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Pharmacological characterization of the inhibitory activity of βh-endorphin (βh-EP),[Arg^{9,19,24,28,29}]-βh-EP,[Gln^{8},Gly^{31}]-βh-EP-Gly-Gly-NH_2, in the neuroeffector junction of the mouse vas deferens

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Abstract—The inhibitory opioid activities of βh-endorphin (βh-EP), its structurally related peptide analogues [Gln^{8}, Gly^{31}]-βh-EP-Gly-Gly-NH_2 (Gly-Gly-βh-EP), [Arg^{9,19,24,28,29}]-βh-EP (Arg-βh-EP) and methionine enkephalin have been examined in the electrically stimulated mouse vas deferens bioassay. All four peptides behaved as full agonists; methionine enkephalin was the most potent followed by Arg-βh-EP, βh-EP and Gly-Gly-βh-EP. Neither Gly-Gly-βh-EP nor Arg-βh-EP antagonized the inhibitory action of βh-EP or methionine enkephalin. An hour of tissue exposure to 30 nM β-funaltrexamine followed by thorough washing, displaced to the right, in a parallel fashion, the concentration-response curves of βh-EP and analogues. Whereas the displacement of the concentration response curves was 8 to 10-fold for βh-EP and Arg-βh-EP, it was only about 3-fold for Gly-Gly-βh-EP and methionine enkephalin. Naltrindole was the most potent antagonist of methionine enkephalin with an apparent pA_2 of 9·4; its potency as an antagonist of βh-EP and related analogues was approximately one-tenth of this with pA_2 values approximately 8·5. Norbinaltorphimine also antagonized the action of the opioid peptides with pA_2 values close to 7·8.

Major advances in our understanding of the pharmacology of the opioids have been based on differentiation of multiple subtypes of opioid receptor sites. Following the first demonstration of opioid receptors by radioligand binding (Pert & Snyder 1973), it was presumed that only one type of opiate receptor existed in the brain. The use of various drugs of markedly different chemical structures demonstrated that more than one opioid receptor type might co-exist in brain and periphery (Martin et al 1976; Lord et al 1977). Efforts to synthesize opioid agonists and antagonists with selectivity for the different opioid receptor subclasses have been successful (Morley 1980; Portoghese 1989). It is now generally accepted based on these findings that at least three distinct types of opioid receptors are present in mammalian tissues: μ, δ, κ. Opioid antagonists with marked selectivity for the opioid receptor subtypes are now available, surpassing the limited selectivity of naloxone (Huidobro-Toro & Way 1985). Considering that the mode of action of naloxone to displace the opioid peptides from the opioid receptor might be different from that of the alkaloids and naturally occurring peptides, much effort has been devoted to the synthesis of peptides with antagonist activity. Nicolas et al (1984) reported that two βh-endorphin (βh-EP) related peptides [Gln^{8}, Gly^{31}]-βh-EP-Gly-Gly-NH_2 (Gly-Gly-βh-EP) and [Arg^{9,19,24,28,29}]-βh-EP (Arg-βh-EP) are weak analgesics but at concentrations one-tenth those required to cause analgesia, antagonized the βh-EP-induced antinociception.

An explanation compatible with these observations argued in favour of the interpretation that these βh-EP-derived peptides could act as partial agonists. This hypothesis encouraged us to further characterize their potential as opioid antagonists in peripheral neurons having different opioid receptor subtypes. Valenzuela et al (1989) demonstrated that these opiate peptides lack antagonist activity in the rat vas deferens, a tissue rich in opioid receptors for βh-EP. Since the analgesia caused by βh-EP might involve interactions with the δ-opioid receptor subtype, the aim of this investigation was to characterize the pharmacology of βh-EP in the mouse vas deferens neurotransmission, and to ascertain whether these βh-EP analogues might exert antagonism by acting on δ-opiate receptors in this tissue. With this hypothesis, we studied the inhibitory action of these peptides in the mouse vas deferens bioassay, a preparation classically accepted as containing mainly the δ-opioid receptor subtype (Lord et al 1977). In addition, we have explored interactions of selective opioid antagonists such as β-funaltrexamine (β-FNA), naltrindole and norbinaltorphimine with these βh-EP analogues.

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Materials and methods

Bioassay and quantification of opioid potency. Swiss-Webster mice were killed by cervical dislocation. The vasa deferentia were removed and mounted in bath chambers maintained at 37°C with Krebs-Ringer solution gassed with 95% O_2–5% CO_2 (Huidobro et al 1980). Four tissues were bioassayed concurrently; transmural field stimulation used rectangular pulses of 1 ms duration, supramaximal voltage (70 V) and 0·15 Hz. Isometric contractions were recorded on a Grass oscillograph. Tissues were set under 0·7 g of basal tension without electrical stimulation for 1 h before the performance of non-cumulative concentration-response experiments. For details of the experimental conditions and quantitative analysis see Valenzuela et al (1989). Median inhibitory concentrations (IC50) were obtained by interpolation using linear regression analysis. Results are expressed as mean IC50 ± s.e.; IC50 ratios were determined for each bioassay, dividing the IC50 obtained after drug treatment by the IC50 before drug antagonism.

Agonist-antagonist properties. These experiments were performed as described by Valenzuela et al (1989). A test concentration of 30 nM βh-EP was assayed either alone or in the presence of 0·09–29 nM Gly-Gly-βh-EP or in the presence of 0·009–3 nM Arg-βh-EP. Likewise, tissues were challenged with 1·7 nM methionine enkephalin alone or in the presence of 0·09–29 nM Gly-Gly-βh-EP or 0·009–3 nM Arg-βh-EP.

Studies with antagonists. (i) β-Funaltrexamine (β-FNA). Tissues were preincubated with 10, 30 or 100 nM of this non-equilibrium μ-selective opioid antagonist for 60 min following the performance of a concentration-response experiment (Takemori et al 1981; Huidobro-Toro et al 1982). The tissues were then rinsed extensively with drug-free Krebs-Ringer buffer for 20 min and concentration-response determinations were performed with either βh-EP or methionine enkephalin. In the case of Gly-Gly-βh-EP and Arg-βh-EP, concentration-response curves were performed before and following incubation with only 30 nM β-FNA.

(ii) Naltrindole. Concentration-response studies were performed before and after the addition of 30 nM naltrindole, a selective δ-opioid antagonist (Portoghese et al 1988). The antagonist was applied 15 min before application of each of the

concentrations of the opioid peptides tested. Apparent pA_2 values using the single concentration procedure, were derived according to the following expression: $pA_2 = -\log(\text{antagonist}) + \log(\text{IC50 ratio} - 1)$; K_e values refer to the antilog of the pA_2 value (nM).

(iii) Norbinaltorphimine (BNI). To test the possible interaction between this selective competitive κ-opioid antagonist (Portoghese et al 1987; Takemori et al 1988) and βh-EP and related structural analogues, concentration-response studies were performed before and 15 min following tissue incubation with 30 nM BNI. Apparent pA_2 values and their corresponding K_e values were calculated as described in the preceding paragraph.

Statistical analysis. Two-tailed Student's *t*-test was used to compare peptide IC50 values before and following treatment with the different antagonists. $P < 0.05$ was accepted as significant.

Animal and peptide sources. Adult Swiss Webster mice, 30–40 g, raised at the Catholic University Animal Reproduction Laboratories were used.

βh-EP, Arg-βh-EP and Gly-Gly-βh-EP were synthesized as described by Zaoral et al (1981) and Yamashiro et al (1982). Methionine enkephalin as the triacetate salt was purchased from Sigma Chemical Co. (St. Louis, MO). β-FNA was provided by Dr W. K. Schmidt, from du Pont & Nemours (Wilmington, DE). Naltrindole and BNI hydrochlorides were purchased from RBI (Natick, MA).

Results and discussion

The application of βh-EP produced concentration-related inhibition of the electrically induced twitching of the mouse vas deferens. Of the two synthetic substituted βh-EP analogues, the more potent was Arg-βh-EP, with an IC50 of 30 ± 4 nM (n = 16) compared with 22 ± 1 nM (n = 32) for βh-EP. Gly-Gly-βh-EP had about one-ninth the potency of βh-EP (IC50, 191 ± 13 nM, n = 12). Methionine enkephalin was 3-fold more potent than βh-EP (IC50, 7 ± 1 nM (n = 42)). All concentration-response curves exhibited parallelism.

No evidence of agonist-antagonist properties was detected for Gly-Gly-βh-EP or Arg-βh-EP. Tissue pretreatment with Gly-Gly-βh-EP in concentrations ranging from 0·09–29 nM did not significantly modify the inhibitory activity induced by the challenge test with methionine enkephalin or βh-EP (data not shown). Likewise, tissue pretreatment with 0·009–3 nM Arg-βh-EP did not significantly modify the inhibitory response elicited by the challenge with methionine enkephalin or βh-EP. In one particular series of these experiments, 30 nM βh-EP caused a 38 ± 2% (n = 8) inhibition of the muscle twitching. In the presence of 0·009, 0·03, 0·09, 0·3, 0·9 or 3 nM Arg-βh-EP the inhibitory effect of βh-EP was 42 ± 3, 37 ± 2, 38 ± 2, 42 ± 4, 45 ± 4, and 41 ± 6%, respectively.

These results allow some interesting comparisons between the in-vivo and in-vitro studies with regard to pharmacological properties of these peptides. The replacement of a methylamine group present in the lysine side chain of βh-EP by a guanidino group in the arginines of Arg-βh-EP, did not impose a significant change in peptide potency for the opioid receptor present in this tissue. Gly-Gly-βh-EP which does not have a negatively charged side chain residue in position 8 and in addition has an extended amidated glycine tail at the carboxy end is about one-ninth as active as βh-EP. Thus the carboxy end of the peptide may make an important contribution to the affinity of βh-EP for the opiate receptor. It is of interest to note that both Arg-βh-EP and Gly-Gly-βh-EP are one-sixth to one-eighth as potent as βh-EP as analgetics, but 13- to 19-fold more potent than βh-EP in displacing the binding of [^{3}H]βh-EP to rat brain membranes (Nicolas et al 1984). While Arg-βh-EP and Gly-Gly-βh-EP exhibited antagonist properties in nociceptive tests with pA_2 values of about 11·5, neither showed antagonist properties in the rat vas deferens bioassay (Valenzuela et al 1989). Based on these results, we conclude that the opiate receptors involved in the analgesic effects of βh-EP and related peptides have different pharmacodynamic properties when compared with those found in the mouse or rat vas deferens neuroeffector junction.

Table 1. Potency of βh-endorphin (βh-EP) and related peptides to inhibit electrically induced muscle twitching; effect of selective μ-, δ- and κ-antagonists.

		IC50 (mean ± s.e.) nM				
	n	Control	after 30 nM β-FNA	Ratio		
Met-enkephalin	(22)	7·4 ± 0·3	21 ± 7	2·8		
Arg-βh-EP	(4)	12·5 ± 2·0	112 ± 17**	9·0		
βh-EP	(19)	21·0 ± 1·0	137 ± 19***	6·5		
Gly-Gly-βh-EP	(4)	157·0 ± 2·0	518 ± 23***	3·3		
		IC50 (mean ± s.e.) nM				
	n	Control	after 30 nM naltrindole	Ratio	pA_2	K_e (nM)
Met-enkephalin	(8)	3·6 ± 0·2	277 ± 19***	77·0	9·40 ± 0·01	0·4
Arg-βh-EP	(7)	39·0 ± 5·2	295 ± 33***	7·6	8·43 ± 0·07	3·7
βh-EP	(7)	22·0 ± 2·0	286 ± 45***	13·0	8·58 ± 0·05	2·6
Gly-Gly-βh-EP	(4)	174·0 ± 33·0	1939 ± 423**	11·0	8·49 ± 0·05	3·2
		IC50 (mean ± s.e.) nM				
	n	Control	after 30 nM BNI	Ratio	pA_2	K_e (nM)
Met-enkephalin	(8)	7 ± 1	11 ± 1*	1·6	(a)	> 30 (b)
Arg-βh-EP	(5)	32 ± 2	99 ± 15**	3·1	7·78 ± 0·06	16·6
βh-EP	(6)	22 ± 1	74 ± 7***	3·4	7·89 ± 0·06	12·8
Gly-Gly-βh-EP	(4)	244 ± 15	658 ± 94**	2·7	7·71 ± 0·10	19·5

(a) pA_2 and (b) K_e values could not be calculated due to a ratio less than 2.
* $P < 0.05$ (two tailed Student's *t*-test); ** $P < 0.01$; *** $P < 0.001$ compared with the IC50 value before drug antagonism. β-Funaltrexamine, β-FNA; norbinaltorphine, BNI.

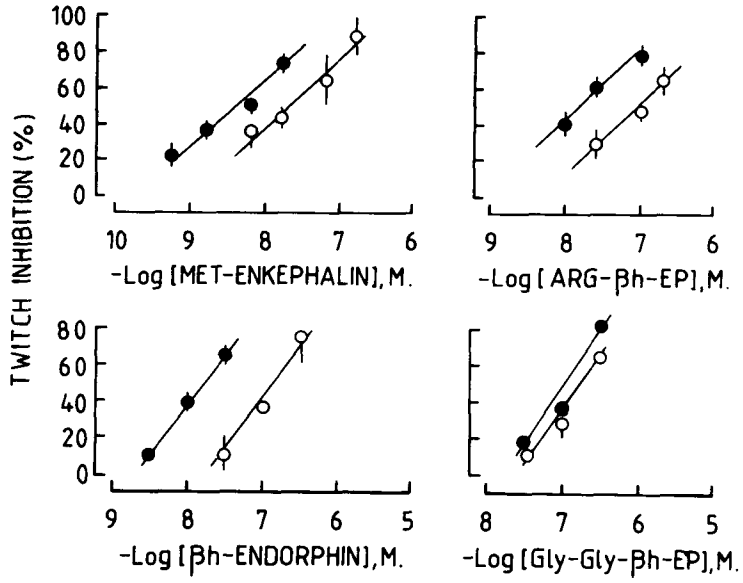

FIG. 1. Blockade of the electrically-induced muscle twitching by βh-endorphin (βh-EP) and related peptides in the neuroeffector junction of the rat vas deferens. Concentration-response experiments were performed before (control) and 20 min following a 60 min tissue exposure to 30 nM β-funaltrexamine (β-FNA). Four separate tissues were tested with either Gly-Gly-βh-EP or Arg-βh-EP, 8 tissues were challenged with methionine enkephalin and 6 with βh-EP. Symbols indicate mean values; bars indicate s.e. ●, Control; ○, following treatment with 30 nM β-FNA.

To further characterize the pharmacological nature of the inhibitory action of β-EP and related peptides, we examined the potency of the peptides in the presence of a variety of selective opioid antagonists.

β-FNA. Pre-exposure of tissues to 10, 30 or 100 nM of this agent displaced to the left, in a concentration-dependent and parallel fashion, the concentration-response curves of βh-EP and methionine enkephalin. Whereas the IC50 for βh-EP and Arg-βh-EP increased 7- to 9-fold, that of methionine enkephalin and Gly-Gly-βh-EP increased only 2- to 3-fold following tissue exposure to 30 nM β-FNA (Table 1, Fig. 1).

Naltrindole. The potency of this selective δ-opiate receptor antagonist was revealed by the 77-fold rightward displacement of the methionine enkephalin concentration-response curve; its pA_2 was 9·4 and the associated K_e was 0·4 nM (Table 1). In contrast, the βh-EP and Gly-Gly-βh-EP IC50 were reduced to about one-twelfth while that of Arg-βh-EP was reduced by only one-eighth. The naltrindole-pA_2 and K_e values for βh-EP and related peptides are summarized in Table 1.

BNI. This antagonist caused a modest yet significant blockade of the inhibitory response of βh-EP, quantitatively of about the same magnitude as that observed with Arg-βh-EP and Gly-Gly-βh-EP; pA_2 and K_e values of BNI are detailed in Table 1.

These studies with selective opiate antagonists add to the characterization of the possible receptor subtypes present in the vas deferens which are sensitive to the action of βh-EP and related peptides. The inhibitory effect of βh-EP is antagonized more potently by naltrindole than by BNI; in the case of β-FNA, the pA_2 cannot be calculated as it does not obey the law of mass action; however, the degree of curve displacement is similar to that found with naltrindole. These results might suggest that the action of βh-EP may involve the occupation of μ- and δ-opiate receptors being least sensitive to κ occupation. In the case of methionine enkephalin, its effects are potently antagonized by naltrindole with a K_e value of 0·4 nM; much larger concentrations of β-FNA or BNI are required to block its effects. These observations confirm the existence of a population of δ-opiate receptors in this tissue. Based on the selectivity of the antagonists and accepting that in the neuroeffector junction of the mouse vas deferens μ-, δ- and κ-opiate receptors might co-exist, a parsimonious explanation for the pharmacodynamics of βh-EP is that it may occupy with similar affinity δ- and μ-opiate receptors, but with a reduced affinity for κ-sites. The present results also demonstrate that Arg-βh-EP behaves more like βh-EP than Gly-Gly-βh-EP. In contrast, Gly-Gly-βh-EP apparently has a higher affinity for δ- than for μ- or κ- sites; in this regard its pharmacological profile is closer to that of methionine enkephalin than β-EP. Our previous study on the rat vas deferens bioassay also concluded that the profile of Gly-Gly-βh-EP showed more of a δ-opiate receptor agonism than Arg-βh-EP (Valenzuela et al 1989). The molecular theory to explain how these minor changes in primary structure determine measurable differences in potency for the different opiate-receptor subtypes is a matter of great importance that might be revealed by a detailed knowledge of the molecular characteristics of the opiate receptors.

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Permeability of the blood-brain barrier for atenolol studied by positron emission tomography

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Abstract—The permeability of the blood-brain barrier for atenolol, a hydrophilic β-adrenergic blocking agent, has been assessed in dogs, by studying the distribution of [^{11}C]atenolol in brain tissue with positron emission tomography. The passage of atenolol into the brain was very limited, but a measurable small net influx into the brain tissues did occur. Osmotic opening of the blood-brain barrier resulted in a marked increase of the atenolol concentrations in brain tissue. The approach described, with sequential non-invasive measurements in brain tissue, is applicable to pharmacokinetic studies of atenolol in man.

The β-adrenergic blocking agent atenolol is a hydrophilic compound which binds to only a limited extent to plasma proteins (Barber et al 1978). Both in dog and man, only 10 to 15% of the dose administered undergoes biotransformation (McAinsh 1977; Reeves et al 1978). After a single injection of atenolol, the drug is rapidly distributed to most tissues, but only a small fraction of the dose reaches the brain (Reeves et al 1978; Heel et al 1979). Higher concentrations were found in brain after chronic administration (Day et al 1977; Street et al 1979; Taylor et al 1981; Davies & McAinsh 1986). Treatment with atenolol was reported to result in a lower incidence of central nervous system side effects than with the more lipophilic compound propranolol. This was ascribed to the different physicochemical properties of the compounds and their different abilities to cross the blood-brain barrier (Cruickshank 1980; Fitzgerald 1980; Neil-Dwyer et al 1981; Westerlund 1983; Frcka & Lader 1988, McAinsh & Cruickshank 1990). With regard to the hypothesis of a central site of action for the antihypertensive effects of β-adrenoceptor blockers, the brain concentrations achieved are a point of discussion in the literature (Street et al 1979; Cruickshank et al 1980; Taylor et al 1981).

We have used positron emission tomography with [^{11}C]atenolol to assess, in anaesthetized dogs, the passage of the drug through the blood-brain barrier. This technique allows non-invasive measurements of drug levels at any desired organ site and is therefore particularly suited to study the early phenomena of drug distribution in brain tissue (Agon et al 1988a). Osmotic opening of the blood-brain barrier was used as an experimental model for the study of the distribution of atenolol in conditions with loss of integrity of the blood-brain barrier.

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Materials and methods

Animals and preparation. Adult mongrel dogs, 9·5–16 kg, were anaesthetized with intravenous sodium pentobarbitone (30 mg kg^{-1}). The dogs were ventilated after the administration of gallamine (2 mg kg^{-1}). Catheters were inserted in the right femoral artery for blood sampling, and in the left femoral vein for drug administration. In experiments where the distribution of atenolol in brain tissue was studied after blood-brain barrier disruption, an additional catheter was placed into the left internal carotid artery. The head of the dog was immobilized in a custom made headholder, with the orbito-meatal plane in a horizontal position.

Radiopharmaceuticals and positron emission tomography. [^{11}C]Atenolol, (2-[*p*-[2-hydroxy-3-([2-^{11}C]isopropylamino)propoxy]phenyl]acetamide), was synthesized according to a modified literature procedure (Antoni et al 1989) by alkylation of the corresponding desisopropyl compound with [2-^{11}C]isopropyl iodide (Goethals et al 1988) in *N,N*-dimethylformamide (DMF) at 120°C for 12 min. Purification was carried out by HPLC on a 10 μm RSil C18HL reversed-phase column with 0·02 M ammonium carbonate-methanol 1 : 1 as mobile phase. The [^{11}C]ateno-