Br. J. Pharmac. (1978), **64**, 223–227.

ADRENOCORTICOTROPHIC HORMONE FRAGMENTS MIMIC THE EFFECT OF MORPHINE *in vitro*

GERDA J.J. PLOMP & JAN M. VAN REE*

Centre for Human Toxicology, University of Utrecht, Vondellaan 14, Utrecht, The Netherlands & *Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, Utrecht, The Netherlands

1 Fragments of the N terminal part of adrenocorticotrophic hormone (ACTH) inhibited the electrically evoked contractions of the mouse vas deferens. This inhibition could be antagonized by naloxone.

2 The same fragments displaced radiolabelled morphine from morphine antiserum.

3 Structure-activity relationship studies showed that in both assay systems the active core is located within the sequence ACTH 7-10.

4 It is postulated that the Trp^9 residue and the peptide bond between Trp^9 and Gly^{10} are particularly important for interaction of ACTH fragments with morphine receptors.

Introduction

Fragments from the N-terminal part of adrenocorticotrophic hormone (ACTH) show affinity for specific opiate binding sites in rat brain (Terenius, 1975; Terenius, Gispen & De Wied, 1975), counteract morphine-induced analgesia (Gispen, Buitelaar, Wiegant, Terenius & De Wied, 1976a) and antagonize the morphine-induced reduction of spinal reflex activity (Zimmerman & Krivoy, 1973). These fragments also elicit excessive grooming activity in the rat after intracerebroventricular application (Gispen, Wiegant, Greven & De Wied, 1975). This activity of the N-terminal ACTH fragments may be mediated by opiate receptors since it is blocked by opiate antagonists. Moreover morphine and the C-fragment of β-LPH produced similar behavioural effects to ACTH-fragments (Gispen & Wiegant, 1976; Gispen, Wiegant, Bradbury, Hulme, Smyth, Snell & De Wied, 1976b).

The aim of the present study was to investigate the interaction between ACTH-like peptides and the opiate receptors in the mouse vas deferens preparation. In addition, the ability of these peptides to displace radiolabelled morphine from morphine antiserum was studied.

Methods

Mouse vas deferens

Male albino Swiss mice (Cpb, The Netherlands), weighing 25 to 30 g were killed by decapitation and their vasa deferentia were dissected out. A single vas was set up in an organ bath (5 ml) containing modified Krebs solution (Hughes, Kosterlitz & Leslie, 1975) at 37° C and gassed with 95% O_2 and 5% CO_2. Longitudinal contractions were monitored by attaching a thread from the upper end of the preparation to a Grass isometric transducer, which was coupled to a Grass polygraph. Tension (100 mg) was applied to the muscle preparation. The intramural nerves were stimulated continuously with rectilinear pulses (2 ms duration, 0.1 Hz, supramaximal voltage) passing between a platinum point source at the bottom and a platinum ring set in the top of the bath. Two or three doses of morphine hydrochloride were tested before and after peptides were added to the bath. The bath fluid was renewed several times once the contractions had reached a constant size after the addition of a compound. The volume added to the bath never exceeded 50 µl. The highest concentration of peptides tested was 2×10^{-4} M. To quantitate the effect of the peptides on a preparation, the effectiveness of morphine on that preparation was used as standard.

Morphine antibodies

Commercially available morphine antibodies (Abuscreen, Hoffmann-La Roche) were used. The test pro-

cedure was similar to that used for measuring morphine levels in urine. Briefly, 100 µl 0.001 N HCl containing morphine or peptide, 200 µl of the ^{125}I-morphine reagent and 200 µl of the morphine antiserum were mixed and incubated for 2 h. Saturated ammonium sulphate (500 µl) was added and the mixture allowed to stand for 10 min. The mixture was then centrifuged at 3000 *g* and a sample of the supernatant (500 µl) was used for counting in a gamma scintillation counter. Several concentrations of the peptides, up to 10^{-4} M, or morphine were tested in duplicate.

Drugs

The following drugs were used: morphine hydrochloride (OPG, Utrecht, The Netherlands) and naloxone hydrochloride (Endo, Brussels, Belgium). All chemicals were of analytical grade or the best commercially available. Highly purified synthetic peptides were obtained from Organon, B.V., Oss, The Netherlands. The peptides were stored in bottles as the dry form at room temperature.

Results

Mouse vas deferens

A number of ACTH-like peptides depressed the electrically-induced contractions of the mouse vas deferens (Table 1). The most active peptide appeared to be ACTH 1–24. The activity seemed to be located in the N-terminal part of this molecule, since the sequence (1–10) exhibited similar activity and the sequence (11–24) was inactive. Shortening of the sequence (1–10) by removal of N-terminal amino acids up to the sequence (8–10) revealed peptides with gradually decreasing but still definite activity. In contrast, the peptides ACTH 4–7 and 4–9 appeared to be inactive. Amidation of the C-terminal Trp^9 of the sequence (4–9) yielded an active peptide. Acetylation of the N-terminal Ser residue of the sequence (1–10), substitution of the D-isomer of Phe^7 in the sequence (4–10) or C-terminal elongation of the sequence (7–10) to (7–16) did not affect the activity.

The effect of ACTH 4–10 was dose-dependent. The dose-response curve was parallel to that found for morphine. To study the effect of naloxone on the inhibitory action of ACTH 4–10, graded doses of naloxone (1 to 100 nM) were added to the bath in the presence of ACTH 4–10 (2×10^{-4} M). Naloxone was able to abolish completely the peptide-induced inhibition. The concentration of naloxone needed to reduce the effect of the peptide by 50% was 31 ± 8 nM ($n = 6$). Under similar conditions the morphine*(0.4 × 10^{-6} M)-induced depression was reduced to 50% by 11 ± 2 nM naloxone ($n = 7$). This amount of naloxone is sig-

Table 1 Relative inhibitory potencies of adrenocorticotrophic hormone (ACTH) fragments on the contractions of mouse vas deferens

Compounds	*No. of observations*	*Potency**
Morphine HCl		100
ACTH 1–24**	6	0.78 ± 0.09
ACTH 1–10	5	0.30 ± 0.03
ACTH 1–10 (Ac-Ser^1)	4	0.30 ± 0.04
ACTH 4–10	8	0.20 ± 0.01
ACTH 4–10 (D-Phe^7)	4	0.20 ± 0.01
ACTH 4–9 –NH_2	5	0.16 ± 0.01
ACTH 5–10	7	0.15 ± 0.02
ACTH 7–16–NH_2	6	0.14 ± 0,01
ACTH 7–10	7	0.13 ± 0.02
ACTH 8–10	6	0.06 ± 0.01
ACTH 11–24	4	—†
ACTH 4–9	6	—†
ACTH 4–7	5	—†

* The relative potencies were determined on a molar base with morphine HCl (mean ED_{50} = 470 nM) as the internal standard.

** The amino acid sequence of $ACTH_{1-24}$ is

H– Ser– Tyr– Ser– Met– Glu– His– Phe– Arg– Trp– Gly– Lys– Pro– Val– Gly– Lys– Lys–
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Arg– Arg– Pro– Val– Lys– Val– Tyr– Pro– OH
17 18 19 20 21 22 23 24

† No activity observed with the highest dose tested (2×10^{-4} M).

nificantly lower ($P < 0.05$, Student's t test), than that found in the case of ACTH 4–10. The dose-response curve of naloxone to counteract the peptide effect was parallel to that found for morphine. To investigate the interaction of ACTH 4–10 with morphine, experiments were performed in which morphine was added to the medium in the presence of ACTH 4–10 (10^{-4} M), the inhibitory effect of which was comparable to that of 194 ± 9 nM morphine ($n = 9$). Addition of 100 nM morphine ($n = 5$) led to further depression of the contractions to a level which could also be obtained with 288 ± 15 nM morphine alone. Addition of 400 nM morphine ($n = 4$) in the presence of ACTH 4–10 (10^{-4} M) caused a total inhibition of the contraction equal to that observed after 610 ± 34 nM morphine alone. Thus, morphine exhibited normal activity in the presence of ACTH 4–10 and neither antagonized nor potentiated the peptide effect.

Antibody displacement

Like morphine, ACTH 1–24 displaced radiolabelled morphine from its antibodies (Figure 1). Increasing amounts of this peptide yielded a displacement curve comparable to that obtained with graded doses of morphine. ACTH 4–10 while somewhat less potent was also active in this system. However, ACTH 7–10 had approximately the same activity on a molar basis as did the sequence (1–24). Other ACTH-like peptides tested up to a dose of 10^{-4} M were inactive e.g. had a lower displacement activity than 0.3×10^{-8} M morphine. α-MSH and the sequences (1–13), (1–10), (4–10 (D-Phe^7)), (4–7), (5–10), (7–16–NH_2) and (11–24) were inactive; the sequence (4–9) showed borderline activity.

Discussion

The present data suggest that ACTH fragments mimic the effect of morphine in the mouse vas deferens preparation and show affinity for morphine antibodies. In both test systems the potency of these peptides appears rather weak as compared to morphine. As both these antibodies and tissue receptors for morphine recognize the drug in a specific manner, there must be some similarities in their conformations. With respect to both the vas deferens and the radioimmunoassay, the active core of the peptides is located within the sequence (4–10) or, more precisely, within the sequence (7–10). This is consistent with other interactions between ACTH-like peptides and opiates. Morphine analgesia is counteracted by ACTH fragments administered subcutaneously. In this case the active core is located in the sequence (5–10), with the most active peptide being $ACTH_{5-14}$ while $ACTH_{11-24}$ and $ACTH_{11-16}$ were inactive (Gispen *et al.*, 1976a). ACTH fragments also have an affinity for stereospecific opiate binding sites in rat brain synaptosomal plasma membrane fractions (Terenius *et al.*, 1975). The active part of the ACTH molecule responsible for displacing dihydromorphine from its binding sites appeared to be located within the sequence (7–10) as (4–10), (5–14) and (7–16) were active, while the sequences (11–24) and (11–16) were inactive (Terenius *et al.*, 1975; Gispen *et al.*, 1976a; Wiegant, Gispen, Terenius & De Wied, 1977). These data suggest that the part of the ACTH molecule which interferes with opiate receptors is located in the sequence (7–10) and presumably in the sequence (8–10). Substitution of Phe^7 by its D-isomer did not substantially affect the activity of ACTH fragments, except in the assay in which morphine antibodies were used. Furthermore, $ACTH_{8-10}$ appeared to be active in the mouse vas deferens preparation. Removal of the C-terminal amino acid (Gly^{10}) from the

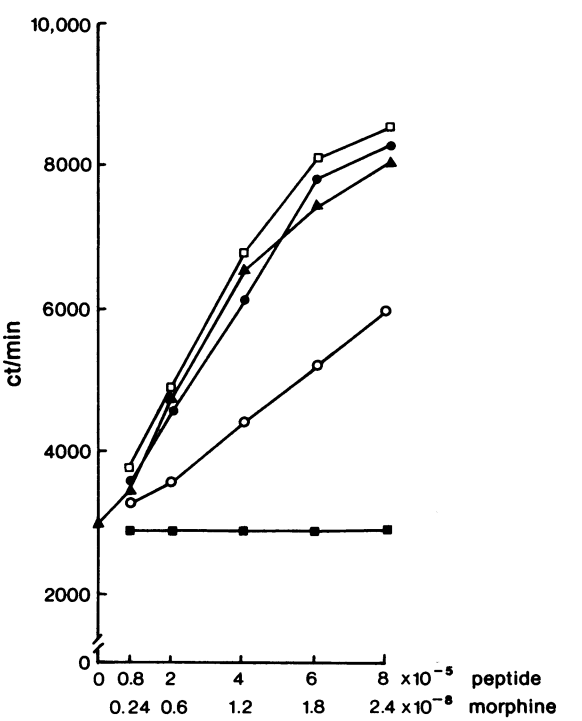

Figure 1 Displacement curves obtained with morphine or adrenocorticotrophic hormone (ACTH)-like peptides by means of a radioimmunoassay for morphine. The concentration of morphine or peptide in the incubation medium was plotted versus the amount (in counts per minute, ct/min) of radioactive morphine displaced from the antibodies. (▲) Morphine; (□) $ACTH_{1-24}$; (○) $ACTH_{4-10}$; (■) $ACTH_{4-10\,(D\text{-}Phe^7)}$; (●) $ACTH_{7-10}$.

sequence (4–10) resulted in a marked loss of activity on the mouse vas deferens preparation, with respect to affinity to both brain opiate binding sites (Terenius *et al.*, 1975) and morphine antibodies. Amidation of the Trp^9 residue of the (4–9) fragment resulted in a more active peptide (Table 1). It might thus be postulated that the tryptophan residue together with the peptide bond between Trp^9 and Gly^{10} are responsible for the interaction of ACTH fragments with the morphine receptor.

The data obtained with morphine antibodies suggest that $ACTH_{7-10}$ contains groups that are recognized by the antibodies and thus probably has structural similarities with morphine. The determinant portion of the morphine molecule for recognition by the antibodies seems to be at least the N-methyl group together with the hydroxyl group on C6, since modifications in these parts of the molecule led to a substantial loss of affinity (see Hoffman-La Roche report supplied with the Abuscreen kit). In contrast, modification of the phenolic hydroxyl group at C3 does not materially affect affinity. The parts of $ACTH_{7-10}$, which interact with the morphine antibodies, could be the N atom of the Trp^9 residue and the O atom of the peptide bond between Trp^9 and Gly^{10}, which are, as in the morphine molecule, separated by 5 C atoms. However, the exact steric configurations of the morphine and $ACTH_{7-10}$ molecules, whether *in vitro* or *in vivo*, is still unknown. It must also be kept in mind that a population of antibodies is used in the radioimmunoassay rather than a single antibody.

Although there are similarities in the structure-activity relationships in the two assay systems, there are also differences, e.g. acetylation of the N-terminal Ser residue led to a substantial loss of affinity of the ACTH-like peptides to brain opiate binding sites. (Terenius *et al.*, 1975) but (Ac-Ser^1) $ACTH_{1-10}$ and $ACTH_{1-10}$ were equipotent in the mouse vas deferens preparation. The differences may not only be accounted for by the morphine antibodies, but also by multiple morphine receptors (Martin *et al.*, 1976; Lord *et al.*, 1977) or conformational changes in the peptide structure due to the biological environment of the assay system (e.g. *in vivo* versus *in vitro*) (Greven & de Wied, 1977).

Multiple opioid receptors may exist (Martin, Eades, Thompson, Huppler & Gilbert, 1976; Lord, Waterfield, Hughes & Kosterlitz, 1977) and ACTH fragments may interfere with one population of these receptors, namely, those present in the mouse vas deferens. These δ receptors, seem to be almost absent from the guinea-pig ileum which may contain more μ-receptors (Lord *et al.*, 1977). Indeed ACTH fragments did not alter the electrically induced contractions of guinea-pig ileum *in vitro* (data not shown). In view of the model proposed for opiate receptors by Feinberg, Creese & Snijder (1976), it is tempting to speculate that δ and μ receptors differ with respect to lipophilic binding sites and that ACTH fragments bind to these sites of the δ receptor and to the amino binding site of the opioid receptor.

ACTH fragments exhibit agonist activity in the mouse vas deferens preparation. However, in the assay determining affinity to brain opiate binding sites, $ACTH_{1-24}$ appears to have mixed agonist-antagonist properties as does nalorphine (Terenius, 1976). This might explain the counteraction of morphine effects (analgesia, spinal reflex reduction) by the peptide (Zimmermann & Krivoy, 1973; Gispen *et al.*, 1976a). Furthermore, the degree of affinity of ACTH fragments for morphine antiserum is approximately the same as that observed for nalorphine (Hoffmann-La Roche report Abuscreen kit). The data thus far suggest that, in the CNS, the opiate antagonist action of ACTH-like peptides might be more pronounced than their agonist activity, especially when it is assumed that these peptides interfere with one particular class of opioid receptors.

C-terminal fragments of β-LPH, starting with the Tyr^{61} residue, have a highly potent morphine-like action (Snyder & Simantov, 1977). Met-enkephalin (sequence 61–65 of β-LPH) appeared to be 49 ± 2 ($n = 8$) times more potent than morphine in the mouse vas deferens preparation. Approximately similar activities were found for α- and β-endorphin (61–76 and 61–91 of β-LPH). On the basis of structural similarities between this part of the endorphins and morphine (an aromatic ring bearing a *p*-hydroxyl group, separated from an N atom by two C atoms) it has been argued that the Tyr^{61} residue is important for interaction with the opiate receptor (Roques, Carbay-Jaureguibeery, Oberlin, Anteunis & Lala, 1976). Interestingly, in most batches of morphine antibodies, met- and leu-enkephalin and α-endorphin were unable to displace morphine. This fits rather well with the characteristics of the antibodies since the phenolic hydroxyl group of morphine only contributes to a minor degree to the affinity of the drug for its antibodies (see above). β-Endorphin showed some affinity to the antibodies, although this peptide was, on a molar basis, about 6 times less potent than $ACTH_{1-24}$.

ACTH fragments delay the extinction of active avoidance behaviour (De Wied, 1974) and after intracerebroventricular application induce excessive grooming (Gispen *et al.*, 1975). Structure-activity relationship studies have shown that in both behavioural paradigms, the active core is located within the sequence (4–10), and presumably in the sequence (4–7) (De Wied *et al.*, 1975; Wiegant & Gispen, 1977). The effect of ACTH fragments on avoidance behaviour is not related to their interaction with opiate receptors, since naltrexone was unable to block the effects

of the peptides (De Wied, Bohus, Van Ree & Urban, 1978). Interestingly, excessive grooming induced by ACTH fragments can be blocked by pretreatment with opiate antagonists (Gispen & Wiegant, 1976). This leads to the conclusion either that (1) opiate antagonists and ACTH fragments exert their effect on grooming at different levels in the brain or (2) that different types of opiate receptors are involved in the excessive grooming response elicited by ACTH fragments and in the effect of ACTH on mouse vas deferens and on brain opiate binding sites.

We thank Willy Spaapen-Kok and Ankie Renkema-Wind for their assistance with the experiments, and Organon International B.V. Oss, The Netherlands, in particular Dr H.M. Greven, for supplying the various peptides. β-Endorphin was supplied by Dr D.G. Smyth, National Institute for Medical Research, Mill Hill, London, England. Naloxone was generously supplied by Endo, Brussels, Belgium.

References

DE WIED, D. (1974). Pituitary-adrenal system hormones and behaviour. In *The Neurosciences Third Study Program*, ed. Schmitt, F.O. & Worden, F.G. pp. 653–666. Cambridge: Mit Press.

DE WIED, D., WITTER, A. & GREVEN, H.M. (1975). Behaviourally active ACTH analogues. *Biochem. Pharmac.*, **24**, 1463–1468.

DE WIED, D., BOHUS, B., VAN REE, J.M. & URBAN, I. (1978). Behavioral and electrophysiological effects of peptides related to lipotropin (β-LPH). *J. Pharmac. exp. Ther.* (in press).

FEINBERG, A.P., CREESE, I. & SNIJDER, S.H. (1976). The Opiate receptor: A model explaining structure-activity relationships of opiate agonists and antagonists. *Proc. natn. Acad. Sci., U.S.A.*, **73**, 4215–4219.

GISPEN, W.H., BUITELAAR, J., WIEGANT, V.M., TERENIUS, L. & DE WIED, D. (1976a). Interaction between ACTH fragments, brain opiate receptors and morphine-induced analgesia. *Eur. J. Pharmac.*, **39**, 393–397.

GISPEN, W.H. & WIEGANT, V.M. (1976). Opiate Antagonists suppress $ACTH_{1-24}$ induced grooming in the rat. *Neurosci. Letters*, **2**, 159–164.

GISPEN, W.H., WIEGANT, V.M., BRADBURY, A.F., HULME, E.C., SMYTH, D.G., SNELL, C.R. & DE WIED, D. (1976b) Induction of excessive grooming in the rat by fragments of lipotropin. *Nature*, **264**, 794–795.

GISPEN, W.H., WIEGANT, V.M., GREVEN, H.M. & DE WIED, D. (1975). The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: structure-activity studies. *Life Sci.*, **17**, 645–652.

GREVEN, H.M. & DE WIED, D. (1977). Influence of peptides structurally related to ACTH and MSH on active avoidance behavior in rats. In *Frontiers of Hormone Research*. ed. Van Wimersma Greidanus, Tj.B. Vol. 4, pp. 140–152. Basel: Karger.

HUGHES, J., KOSTERLITZ, H.W. & LESLIE, F.M. (1975). Effect of morphine on adrenergic transmission in the mouse van deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmac.*, **53**, 371–381.

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

MARTIN, W.R., EADES, C.G., THOMPSON, J.A., HUPPLER, R.E. & GILBERT, P.E. (1976). The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmac. exp. Ther.*, **197**, 517–532.

ROQUES, B.P., CARBAY-JAUREGUIBEERY, C., OBERLIN, R., ANTEUNIS, M. & LALA, A.K. (1976). Conformation of Met^5-enkephalin determined by high field PMR Spectroscopy. *Nature*, **262**, 778–779.

SNYDER, S.H. & SIMATOV, R. (1977). The opiate receptor and opioid peptides. *J. Neurochem.*, **28**, 13–20.

TERENIUS, L. (1975). Effect of peptides and amino acids on dihydromorphine binding to the opiate receptor. *J. Pharm. Pharmac.*, **27**, 450–452.

TERENIUS, L. (1976). Somatostatin and ACTH are peptides with partial antagonist-like selectivity for opiate receptors. *Eur. J. Pharmac.*, **38**, 211–213.

TERENIUS, L., GISPEN, W.H. & DE WIED, D. (1975). ACTH-like peptides and opiate receptors in the rat brain: structure-activity studies. *Eur. J. Pharmac.*, **33**, 395–399.

WIEGANT, V.M. & GISPEN, W.H. (1977). ACTH-induced excessive grooming in the rat: latent activity of $ACTH_{4-10}$. *Behav. Biol.*, **19**, 554–558.

WIEGANT, V.M., GISPEN, W.H., TERENIUS, L. & DE WIED, D. (1977). ACTH-like peptides and morphine: interaction at the level of the CNS. *Psychoneuroendocrinology*, **2**, 63–69.

ZIMMERMANN, E. & KRIVOY, W. (1973). Antagonists between morphine and polypeptides ACTH, $ACTH_{1-24}$ and β-MSH in the nervous system. In *Drug Effects on Neuroendocrine Regulation*. ed. Zimmermann, E., Gispen W.H., Marks, B.H. & De Wied, D. pp. 383–392. Progress in Brain Research Vol. 39. Amsterdam: Elsevier.

(Received January 23, 1978. Revised April 6, 1978)