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PHARMACOLOGICAL DIFFERENTIATION OF PRESYNAPTIC INHIBITORY α-ADRENOCEPTORS AND OPIATE RECEPTORS IN THE CAT NICTITATING MEMBRANE

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1 The action of morphine, naturally occurring and synthetic opiate peptides on [^{3}H]-noradrenaline release induced by nerve stimulation was studied in the isolated nerve muscle preparation of the cat nictitating membrane under experimental conditions in which the α-presynaptic receptors were blocked by phentolamine 1 μM.

2 Morphine and the naturally occurring peptides: [Met^5]-enkephalin, [Leu^5]-enkephalin and β-endorphin reduced ^{3}H-transmitter overflow and responses to nerve stimulation from the cat nictitating membrane, effects which were completely antagonized by naloxone 0.3 μM. The relative order of potency for the inhibition of the stimulation-induced ^{3}H-transmitter overflow at the level of the IC_{50} (μM) was as follows: [Met^5]-enkephalin (0.020 μM) ≥ [Leu^5]-enkephalin (0.036 μM) > morphine (0.3 μM) > β-endorphin (1 μM).

3 The synthetic opiate pentapeptides: BW 180 C (Tyr-D-Ala-Gly-Phe-D-Leu), and BW834 C (Tyr-D-Ala-Gly-pC1Phe-DLeu), which are resistant to enzymatic degradation were more potent than the enkephalins in reducing the stimulation-evoked transmitter overflow from the cat nictitating membrane. On the other hand, the tetrapeptide BW832 C, which lacks the D-leucine terminal of BW180 C was less potent than the enkephalins in inhibiting neurotransmission.

4 In the presence of phenoxybenzamine 1 μM, ^{3}H-transmitter overflow was increased 8 fold and the inhibition of neurotransmission by methionine-enkephalin was not affected. Exposure to phenoxybenzamine 10 μM increased [^{3}H]-noradrenaline overflow 15 fold and antagonized the effects of methionine enkephalin on transmitter release.

5 In the cat nictitating membrane the inhibitory presynaptic opiate receptors are different from the presynaptic α-autoreceptors which regulate the release of noradrenaline elicited by nerve depolarization through a negative feed-back mechanism.

Introduction

Activation of presynaptic opiate receptors by morphine and its analogues results in a decrease of noradrenaline release during nerve stimulation in some tissues of the peripheral nervous system (Henderson, Hughes & Kosterlitz, 1972a; 1975; Henderson, Hughes & Thompson, 1972b; Henderson & Hughes, 1974; Hughes, Kosterlitz & Leslie, 1975a; Henderson & Hughes, 1976).

In the mouse vas deferens, the two endogenous morphine-like pentapeptides methionine [Met^5] and leucine [Leu^5]-enkephalin act like morphine and its analogues (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975b; Morgan, Smith, Waterfield, Hughes & Kosterlitz, 1976). On the other hand, it has recently been shown that in the central ear artery of the rabbit and rat vas deferens, both [Met^5]- and [Leu^5]-enkephalin reduce the responses to nerve stimulation through a mechanism which is blocked by the classical opiate receptor antagonists, naloxone or azidomorphine (Knoll, 1976; Lemaire, Magnan & Regoli, 1978). However, in these tissues exposure to morphine does not affect noradrenergic neurotransmission.

The presynaptic regulation of noradrenaline released during nerve stimulation which operates under physiological conditions is the negative feedback mechanism mediated by presynaptic α-adrenoceptors (for reviews, see Langer, 1974; 1977; 1979; Starke, 1977; Westfall, 1977). Yet, most of the studies on the inhibition of noradrenaline release by activation of presynaptic opiate receptors were carried out under conditions in which the regulatory mechanism mediated by presynaptic α-adrenoceptors was

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operative (Henderson *et al.*, 1972a, b; Henderson & Hughes, 1974; 1976; Montel, Starke & Weber, 1974a, b; Montel, Starke & Taube, 1975a, b). Consequently, it was considered of interest to study the presynaptic effects of morphine, the two endogenous morphine-like pentapeptides, [Met^5]- and [Leu^5]-enkephalin (Hughes *et al.*, 1975b) and some synthetic analogues on [^{3}H]-noradrenaline release induced by nerve stimulation. These experiments were carried out in the isolated nerve muscle preparation of the cat nictitating membrane under experimental conditions in which phentolamine or phenoxybenzamine were employed to block the presynaptic inhibitory α-adrenoceptors.

Methods

Cats of 2.0 to 2.5 kg body weight and of either sex were used. Each animal was anaesthetized with sodium pentobarbitone (35 mg/kg) and the trachea was cannulated. The eyeballs were excised and the medial muscle of the nictitating membrane was dissected together with the postganglionic sympathetic fibres arising from the infratrochlear nerve, under a binocular dissecting microscope. The tissue was kept in modified Krebs solution previously equilibrated with 95% O_2 and 5% CO_2. The composition of the Krebs solution was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 25.0, glucose 11.1, sodium ethylenediamine tetraacetic acid 0.004 and ascorbic acid, 0.11.

The cartilage on which the fibres of the medial muscle are inserted was fixed to the bottom of a 5 ml organ bath. The upper end of the muscle was connected to a force-displacement transducer (Grass FT 03) and the tension developed by the muscle was recorded with a Grass polygraph. The temperature was maintained at 37°C and the organ bath was bubbled with a 95% O_2/5% CO_2 mixture. The infratrochlear nerve was pulled through shielded bipolar platinum electrodes for stimulation with monophasic square pulses of 0.5 ms duration and supramaximal voltage.

The resting tension of the muscle was repeatedly adjusted to 2.5 g. Thirty minutes after the tissue was set up in the organ bath it was incubated for 30 min with (±)-[7-^{3}H]-noradrenaline 0.5 μM (Radiochemical Centre, Amersham; specific activity 19.3 Ci/mmol). At the end of the incubation period the tissue was washed 8 times for 1 min and 3 times for 10 min each with fresh Krebs solution.

The stimulation periods were started 48 min after the end of the incubation with [^{3}H]-noradrenaline. Samples of 1.0 ml of the Krebs solution which had been in contact with the tissue for 2.5 min were collected before, during and after the periods of nerve stimulation. Total radioactivity was measured by scintillation spectrometry. When used, phentolamine or phenoxybenzamine was added to the incubation medium 30 min before the first period of nerve stimulation and maintained throughout the rest of the experiment.

In the control group, four periods of nerve stimulation were applied every 20 min (4 Hz for 2 min, 0.5 ms duration and supramaximal voltage). Morphine, the opiate-like peptides or naloxone were added 10 min before the corresponding period of nerve stimulation.

At the end of each experiment the smooth muscle of the nictitating membrane was blotted dry, weighed and dissolved in 0.5 ml of Soluene 350. An aliquot of this solution was taken for measuring the total radioactivity in the tissue.

The total outflow of neurotransmitter in each sample was expressed as the fraction of the total tissue radioactivity present at the start of the corresponding sample collection: total ^{3}H-released per sample, divided by total ^{3}H-present in the tissue. The total radioactivity present in the tissue at the start of each sample collection was determined by adding the radioactivity present in the tissue at the end of the experiment to the radioactivity collected in each sample until the end of the sample collections.

The 'total overflow of the transmitter' was the sum of all increases in fractional release above spontaneous levels induced by the period of nerve stimulation. These increases were expressed as fractional release per shock (Langer & Enero, 1974).

Statistical calculations were performed according to conventional procedures (Snedecor & Cochran, 1967).

The following drugs were used: pehentolamine hydrochloride, phenoxybenzamine hydrochloride, naloxone hydrochloride, morphine hydrochloride. The opiate like peptides: [Met^5]-enkephalin, [Leu^5]-enkephalin, BW 180 C (Tyr-D-Ala-Gly-Phe-D-Leu); BW 227 C (DTyr-DAla-Gly-Phe-Leu); BW 832 C (Tyr-D-Ala-Gly-Phe) and BW 834 C (Tyr-D-Ala-Gly-pCl Phe-D-Leu) were synthesized by Dr S. Wilkinson in the Department of Chemistry at the Wellcome Research Laboratories, Beckenham, Kent. β-Endorphin was purchased from Peninsula Laboratories, Inc. (California). All concentrations are expressed on a molar basis.

Results

Inhibition of the stimulation-induced ^{3}H-transmitter overflow from the nictitating membrane with morphine, the morphine-like pentapeptides and β-endorphin in the presence of phentolamine

Under control conditions there was no significant decline in ^{3}H-transmitter overflow or in the mechanical

responses to nerve stimulation during four consecutive periods of nerve stimulation obtained in the presence of phentolamine 1 μM (Table 1). The concentration of phentolamine employed in these experiments produced a 3.5 fold increase in ^{3}H-transmitter overflow when compared with the untreated controls. The fractional release per shock in the controls was $1.12 \pm 0.16(\times 10^{-5})$ ($n = 14$) and after exposure to phentolamine 1 μM it was significantly increased to $3.53 \pm 0.47(\times 10^{-5})$ ($n = 18$, $P < 0.001$). Under these experimental conditions the responses to nerve stimulation were reduced from 8.99 ± 0.51 g in the controls ($n = 14$) to 4.09 ± 0.46 g in the presence of phentolamine ($n = 18$, $P < 0.001$).

The addition of naloxone 0.3 μM during the fourth period of stimulation (S_4) in the presence of phentolamine did not affect either ^{3}H-transmitter overflow (1.99 ± 0.39 ($\times 10^{-5}$), $n = 4$) or the postsynaptic responses (2.95 ± 1.19 g, $n = 4$) obtained during nerve stimulation when compared with the corresponding controls (Table 1).

As shown in Figure 1, exposure to morphine 1.0 and 3.0 μM produced a concentration-dependent reduction in ^{3}H-transmitter overflow when nerve stimulation was applied at 4 Hz in the presence of phentolamine. The inhibition of ^{3}H-transmitter overflow during exposure to morphine was antagonized in the presence of naloxone 0.3 μM (Figure 1).

Similar results were obtained during exposure to the two endogenous morphine-like pentapeptides, [Met^5] and [Leu^5]-enkephalin in concentrations of 0.03 and 0.3 μM (Figure 1). These effects were also naloxone-sensitive. The inhibition of [^{3}H]-noradrenaline release obtained with morphine, [Met^5]- or [Leu^5]-enkephalin was reflected in the reduction of the responses to nerve stimulation, and these effects were also antagonized by naloxone (Figure 2). The natural neuropeptide, β-endorphin 1 μM, reduced significantly the release of ^{3}H-neurotransmitter induced by nerve stimulation from the nictitating membrane in the presence of phentolamine (Table 2). The inhibition of ^{3}H-transmitter overflow during exposure to β-endorphin was antagonized in the presence of naloxone 0.3 μM (Table 2).

These results indicate that the two enkephalins and the polypeptide, β-endorphin, inhibit [^{3}H]-noradrenaline release during nerve stimulation through the same presynaptic opiate receptor that is activated by morphine. The relative order of potency for the inhibition of the stimulation-induced ^{3}H-transmitter overflow was as follows: [Met^5]-enkephalin ≥ [Leu^5]-enkephalin > morphine > β-endorphin (Table 3). With the concentration of β-endorphin tested (1 μM), the inhibition of stimulation-evoked release of [^{3}H]-noradrenaline was $53.0 \pm 16.0\%$.

Inhibition of ^{3}H-transmitter overflow by synthetic morphine-like penta- and tetrapeptides in the presence of phentolamine

Three synthetic pentapeptides and one tetrapeptide were tested for their effects on [^{3}H]-noradrenaline release during nerve stimulation in the presence of phentolamine. Their structures are given in the Methods section.

The synthetic opioid pentapeptides, BW 180 C (Baxter, Goff, Miller & Saunders, 1977) and BW 834 C, which are potent analogues of [Leu^5]-enkephalin, decreased, in a concentration-dependent manner, the release of [^{3}H]-noradrenaline elicited by nerve stimulation from the cat nictitating membrane (Figure 3a, Table 4). The effects of BW 180 C and BW 834 C on the inhibition of the stimulation-evoked ^{3}H-transmitter release were antagonized in the presence of naloxone 0.3 μM with the exception of the highest concentration of BW 834 C for which the antagonism by naloxone was not complete (Figure 3a, Table 4). The comparison of the concentrations of the opiate ago-

Table 1 Effects of repeated periods of nerve stimulation on ^{3}H-transmitter overflow and responses to nerve stimulation of the cat nictitating membrane in the presence of phentolamine

Experimental group	*n*	*Periods of nerve stimulation* S_1	S_2	S_3	S_4
Fractional release [a] per shock $\times 10^{-5}$	5	2.97 ± 0.39	2.84 ± 0.62	2.46 ± 0.68	2.83 ± 0.80
Responses (g)[b]	5	3.89 ± 0.86	3.95 ± 0.82	3.45 ± 0.79	3.65 ± 0.71

[a]Total radioactivity released by nerve stimulation expressed as fractional release per shock ($\times 10^{-5}$).
[b]Maximal development of tension expressed in grams (g) of the isolated medial muscle of the nictitating membrane in response to nerve stimulation.
The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage) every 20 min (S_1 to S_4). Phentolamine 1 μM was present in the medium from 30 min before S_1 until the end of the experiment. Shown are mean values ± s.e. mean; n = number of experiments.

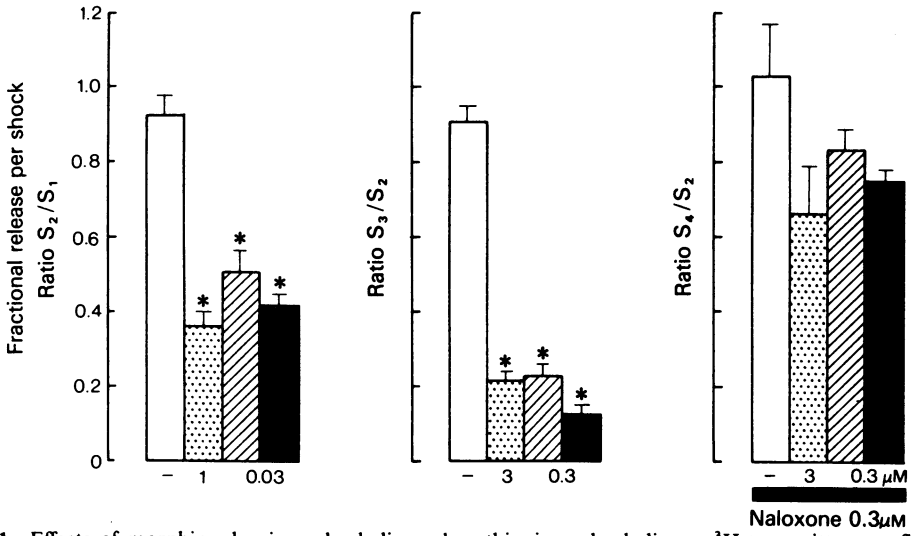

Figure 1 Effects of morphine, leucine-enkephalin and methionine-enkephalin on ^{3}H-transmitter overflow from the cat nictitating membrane elicited by nerve stimulation in the presence of phentolamine. Ordinates: fraction of the total tissue radioactivity released per shock expressed as the ratio obtained between each period of nerve stimulation (S_2, S_3 or S_4) and the first one (S_1) within each individual experiment. The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage) every 20 min. Phentolamine 1 μM was present in the medium from 30 min before S_1 until the end of the experiment. Morphine, leucine-enkephalin or methionine-enkephalin in the concentration indicated were present in the incubation medium 10 min before the corresponding stimulation period. Naloxone, 0.3 μM was added 10 min before S_4. Drug concentrations are indicated under the abscissae. Open columns = control; stippled columns = morphine; hatched columns = leucine-enkephalin; solid columns = methionine enkephalin. Mean values of 4 to 18 experiments per group are shown; vertical lines indicate s.e. mean. *$P < 0.001$ when compared with the corresponding control.

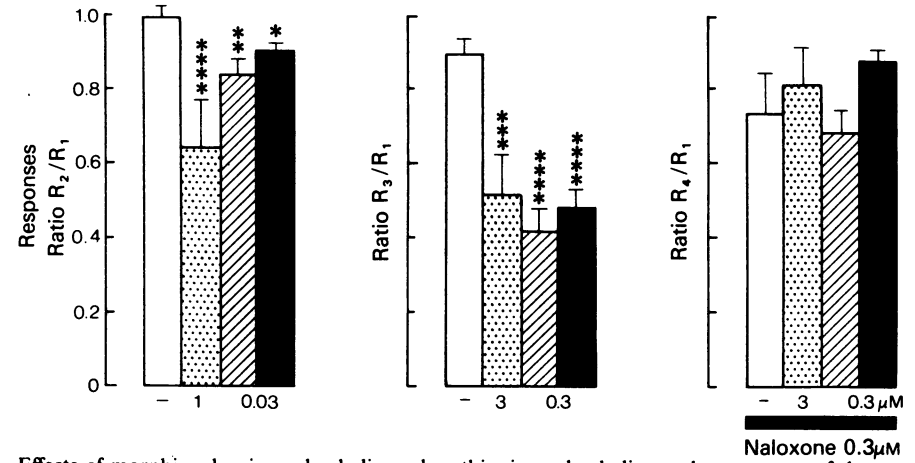

Figure 2 Effects of morphine, leucine-enkephalin and methionine-enkephalin on the responses of the cat nictitating membrane elicited by nerve stimulation in the presence of phentolamine. Ordinates: responses to nerve stimulation (maximal development of tension) expressed as the ratio obtained between each period of nerve stimulation (R_2, R_3 or R_4) and the first one (R_1) within each individual experiment. The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage) every 20 min. Phentolamine 1 μM, was present in the medium from 30 min before R_1, until the end of the experiment. Morphine, leucine-enkephalin or methionine-enkephalin in the concentration indicated were present in the incubation medium 10 min before the corresponding stimulation. Naloxone, 0.3 μM, was added 10 min before R_4. Drug concentrations are indicated under the abscissae. Open columns = control; stippled columns = morphine; hatched columns = leucine-enkephalin; solid columns = methionine- enkephalin. Mean values of 4 to 19 experiments per group are shown; vertical lines indicate s.e. mean. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$ when compared with the corresponding controls.

Table 2 Effects of β-endorphin on ^{3}H-transmitter overflow elicited by nerve stimulation from the cat nictitating membrane in the presence of phentolamine

Experimental group	n	Fractional release per shock × 10^{-5} [a] S_1	S_2	S_3 Naloxone 0.3 μM	Ratio [b] S_2/S_1	S_3/S_1
Control	4	2.00 ± 0.59	2.05 ± 0.35	1.99 ± 0.39	1.05 ± 0.11	1.03 ± 0.14
β-Endorphin 1 μM	5	3.10 ± 0.71	1.84 ± 0.70	4.23 ± 1.56	0.47 ± 0.16*	1.27 ± 0.33

[a] Total radioactivity released by nerve stimulation expressed as fractional release per shock ($\times 10^{-5}$).
[b] Ratio obtained between the values of fractional release in S_2 or S_3 and the first control (S_1).
The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage) every 20 min (S_1 to S_3). Phentolamine, 1 μM, was present in the medium from 30 min before S_1 until the end of the experiment β-Endorphin was present in the incubation medium from 10 min before S_2 until the end of the experiment. Naloxone, 0.3 μM was added 10 min before S_3. Shown are mean values ± s.e. mean. n = number of experiments.
* $P < 0.05$ when compared with control.

nists required to produce 50% inhibition (IC_{50}) of the stimulation-evoked release of [^{3}H]-noradrenaline, indicates that both BW 180 C and BW 834 C are more potent that either [Leu^5]- or [Met^5]-enkephalin in reducing neurotransmission from the cat nictitating membrane (Table 3).

The reduction in [^{3}H]-noradrenaline release during nerve stimulation in the presence of both synthetic compounds was accompanied by a significant reduction in the responses to nerve stimulation and this effect was also antagonized by naloxone (Figure 3b; data not shown for BW 834 C).

Table 3 Relative order of potency of natural and synthetic opiate agonists on the presynaptic opiate receptors of the cat nictitating membrane

Peptide	IC_{50} (M) [a]
BW 834 C	2.8×10^{-10}
BW 180 C	6.7×10^{-9}
Methionine-enkephalin	2.0×10^{-8}
Leucine-enkephalin	3.6×10^{-8}
BW 832 C	1.6×10^{-7}
Morphine	3.0×10^{-7}
β-Endorphin	1.0×10^{-6}
BW 227 C	7.0×10^{-6}

[a] IC_{50}: Concentration producing a 50% decrease in the release of [^{3}H]-noradrenaline elicited by nerve stimulation from the cat nictitating membrane. The IC_{50} was determined by graphical extrapolation of mean values of at least 4 experiments per group. Only one concentration of β-endorphin (1 μM) was tested, which gave an inhibition of 53.0 ± 16.0%. The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage). Phentolamine, 1 μM was present in every experiment.

The synthetic pentapeptide, BW 227 C, at a concentration of 3 μM reduced by 40% the overflow and by 30% the responses to nerve stimulation (Table 4). These effects were also blocked by naloxone 0.3 μM.

Exposure to 0.3 and 3 μM of the tetrapeptide, BW 832 C, produced a concentration-dependent reduction of ^{3}H-transmitter overflow induced by nerve stimulation (Table 4) and decreased significantly the postsynaptic responses to nerve stimulation. Both effects were completely antagonized by naloxone 0.3 μM.

Effects of phenoxybenzamine on the inhibition of the stimulation-induced release of ^{3}H-transmitter elicited by morphine and the morphine-like pentapeptides

Two concentrations of phenoxybenzamine were employed in these studies, 1 and 10 μM. The increases in ^{3}H-transmitter overflow obtained during nerve stimulation were approximately 8 and 15 fold respectively. In the controls the overflow of ^{3}H-transmitter was 1.12 ± 0.16 ($\times 10^{-5}$) $n = 14$; during exposure to 1 μM phenoxybenzamine these values were 8.64 ± 1.12($\times 10^{-5}$) ($n = 7$, $P < 0.001$) and in the presence of 10 μM phenoxybenzamine 15.2 ± 1.94 ($\times 10^{-5}$) ($n = 9$, $P < 0.001$). As shown in Table 5 there was no decline in ^{3}H-transmitter overflow during four consecutive periods of nerve stimulation in the presence of 1 μM phenoxybenzamine. On the other hand, during exposure to 10 μM phenoxybenzamine there was a progressive decline in ^{3}H-transmitter overflow particularly after the second period of nerve stimulation (Table 5). The addition of naloxone 0.3 μM in the presence of either concentration of phenoxybenzamine did not affect ^{3}H-transmitter overflow elicited by nerve stimulation (Table 5).

As shown in Figure 4a, in the presence of phenoxybenzamine 1 μM exposure to 0.03 and 0.3 μM [Met^5]-enkephalin produced a concentration-dependent inhi-

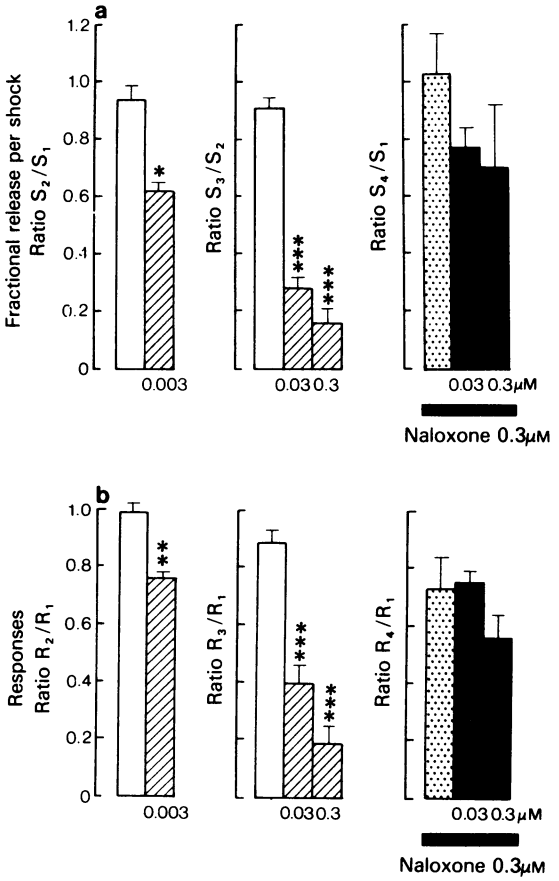

Figure 3 Effects of BW 180C on ^{3}H-transmitter overflow and responses elicited by nerve stimulation of the cat nictitating membrane in the presence of phentolamine. (a) Ordinates: fraction of the total tissue radioactivity released per shock expressed as the ratio obtained between each period of nerve stimulation (S_2, S_3 or S_4) and the first period (S_1) within each individual experiment. (b) Ordinates: responses to nerve stimulation (maximal development of tension) expressed as the ratio obtained between each period of nerve stimulation (R_2, R_3 or R_4) and the first one (R_1) within each individual experiment. The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage) every 20 min. Phentolamine 1 μM was present in the medium from 30 min before the first period of nerve stimulation until the end of the experiment. BW 180 C in the concentration indicated was present in the incubation medium from 10 min before the corresponding stimulation period. Naloxone, 0.3 μM was added 10 min before S_4. Drug-concentrations are indicated under the abscissae. Open columns = control; hatched columns = BW 180 C; stippled columns = naloxone; solid columns = BW 180 C plus naloxone. Mean values of 3 to 18 experiments per group are shown; vertical lines indicate s.e. mean. *$P < 0.01$; **$P < 0.005$; ***$P < 0.001$, when compared with the corresponding control.

bition of ^{3}H-transmitter overflow during nerve stimulation, which was antagonized by naloxone. These effects were reflected in a reduction of the responses to nerve stimulation which reached levels of statistical significance only for 0.3 μM [Met^5]-enkephalin ($R_3/R_1 = 0.58 \pm 0.06$, $n = 4$, $P < 0.05$ when compared with the corresponding control $R_3/R_1 = 0.96 \pm 0.08$, $n = 3$).

When similar experiments were carried out in the presence of 10 μM phenoxybenzamine, [Met^5]-enkephalin (0.03 to 0.3 μM) failed to inhibit ^{3}H-transmitter overflow (Figure 4b) and the postsynaptic responses to nerve stimulation were not decreased (data not shown).

Table 4 Effects of synthetic opiate peptides on ^{3}H-transmitter overflow elicited by nerve stimulation in the presence of phentolamine

Experimental group	*Ratio S_2/S_1*[a]
Control	0.94 ± 0.05 (18)
BW 834 C, 0.003 μM	0.62 ± 0.03 (4)**
BW 227 C, 0.3 μM	0.80 ± 0.04 (4)
BW 832 C, 0.3 μM	0.38 ± 0.06 (4)**
	S_3/S_1
Control	0.91 ± 0.05 (18)
BW 834 C, 0.03 μM	0.18 ± 0.04 (8)**
BW 834, C, 0.3 μM	0.10 ± 0.02 (4)**
BW 227 C, 3 μM	0.58 ± 0.06 (5)**
BW 832 C, 3 μM	0.05 ± 0.02 (4)**
	S_4/S_1
Naloxone, 0.3 μM	1.03 ± 0.14 (4)
Naloxone, 0.3 μM plus BW 834 C, 0.03 μM	0.75 ± 0.12 (4)
Naloxone, 0.3 μM plus BW 834 C, 0.3 μM	0.54 ± 0.02 (4)*
Naloxone 0.3 μM plus BW 227 C, 3 μM	0.82 ± 0.08 (4)
Naloxone 0.3 μM plus BW 832 C, 3 μM	0.97 ± 0.17 (3)

[a] Ratio obtained between the values of fractional release in S_2, S_3 or S_4 and the first control (S_1). The nerves were stimulated at 4 Hz for 2min (0.5 ms duration, supramaximal voltage) every 20 min (S_1 to S_4). Phentolamine, (1 μM) was present in the medium from 30 min before S_1 until the end of the experiment. The synthetic opiate peptides: BW 227 C, BW 832 C and BW 834 C in the concentrations which are indicated were present in the incubation medium from 10 min before the corresponding stimulation period. Naloxone 0.3 μM was added 10 min before S_4. The numbers in parentheses correspond to the number of experiments per group. Shown are mean values ± s.e. mean.
* $P < 0.05$; ** $P < 0.001$ when compared with the corresponding control.

Discussion

It was shown many years ago that morphine inhibits contractions of the nictitating membrane elicited by nerve stimulation both under *in vivo* and *in vitro* conditions (Trendelenburg, 1957; Cairne, Kosterlitz & Taylor, 1961). Yet it is only in recent years that the inhibition of sympathetic neurotransmission by morphine and other opiate-like compounds has been identified with an effect at the level of presynaptic inhibitory opiate receptors on noradrenergic nerve terminals of the peripheral and central nervous system (Henderson *et al.*, 1972a, b; 1975; Henderson & Hughes, 1974; 1976; Montel, Starke & Weber, 1974a, b; Montel, Starke & Taube, 1975a, b,; Arbilla & Langer, 1978). Inhibition of noradrenergic neurotransmission can also be observed when the presynaptic α-adrenoceptors involved in a negative feedback mechanism regulating noradrenaline release are activated by α-receptor agonists (for recent reviews, see Langer, 1974; 1977; 1979; Starke, 1977; Westfall, 1977).

So far, most studies on the inhibition of noradrenaline release by activation of presynaptic opiate-receptors were carried out under conditions in which the regulatory mechanism mediated by presynaptic α-adrenoceptors was operative (Henderson *et al.*, 1972a, b,; Henderson & Hughes, 1974; 1976; Montel, Starke & Weber, 1974a, b; Montel, Starke & Taube, 1975a, b) while in a few studies a very high concentration (30 μM) of the α-adrenoceptor blocking agent, phentolamine, was employed (Henderson *et al.*, 1975; Hughes *et al.*, 1975a). In the present study the presynaptic effects of morphine and the morphine-like peptides on the stimulation-evoked release of [^{3}H]-noradrenaline were investigated in the cat nictitating membrane under conditions in which the presynaptic α-adrenoceptors were blocked by either phentolamine or phenoxybenzamine.

In the cat nictitating membrane, phentolamine, 1 μM, increased by 3 fold the overflow of [^{3}H]-noradrenaline induced by nerve stimulation by blocking the presynaptic α-adrenoceptors and at the same time reduced the end-organ responses to nerve stimulation by 50% as a result of the blockade of postsynaptic α-receptors. In the presence of phentolamine, morphine, [Met^5]-enkephalin and [Leu^5]-enkephalin produced a concentration-dependent inhibition of [^{3}H]-noradrenaline release and of responses elicited by nerve stimulation. Since these effects were completely antagonized by naloxone, it follows that morphine and the naturally occurring pentapeptides decrease [^{3}H]-noradrenaline overflow by acting on presynaptic inhibitory opiate receptors, which are different from presynaptic α-adrenoceptors.

The inhibition of the stimulation-evoked release of [^{3}H]-noradrenaline elicited by 1 μM morphine in the presence of phentolamine (S_2/S_1: 0.36 ± 0.04, $n = 4$) was practically the same as that obtained under identical experimental conditions in the absence of the α-adrenoceptor antagonist (S_2/S_1: 0.45 ± 0.09, $n = 9$) (Luchelli-Fortis & Langer, unpublished). It therefore appears that blockade of the presynaptic α-adrenoceptors by phentolamine does not modify the effectiveness of morphine in reducing the release of the neurotransmitter.

Under conditions in which the α-presynaptic regulatory mechanism is operative, morphine reduces the release of [^{3}H]-noradrenaline elicited by nerve stimulation from the cat nictitating membrane (Henderson *et al.*, 1972b; 1975; Luchelli-Fortis & Langer, unpublished), and from the mouse vas deferens (Hughes *et al.*, 1975a; Henderson & Hughes, 1974; 1976). A presynaptic effect of the naturally occurring pentapeptides was also demonstrated in the rat vas deferens, mouse vas deferens and guinea-pig ileum and its myenteric plexus by measuring end organ responses to nerve stimulation (Hughes *et al.*, 1975b; Lord, Waterfield, Hughes & Kosterlitz, 1977; Lemaire *et al.*, 1978; Waterfield, Lord, Hughes & Kosterlitz, 1978). It is of interest to note that a presynaptic site of action of morphine has been demonstrated by intracellular recording from the smooth muscle of the mouse vas deferens during nerve stimulation (North & Henderson, 1975; Henderson & North, 1976).

In addition to the effects observed for opiate agonists in peripheral tissues, morphine-like drugs and [Met^5]-enkephalin have been shown to reduce the electrically and potassium-evoked [^{3}H]-noradrenaline overflow from slices of different regions of the rat brain: occipital cortex, hypothalamus and cerebellar cortex (Montel *et al.*, 1974a, b; 1975a, b; Taube, Borowski, Endo & Starke, 1976; Taube, Starke & Borowski, 1977; Arbilla & Langer, 1978). Naloxone blocked the inhibition of ^{3}H-transmitter overflow obtained in the presence of morphine or [Met^5]-enkephalin, but did not antagonize the inhibitory effect of the α-agonist, tramazoline (Taube *et al.*, 1977). Moreover, a high concentration of phentolamine failed to reduce the inhibitory effect of morphine and methionine-enkephalin (Taube *et al.*, 1977). Consequently, opiate agonists appear to modulate the release of noradrenaline induced by nerve depolarization from the rat brain (Taube *et al.*, 1977) and from the cat nictitating membrane (present data) through activation of presynaptic opiate receptors which differ from the presynaptic inhibitory α-adrenoceptors. It is of interest to note that in the locus coeruleus, the activity of noradrenergic neurones can be inhibited by α_2 agonists like clonidine and opiate agonists through the activation of independent receptors (i.e. α_2-adrenoceptors and opiate receptors) (Aghajanian, 1978).

The polypeptide, β-endorphin, was less potent than

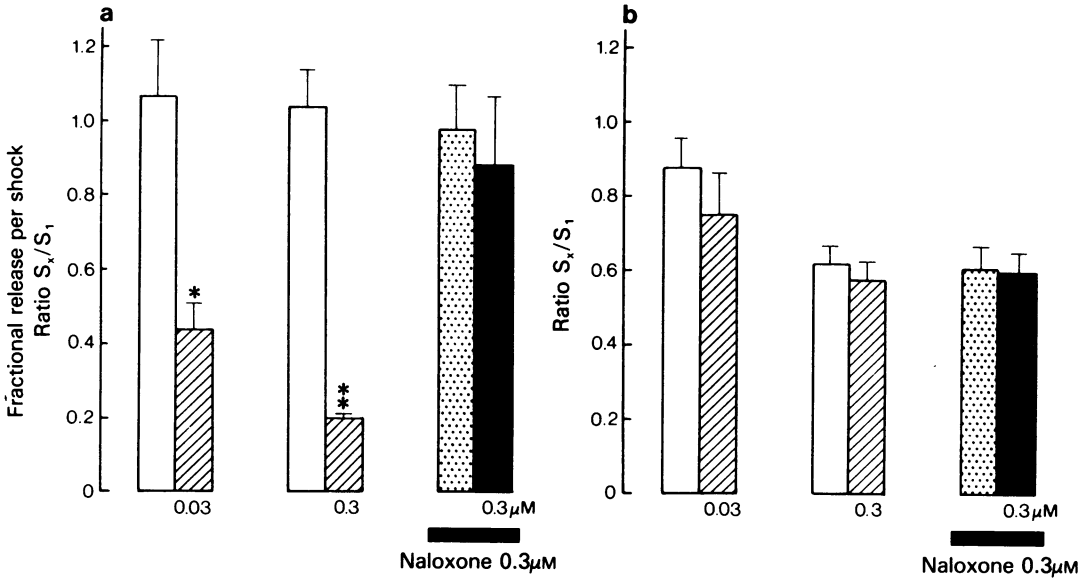

Figure 4 Effects of methionine-enkephalin on ^{3}H-transmitter overflow from the cat nictitating membrane elicited by nerve stimulation in the presence of phenoxybenzamine. Ordinates: fraction of the total tissue radioactivity released per shock expressed as the ratio obtained between each period of nerve stimulation in the presence of the drugs and the first one (S_1), within each individual experiment. The nerves were stimulated at 4 Hz for 2 min (0.5 ms duration, supramaximal voltage), every 20 min. Phenoxybenzamine 1 μM (a) or 10 μM (b) was present in the medium from 30 min before S_1, until the end of the experiment. Methionine-enkephalin in the concentration indicated was present in the incubation medium from 10 min before the corresponding stimulation period. Naloxone, 0.3 μM, was added 10 min before S_4. Drug concentrations are indicated under the abscissae. Open columns = control; hatched columns = methionine enkephalin; stippled columns = naloxone; solid columns = methionine-enkephalin plus naloxone. Mean values of 3 to 5 experiments per group are shown; vertical lines indicate s.e. mean. $^*P < 0.01$; $^{**}P < 0.001$ when compared with the corresponding controls.

the natural enkephalins and morphine in reducing the stimulation-evoked transmitter overflow from the cat nictitating membrane under conditions in which the α-receptors were blocked by phentolamine. It was recently shown that β-endorphin is about 5 times less potent than [Met^5]- and [Leu^5]-enkephalin in reducing the twitch responses of vasa deferentia from two strains of mice (Waterfield *et al.*, 1978). On the other

Table 5 ^{3}H-transmitter overflow elicited by nerve stimulation from the cat nictitating membrane in the presence of phenoxybenzamine

		Fractional release per shock $\times 10^{-5}$[a]			
Experimental group	*n*	S_1	S_2	S_3	S_4 *Naloxone* 0.3 μM
Phenoxybenzamine 1 μM	3	8.16 ± 1.71	8.45 ± 1.71	8.73 ± 2.54	8.19 ± 2.42
Phenoxybenzamine 10 μM	4	18.04 ± 2.86	15.60 ± 2.47	10.91 ± 0.99	10.91 ± 1.49

[a] Total radioactivity released by nerve stimulation expressed as fractional release per shock ($\times 10^{-5}$). The nerves were stimulated at 4 Hz for 2 min (0.5 ms. duration, supramaximal voltage) every 20 min (S_1 to S_4). Phenoxybenzamine 1 μM or 10 μM was present in the medium from 30 min before S_1 until the end of the experiment. Naloxone 0.3 μM was added 10 min before S_4 in both groups. Shown are mean values ±s.e. mean. *n* = number of experiments.

hand, β-endorphin is at least 100 times more potent than the enkephalins in inhibiting the electrically-evoked responses of the rat vas deferens (Lemaire *et al.*, 1978), although this tissue was shown to be insensitive to morphine (Hughes *et al.*, 1975a; Lemaire *et al.*, 1978). In slices of the rat occipital cortex, β-endorphin was shown to be more potent than morphine in decreasing the potassium-induced [^{3}H]-noradrenaline overflow (Arbilla & Langer, 1978) through activation of presynaptic opiate receptors. Rapid metabolism or differences between subclasses of opiate receptors when different tissues and species are compared could account for the relative low affinity of β-endorphin for the presynaptic opiate receptors of the cat nictitating membrane. In fact, the existence of multiple receptors for endogenous opioid peptides has been demonstrated (Lord *et al.*, 1977).

[Met^5]-enkephalin reduced ^{3}H-transmitter overflow and responses elicited by nerve stimulation when the presynaptic α-autoreceptors were blocked either by phenoxybenzamine 1 μM, or phentolamine 1 μM. However, when the concentration of phenoxybenzamine was increased to 10 μM, exposure to [Met^5]-enkephalin failed to inhibit ^{3}H-transmitter overflow, indicating that at high concentrations the non-competitive α-adrenoceptor antagonist can block the inhibitory presynaptic opiate receptors. In support of this view, it was reported that phenoxybenzamine displaces stereospecifically bound [^{3}H]-naloxone from mouse brain homogenates in a concentration-related manner (Spiehler, Fairhurst & Randall, 1978). Since the overflow of the labelled neurotransmitter was increased 15 fold in the presence of 10 μM phenoxybenzamine, it is also possible that the failure of [Met^5]-enkephalin to inhibit release under these conditions was related to a higher output of noradrenaline per shock. Attention should be drawn to the fact that the use of high concentrations of phenoxybenzamine is not a selective pharmacological tool for the analysis of the presynaptic effects of several drugs. In this respect, it was found that phenoxybenzamine antagonizes the inhibition of noradrenaline release mediated by presynaptic dopamine receptors in the cat nictitating membrane (Enero & Langer, 1975). Moreover, Walton, Liepmann & Baldessarini (1978) have shown that phenoxybenzamine blocks the dopamine-induced stimulation of adenylate cyclase activity in homogenates of rat striatum.

Following the identification and synthesis of [Met^5]- and [Leu^5]-enkephalin and the demonstration of their opiate agonist activity in a variety of test situations, it became apparent that the intensity and duration of their actions was limited by rapid inactivation in brain tissue. It has been shown that substitution of D-alanine (D-Ala) for glycine (Gly) on the enkephalin peptide increases the opiate-like activity, both when tested under *in vivo* and *in vitro* experimental conditions (Pert, Bonie, Fong & Chang, 1976; Roemer, Buescher, Hill, Pless, Bauer, Cardinaux, Closse, Hauser & Huguenin, 1977; Lord *et al.*, 1977). The [D-Ala^2] analogues of [Met^5]-enkephalin produce long lasting analgesia in the rat when administered intra-cerebrally (Pert, 1976), and are about 5 to 7 times more potent than [Met^5]-enkephalin in inhibiting the twitch responses in the vasa deferentia of two strains of mice (Waterfield *et al.*, 1978).

In the cat nictitating membrane, the synthetic pentapeptides, BW 180 C and BW 834 C, were more potent thatn [Met^5]-enkephalin in decreasing both ^{3}H-transmitter overflow and responses to nerve stimulation. Replacement of [Gly^2] by [D-Ala^2] and [Leu^5] by [D-Leu^5] in the molecule of [Leu^5]-enkephalin, resulted in the peptide BW 180 C which was about 5 times more potent than the natural peptide for inhibiting the stimulation-evoked release of [^{3}H]-noradrenaline (Table 3).

The difference in potencies for the activation of presynaptic opiate receptors observed for the enkephalins and the synthetic peptides would indicate that the substitution of the natural aminoacids by the corresponding D-isomers stabilizes the peptide molecule. This effect appears to be the result of increased resistance to enzymatic degradation (Pert *et al.*, 1976; Miller, Chang, Cuatrecasas & Wilkinson, 1977; Hill & Pepper, 1978; Lee, Sewell & Spencer, 1978; Lemaire *et al.*, 1978).

When the phenylalanine (Phe) of BW 180 C was substituted by *p*-chlorophenylalanine (pCl Phe^4) as in the compound BW 834 C, an approximately 24 fold increase in potency on the presynaptic opiate receptor of the cat nictitating membrane was obtained. On the other hand, the substitution of phenylalanine by tyrosine in the enkephalin molecule leads to a decrease in opiate activity (Morgan *et al.*, 1976).

When tyrosine (Tyr^1) was replaced in the molecule of BW 180 C by [D-Tyr^1] and [D-Leu^5] by [Leu^5], the compound BW 227 C was obtained, which was less potent than morphine in decreasing ^{3}H-transmitter release and the responses to nerve stimulation in the cat nictitating membrane (Table 3). It has been shown that the presence of tyrosine is essential in the molecule of the pentapeptides for maintaining their opiate-like activity (Morgan *et al.*, 1976). It is possible that the low activity found for the compound BW 227 C is due to the fact that it has the D-isomer of tyrosine in the molecule.

The synthetic tetrapeptide, BW 832 C, was less potent than BW 180 C but more potent than morphine on the presynaptic inhibitory opiate receptors of the cat nictitating membrane (Table 3). The difference in potency between BW 180 C and BW 832 C is probably due to the fact that the latter lacks the terminal leucine.

In the central nervous system the highest concen-

tration of enkephalins is present in the rat caudate nucleus and in the striatum (Yang, Hong, Frata & Costa, 1978), although in this tissue, opiate receptor agonists do not inhibit the stimulation-evoked release of dopamine (Arbilla & Langer, 1978). On the other hand, in the rat cerebral cortex, where the concentration of endogenous enkephalins is among the lowest levels (Yang *et al.*, 1978), there is convincing evidence for the inhibition of the stimulation-evoked release of noradrenaline through presynaptic inhibitory opiate receptors (Taube *et al.*, 1977; Arbilla & Langer, 1978).

In the peripheral tissues in which the presence of presynaptic inhibitory opiate receptors has been clearly established (mouse vas deferens, cat nictitating membrane), there is as yet no evidence for the presence of endogenous enkephalins. Yet, attention should be drawn to the fact that opiate-like peptides were reported to be present in human plasma (Pert, Pert & Tallman, 1976). The possible physiological role of the presynaptic opiate receptors at the level of modulation of the release of noradrenaline in these peripheral tissues is still an open question. The recent finding that in the adrenal medulla an opiate-like material is stored and secreted concomitantly with catecholamines (Viveros, Diliberto, Hazum & Chang, 1979) tempts one to speculate on the possibility that a similar mechanism may also exist in some noradrenergic neurones.

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