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Opioid receptor types on adrenergic nerve terminals of rabbit ear artery

Hiroshi Fukuda, Eiji Hosoki, Yukio Ishida & Hideki Moritoki[1]

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi, Tokushima 770, Japan

1 Methionine enkephalin, leucine enkephalin, [D-Ala^2, D-Leu^5] enkephalin, α-neoendorphin, β-endorphin, dynorphin (1–13) and ethylketocyclazocine inhibited the contractions of rabbit ear artery ring segments elicited by transmural nerve stimulation at 8 Hz.

2 Ethylketocyclazocine, dynorphin (1–13) and leucine enkephalin produced partial inhibition, their apparent intrinsic activities (α) being 0.57, 0.75 and 0.66, respectively.

3 Morphine and normorphine, which are agonists at μ-receptors, did not inhibit the response of the artery.

4 Naloxone antagonized the actions of opioids and ethylketocyclazocine, and was more effective against methionine enkephalin, leucine enkephalin and [D-Ala^2, D-Leu^5] enkephalin than against α-neoendorphin, ethylketocyclazocine and dynorphin (1–13). The pA_2 values of naloxone against so-called δ-agonists were approx. 8.5, and against so-called κ-agonists were approx. 7.7.

5 The supposed κ-antagonist, Mr 2266, was more effective than naloxone in antagonizing the actions of α-neoendorphin, and the κ-agonists dynorphin (1–13) and ethylketocyclazocine. The pA_2 values of Mr 2266 against κ-agonists were 8.5–9.0, and against δ-agonists were 7.8 or less.

6 The opioid peptides and opioids tested did not cause dilatation of the artery previously contracted with histamine.

7 These results suggest that the opioid peptides and ethylketocyclazocine acted on opioid receptors at adrenergic nerve terminals in the ear artery.

8 The opioid receptors appear to be of the δ- and κ-types, not the μ-type.

Introduction

The physiological significance of enkephalins and other opioid peptides as modulators of neuronal activity has recently been suggested from the findings that these peptides are located in or near nervous structures (Hughes *et al.*, 1977; Schultzberg *et al.*, 1979; 1980; Watson *et al.*, 1981) and are released in response to nerve stimulation (Schulz *et al.*, 1977; Corbett *et al.*, 1980). Moreover, there is a report that enkephalins depress the output of acetylcholine (ACh) and noradrenaline, possibly by a presynaptic action in various nervous systems (Waterfield *et al.*, 1977).

Enkephalins and other opioids could also play a role in regulating vascular tone either via a presynaptic or postsynaptic mechanism. This possibility is supported by the findings that enkephalins act on neuronal opioid receptors in the perfused rabbit ear artery (Knoll, 1976; Rònai *et al.*, 1982; Illes *et al.*, 1983), and on opioid receptors in the vessel walls (Hanko & Hardebo, 1978; Altura *et al.*, 1980). In addition to the enkephalins, opioids such as morphine and bremazocine have been shown to cause vasodilatation both *in vivo* (Altura *et al.*, 1980) and *in vitro* (Hanko & Hardebo, 1978) via specific postsynaptic opioid receptors in vascular smooth muscle. However, the classification of opioid receptors in blood vessels has not been studied extensively.

In the present work, we have examined the types of opioid receptors that are present in the rabbit ear artery by assessing the pA_2 values of opioid antagonists against opioid agonists that have some selectivity for the various receptor types.

[1] Author for correspondence.

Methods

Male albino rabbits of 13 to 15 weeks old (about 2.5 kg) were anaesthetized with pentobarbitone (50 mg kg^{-1}, i.v.) and exsanguinated. Segments of the middle portion of the ear artery were isolated and separated from attached connective tissue under a dissecting microscope. A ring segment of 4 mm length was set up in an organ bath by the method of Bevan *et al.* (1975). Briefly, a U-shaped stainless steel wire was passed through the lumen of the vessel segment, and the ends of this wire were anchored to a plastic holder. A second wire was passed through the vessel and connected to an isometric transducer to measure contractions. The modified Krebs solution in the bath had the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 11.1. The medium was maintained at 34°C and bubbled with 95% O_2 and 5% CO_2. The resting tension of the preparation was maintained at 1.0 g. After equilibration for 60 min, the artery was stimulated transmurally by trains of 5 pulses, each of 0.3 ms duration and supramaximal intensity, at a frequency of 8 Hz every 2 min from a stimulator with a constant voltage modulator (Nihon Kohoden SEN 3201) through a pair of platinum electrodes placed on either side of the artery. Contractions were recorded isometrically with a force displacement transducer (Nihon Kohoden SB-1T). The contractions induced by transmural nerve stimulation were abolished by either tetrodotoxin (100 μM), guanethidine (3 μM) or prazosin (1 μM), indicating that the stimulation was indirect and adrenergic in nature.

For construction of dose-response curves, the opioids were added cumulatively in a volume of 10–70 μl to the 10 ml organ bath. The effects of desensitization of the artery and degradation of opioids were reduced by using the shortest possible period of contact with opioids. Therefore, subsequent doses of opioids were added just when the inhibition reached a maximum. The inhibitions were plotted as percentages of the contractions induced by transmural stimulation just before addition of agonists.

The potencies of agonists are expressed as pD_2 values (negative logarithms of ED_{50} values), and the maximal inhibitions attained with the agonists are expressed as intrinsic activities, α-values, taking the inhibition caused by [D-Ala^2, D-Leu^5] enkephalin or methionine enkephalin as 1.0.

Antagonists were applied 10 min before addition of agonists. Pretreatment with naloxone and Mr 2266 for 10 min appeared to be sufficient for equilibration of these compounds with the receptors, because longer periods of pretreatment did not result in greater inhibition by these antagonists. The activities of opioid antagonists are expressed as pA_2 values, calculated from the shift of the dose-response curves for the agonists by the method of Van Rossum (1963).

The drugs used were α-neoendorphin, [D-Ala^2, D-Leu^5] enkephalin, dynorphin (1–13) (Peptide Institute, Osaka, Japan), ethylketocyclazocine (Sterling-Winthrop Research Institute, Rensselaer, N.Y., U.S.A.), guanethidine sulphate (Sigma Chemical Co., St Louis, Mo., U.S.A.), human β-endorphin, leucine enkephalin, methionine enkephalin (Peptide Institute, Osaka), morphine hydrochloride (Takeda, Osaka, Japan), Mr 2266 ([−]-2-(3-furylmethyl)- 5,9-diethyl-2′-hydroxy-6,7-benzomorphan; Boehringer Ingelheim KG, FRD), naloxone (Endo Laboratories, Garden City, N.Y., U.S.A.), normorphine hydrochloride (Takeda, Osaka), prazosin (Taito-Pfeizer, Tokyo, Japan) and tetrodotoxin (Sigma Chemical Co.,).

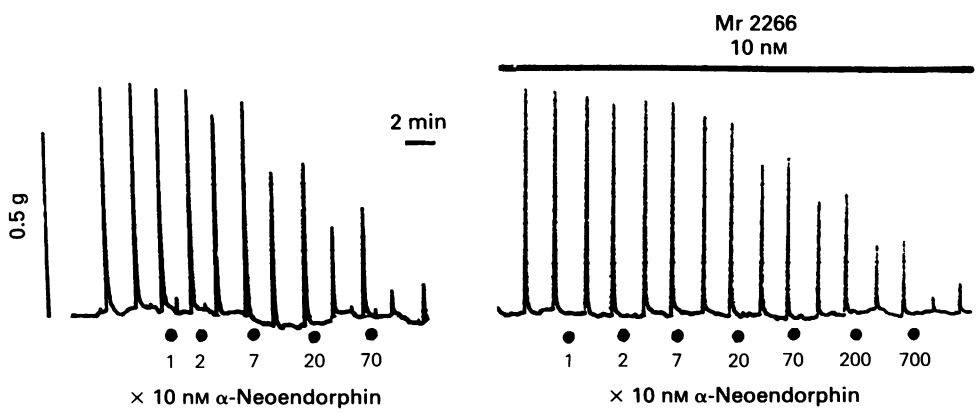

Figure 1 Typical record showing the inhibitory action of α-neoendorphin, and its antagonism by Mr 2266 on contractions of a rabbit ear artery ring segment. The artery was stimulated transmurally by trains of pulses every 2 min; each train consisted of 5 pulses each of 0.3 ms duration delivered at a frequency of 8 Hz. α-Neoendorphin was added cumulatively, and Mr 2266 was applied 10 min before the opioid. The record on the right was obtained in the presence of Mr 2266.

Results

Effects of opioid peptides and opioids on the contractions induced by nerve stimulation

α-Neoendorphin (α-Neoend) at concentrations above 10 nM caused dose-dependent inhibition of the contractions elicited by transmural nerve stimulation (TMS) of rabbit ear artery ring segments (Figure 1). The inhibition developed rapidly reaching a maximum in 2 min that was sustained for a further 2 min, and then decreased slowly. Therefore, subsequent doses of α-Neoend were applied at 4 min intervals. The concentration of α-Neoend causing 50% inhibition of TMS-induced arterial contractions was 150.5 nM. After the ED_{100} concentration of α-Neoend had been applied to the artery for construction of the dose-response curve, at least 40 min was necessary for recovery of the initial sensitivity of the preparation to the peptide.

The apparent potencies of the opioid agonists tested, expressed as pD_2 values, are listed in Table 1, and the dose-response curves for the opioids are shown in Figure 2. β-Endorphin (β-End) was as effective as α-Neoend in inhibiting the arterial contractions. Methionine enkephalin ([Met] enkephalin) and [D-Ala^2, D-Leu^5] enkephalin (DADL) were about 4 and 70 times more potent than α-Neoend, their ED_{50} values being 33.1 and 1.9 nM, respectively. The maximal inhibitions produced by cumulative additions or single additions of the ED_{100} concentrations of the peptides gradually decreased with time in their continuous presence, half recovery taking about 20 min and thereafter the rate of recovery becoming slower.

Dynorphin (1–13) (Dyn (1–13)), ethylketocyclazocine (EKC) and leucine enkephalin ([Leu]enkephalin) partially inhibited the TMS-induced contractions. The threshold concentration of Dyn (1–13) for inhibition was 10 nM. At 1 μM, it produced transient contraction followed by relaxation to the basal level in 60 s, but it inhibited TMS-induced contractions. The maximal inhibition by Dyn (1–13) was about 75% (α = 0.75) of that by DADL, [Met]enkephalin or α-Neoend. The inhibitory effect of Dyn (1–13) persisted much longer than those of α-Neoend and [Met]enkephalin.

The dose-response curves for EKC and [Leu]enke-

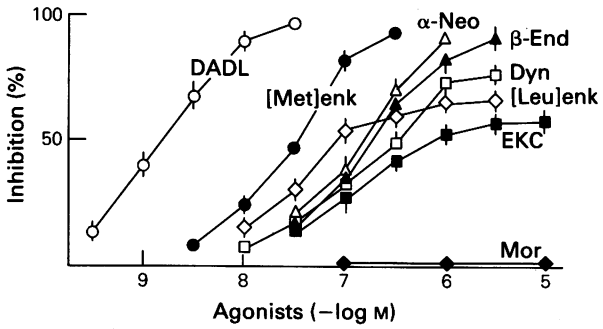

Figure 2 Dose-response curves for the opioid peptides and opioids in inhibiting contractions of rabbit ear artery by transmural nerve stimulation. The ordinate scale shows inhibition of contractions as a percentage of the initial contraction. Values are means for arteries from 5 to 9 animals; s.e.means indicated by vertical lines. DADL, [D-Ala^2, D-Leu^5]enkephalin; [Met]enk, methionine enkephalin; [Leu]enk, leucine enkephalin; α-Neoend, α-neoendorphin; β-End, β-endorphin; Dyn, dynorphin (1-13); EKC, ethylketocyclazocine; Mor, morphine (including normorphine).

Table 1 Potencies of opioids in inhibiting arterial contractions elicited by transmural nerve stimulation and effects of opioid antagonists in antagonizing the actions of opioids

	Intrinsic activities (α)	pD_2	pA_2 *Naloxone*	pA_2 *Mr 2266*	*Ke Mr 2266* / *Ke Naloxone*
α-Neoend	1.00	6.78 ± 0.09 (7)	7.70 ± 0.13 (4)	8.47 ± 0.06 (8)	0.17
β-End	1.00	6.72 ± 0.07 (14)	7.23 (2)	8.13 (3)	0.13
EKC	0.57 ± 0.03	6.93 ± 0.10 (12)	7.81 ± 0.09 (12)	8.95 ± 0.14 (11)	0.07
Dyn (1-13)	0.75 ± 0.05	6.87 ± 0.13 (7)	7.52 ± 0.06 (5)	9.12 ± 0.03 (5)	0.03
[Met]enkephalin	1.00	7.48 ± 0.07 (16)	8.34 ± 0.09 (17)	7.81 ± 0.05 (11)	3.37
DADL	1.00	8.73 ± 0.06 (19)	8.30 ± 0.10 (9)	8.14 ± 0.06 (9)	1.45
[Leu]enkephalin	0.66 ± 0.04	7.45 ± 0.07 (20)	8.05 ± 0.06 (9)	7.92 ± 0.14 (8)	1.35

Stimulation was by trains of pulses as described in the legend to Figure 1. The inhibitory potencies of opioids are expressed as pD_2 values. The antagonistic activities of naloxone and Mr 2266 are expressed as pA_2 values. Appropriate values are expressed as means ± s.e.mean. Numbers in parentheses show numbers of observations.

α-Neoend, α-neoendorphin; β-End, β-endorphin; EKC, ethylketocyclazocine; Dyn (1-13), dynorphin (1-13); [Met]enkephalin, methionine enkephalin; DADL, [D-Ala^2, D-Leu^5]enkephalin; [Leu]enkephalin, leucine enkephalin.

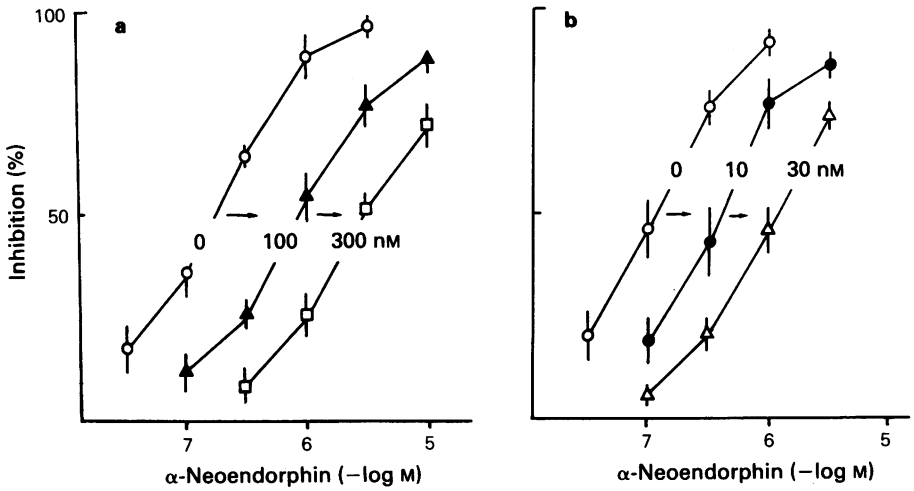

Figure 3 Antagonisms by (a) naloxone and (b) Mr 2266 of the inhibitory action of α-neoendorphin on stimulation-induced arterial contractions. Concentrations of antagonists are indicated in nM on the curves. The ordinate scale shows inhibition of contraction as a percentage of the initial contraction. Values are means for arteries from 5 animals; s.e.means shown by vertical lines.

phalin had low slopes and maxima, their α-values being 0.57 and 0.66, respectively. The effect of EKC developed slowly, reaching a maximum in 6 to 10 min, but it persisted without attenuation. On the other hand, the effect of [Leu]enkephalin was rapid in onset (in 2 min), but in its continuous presence the inhibition gradually became attenuated. A single application of concentrations of [Leu]enkephalin greater than the ED_{100}, but not of EKC, caused almost full inhibition ($94.7 \pm 3.9\%$, $n = 4$), whereas on cumulative application the same concentrations caused partial inhibition ($66.3 \pm 4.2\%$).

After washing, desensitization of the artery to EKC and [Leu]enkephalin persisted; 2 h or more was necessary for the artery to recover its initial sensitivity to these compounds.

In contrast to opioid peptides, opioids, such as morphine and normorphine, at concentrations up to 100 μM, did not inhibit the response of the artery.

Antagonism by naloxone and Mr 2266

Naloxone competitively antagonized the inhibitory actions of opioids on the artery. Occasionally, when high concentrations of the opioids were applied to overcome the antagonism of high concentrations of opioid antagonists, the maximal inhibition was decreased, and therefore the dose-response curves were not parallel over their whole range. To avoid this complication, we measured the pA_2 values in concentration ranges that shifted the dose-response curve to the right up to 30 fold. In the presence of 100 nM naloxone, the dose-response curve for α-Neoend was shifted 4.7 fold in parallel to the right (Figure 3). The pA_2 values with concentrations of 100 and 300 nM naloxone were 7.26 and 7.78, respectively. Naloxone was more effective against [Met]enkephalin, DADL and [Leu]enkephalin than against α-Neoend, Dyn (1–13) and EKC, and at a concentration of 100 nM it shifted the dose-response curve for [Met]enkephalin 24 fold to the right (Figure 4). The pA_2 values of naloxone against [Met]enkephalin and DADL were greater than that against α-Neoend, being 8.34 and 8.30, respectively. The pA_2 values of naloxone against opioid peptides are listed in Table 1. Naloxone also antagonized the actions of Dyn (1–13) and EKC to lesser degrees, its pA_2 values being about 7.5–7.8, compared with 8.3 for [Met]enkephalin and DADL.

Mr 2266 was more effective than naloxone in antagonizing the actions of α-Neoend, EKC and Dyn (1–13). At a concentration of 30 nM, Mr 2266 shifted the dose-response curve for α-Neoend 11 fold in parallel to the right (Figure 3) and that for [Met]enkephalin 3 fold to the right (Figure 4). The pA_2 values of Mr 2266 against EKC and Dyn (1–13) were about 9.0, and that against α-Neoend was about 8.5. On the other hand, Mr 2266 was less effective than naloxone in antagonizing the actions of the so-called δ-agonists DADL, [Leu]enkephalin and [Met]enkephalin, its pA_2 values against these opioids being 7.8–8.1.

Effects of opioids on vascular smooth muscle

To test for possible direct effects of the opioids on vascular smooth muscle, we first induced steady contraction of the artery with 1 μM histamine, and

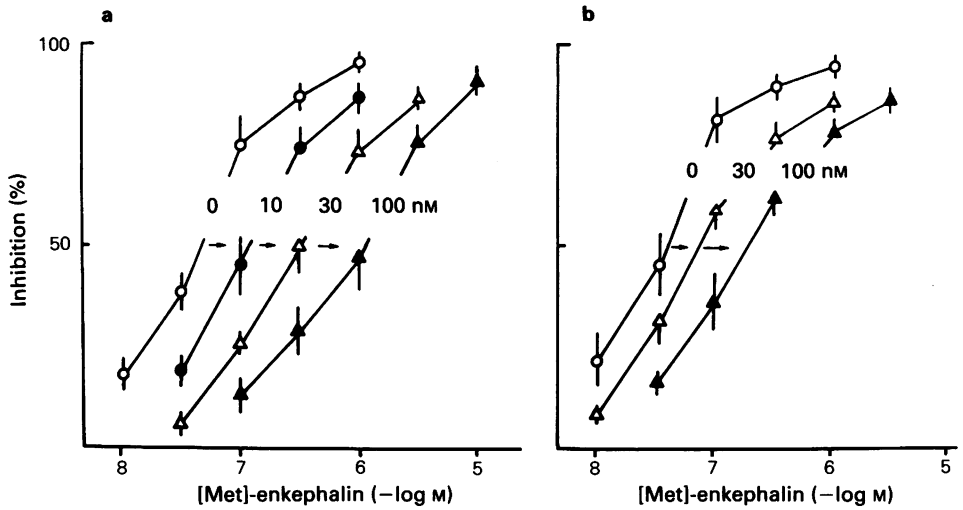

Figure 4 Antagonisms by (a) naloxone and (b) Mr 2266 of the inhibitory action of methionine enkephalin on stimulation-induced contractions. Explanation as for Figure 3.

then applied the opioids cumulatively. None of the peptides that inhibited TMS-induced contractions produced any vasodilatation even at 100 times their ED_{50} concentrations. Opioids, such as EKC, morphine and normorphine at concentrations of up to 100 μM, had no effect on histamine-induced contractions.

Discussion

Dyn (1–13), EKC, α-Neoend, DADL, [Met]enkephalin and [Leu]enkephalin suppressed the contractions of ring segments of rabbit ear artery elicited by TMS. The dose-response curves for opioids in the absence and presence of naloxone and Mr 2266, the pD_2 values of the opioids, and the pA_2 values of the antagonists against opioids are shown in Table 1, and Figures 2, 3 and 4. The values and the dose-response curves are apparent ones and do not necessarily represent real features, since they are influenced by many factors, such as degradation of the peptides, desensitization of the artery to the opioids and possible partial agonist actions of the opioids.

Dyn (1–13), EKC and [Leu]enkephalin caused only partial inhibition of the TMS-induced contractions and their dose-response curves showed lower slopes. Desensitization of the artery to [Leu]enkephalin could be in part responsible for the fact that when added cumulatively [Leu]enkephalin caused only partial inhibition. On the other hand, the partial blockade caused by Dyn (1–13) and EKC seems unlikely to be due to acute desensitization on their cumulative addition, or to degradation in case of Dyn (1–13), because even high single doses of these agonists produced only partial blockade. The possibility that EKC and Dyn (1–13) act as partial agonists cannot be excluded.

Our finding that naloxone and Mr 2266 antagonized the inhibitory effects of the opioid peptides and EKC is strong evidence that specific opioid receptors are involved in the inhibition. The opioids and EKC at concentrations that inhibited TMS-induced contractions maximally did not affect histamine-induced contractions and the TMS-induced contractions were blocked by tetrodotoxin, guanethidine and prazosin. Therefore, these opioids and EKC appear to act on opioid receptors on adrenergic nerves to reduce noradrenaline release, as reported by Illes *et al.* (1983).

There are reports that in the perfused rabbit ear artery, EKC (Rónai *et al.*, 1982), [Met]enkephalin (Knoll, 1976; Rónai *et al.*, 1982) and [Leu]enkephalin (Illes *et al.*, 1983) inhibit stimulation-induced vasoconstriction via presynaptic opioid receptors. In addition to having a neuronal effect, high concentrations of EKC and [Met]enkephalin caused dilatation of dog and cat cerebral arteries previously contracted with prostaglandin $F_{2\alpha}$, their actions being via postsynaptic opioid receptors in the vessel walls (Hanko & Hardebo, 1978). In contrast to the finding of Rónai *et al.* (1982) that EKC caused full inhibition, we found that EKC produced only partial inhibition. This discrepancy may be due to differences in the experimental conditions.

An opioid receptor that is sensitive to morphine, seemingly of the μ-type has been demonstrated at postsynaptic sites in the cerebral artery (Hanko &

Hardebo, 1978) and mesenteric artery (Alture *et al.*, 1980). However, since the rabbit ear artery, unlike the cerebral artery and mesenteric vascular smooth muscle, is not sensitive to μ-agonists, we conclude that μ-receptors cannot be located on the adrenergic nerve terminals.

DADL and [Met]enkephalin or [Leu]enkephalin are known to show preference for the δ-receptor (Lord *et al.*, 1977; Wüster *et al.*, 1979; Kosterlitz *et al.*, 1980), while EKC and Dyn (1–13) show preference for the κ-receptor (Huidobro-Toro *et al.*, 1981; Oka *et al.*, 1982b; Chavkin *et al.*, 1982). Although α-Neoend acts on opioid receptors other than the κ- and μ-types in mouse vas deferens (Oka *et al.*, 1982a), it acts as a κ-agonist in guinea-pig ileum (Wüster *et al.*, 1981; Oka *et al.*, 1982a), rabbit ileum and vas deferens (Oka *et al.*, 1982a) and guinea-pig oesophagus (Kamikawa & Shimo, 1983).

Judging from the activities of the opioids and opiates on rabbit ear artery, and from the reported agonist characteristics of the compounds, the opioid receptors in the ear artery seem to be of the δ- and κ-types; the δ-receptor being predominant. This idea is supported by our findings with naloxone and Mr 2266. In the rabbit ear artery, the pA_2 values of naloxone against [Met]enkephalin, DADL and [Leu]enkephalin were about 8.3 (Ke = 5 nM), a value which is close to those reported for δ-agonists (Ke = 5 nM) on guinea-pig ileum (Yoshimura *et al.*, 1982a,b). In contrast to the high pA_2 values of naloxone against so-called δ-agonists, its pA_2 values against EKC, Dyn (1–13) and α-Neoend in the ear artery were about 7.7 (Ke = 20 nM), a value which is comparable to those reported for κ-agonists (Ke = 24–33 nM) in rabbit ileum (Oka *et al.*, 1982b) and guinea-pig ileum (Oka *et al.*, 1982b; Yoshimura *et al.*, 1982a,b).

On the other hand, the pA_2 values of the supposed κ-antagonist, Mr 2266 (Lord *et al.*, 1977; Römer *et al.*, 1980), against κ-agonists have been shown to be different from those against δ-agonists in mouse vas deferens (Lord *et al.*, 1977) and rat colon (Moritoki *et al.*, 1984). Indeed, in the present experiments, Mr 2266 was more effective against EKC, Dyn (1–13) and α-Neoend than against [Met]enkephalin, [Leu]enkephalin and DADL. Its pA_2 values against the so-called κ-agonists EKC, Dyn (1–13) and α-Neoend were about 9.0 (Ke = 1.0 nM), whereas those against the δ-agonists [Met]enkephalin, [Leu]enkephalin and DADL were about 8.0 (Ke = 10 nM). The greater antagonistic effects of Mr 2266 than those of naloxone against Dyn (1–13), EKC and α-Neoend may be taken as evidence that these compounds act as κ-agonists, thus suggesting the presence of κ-receptors in the ear artery.

Further support for this idea is that the relative effects of Mr 2266 and naloxone, expressed as Ke Mr 2266/Ke naloxone ratio (Oka *et al.*, 1982b), against the κ-agonists EKC, Dyn (1–13) and α-Neoend were about 0.03–0.17, thus differing from the values of 1.4–3.3 for [Met]enkephalin, [Leu]enkephalin and DADL. The pA_2 value of Mr 2266 against α-Neoend was slightly different from those against EKC and Dyn (1–13), even though the Ke Mr 2266/Ke naloxone ratio showed κ-preference, and the shapes of the dose-response curves were different. Therefore, it is possible that either α-Neoend or Dyn (1–13) may act on an as yet undescribed type of opioid receptor.

β-Endorphin was almost as effective as α-Neoend in inhibiting TMS-induced contractions of the rabbit ear artery. Judging from the Ke Mr 2266/Ke naloxone ratio, β-endorphin displayed a preference for the κ-receptor in the ear artery. However, the possible existence of an ε-receptor cannot be excluded, because β-endorphin has been reported to show preference for the ε-receptors in rat vas deferens (Wüster *et al.*, 1980; Schulz *et al.*, 1981).

We suggest, based on the results of the present study, that the opioid peptides and ethylketocyclazocine produced their inhibitory effects on rabbit ear artery through the opioid receptors at adrenergic nerve terminals. The opioid receptors appear to be of the δ- and κ-types. However, the possible involvement of a new type of opioid receptor other than δ- and κ-types cannot be excluded.

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