followed by a complete blockade of the inhibitory effect of β-endorphin. Thus, the potentiation of the morphine response following β-endorphin pretreatment is probably not the result of a simple interaction. We suggest that the sustained activation of the opiate-like peptide receptor by β-endorphin facilitates the opposing action of morphine, whereby the alkaloid causes, under these conditions, an increment in the apparent release of neurotransmitter. Alternatively, occupancy of the opiate-peptide receptor may unmask an inhibitory action on the effect of morphine. Whether the morphine-sensitization produced by pretreatment with β-endorphin is relevant to other morphine effects, especially to its antinociceptive action is a matter of interest. Vaught & Takemori (1978) recently demonstrated that pretreatment of isolated strips of guinea-pig ileum with leucine-enkephalin significantly decreased the median effective dose of subsequent morphine applications in suppressing the electrical twitch. More interestingly, Vaught & Takemori (1979) showed that pretreatment of mice with leucine-enkephalin decreased about 4 fold the median effective dose of morphine required for analgesia. Thus, it appears that the β-endorphin-morphine interaction seen in the rat vas deferens has features in common with other *in vitro* and *in vivo* effects of the opiates.

The precise site of action of the opiates in the rat vas deferens is not known. However, the present results demonstrate that morphine does not sensitize the postsynaptic catecholamine receptor or alter the muscle excitability, since morphine did not affect the contractile responses to exogenous noradrenaline, dopamine or potassium. These results allow the suggestion that morphine modifies presynaptic sites that, in turn, probably modulate the release and/or metabolism of neurotransmitter in the vas deferens.

Present results could be best interpreted by assuming the presence of two types of receptors in the tissue. One type of opiate receptor is apparently specific for the opioid-like peptides. This receptor is similar to that activated by the endogenous opioids in the guinea-pig ileum (Waterfield, Smockum, Hughes, Kosterlitz & Henderson, 1977; Huidobro-Toro, Foree & Way, 1978), and the mouse vas deferens (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975). The other receptor site, activated by morphine and surrogates shows a low degree of stereospecificity, develops tolerance to the effect of chronic morphine, but the responses are not blocked by naloxone. This latter type of receptor bears pharmacological resemblance to that described by Jacquet, Klee, Rice, Iijima, & Minamikawa (1977) and Jacquet (1978) in the central nervous system after stereotaxic injection of the morphine stereoisomers. Thus, the present results support the notion proposed by Lord *et al.*, (1977) indicating the existence of multiple opiate receptors. Experiments are in progress to establish the nature of the opiate receptors in the vas deferens of the rat and the cellular events which they mediate.

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