The action of enkephalins and enkephalin analogues on neurotransmission in the isolated nictitating membrane of the cat

J. KNOLL*, P. ILLÉS, K. MEDZIHRADSZKY†, *Department of Pharmacology, Semmelweis University of Medicine, Budapest, and †Department of Organic Chemistry, Eötvös Lóránd University, Budapest, Hungary*

The field stimulated guinea-pig ileum and vas deferens preparations are widely used for the *in vitro* testing of opiates (see Kosterlitz & Waterfield, 1975) and opioid peptides (Morgan, Smith & others, 1976; Waterfield, Smokcum & others, 1977). Recently a new model, the isolated medial smooth muscle of the cat nictitating membrane, has been introduced for the prediction of the analgesic potency of opiates (Henderson, Hughes & Kosterlitz, 1975; Knoll & Illés, submitted for publication). We have tested the activity of enkephalin analogues on this model to study their relative potencies, and also to compare them with results obtained on the field stimulated longitudinal muscle of the guinea-pig ileum.

The medial smooth muscle of the nictitating membrane of the cat was prepared according to Thompson (1958) and the longitudinal muscle strip of guinea-pig ileum as described by Paton & Vizi (1969). The organs were bathed in Krebs solution at 37° and bubbled with 5% CO_2 in oxygen. The composition of the Krebs solution was (mM): NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $MgSO_4$ 1·2; $NaHCO_3$ 2·5 and glucose 11·5. Responses to field stimulation were recorded isometrically. Supramaximal square wave pulses of 1 ms duration were delivered. The membrane and muscle preparations were stimulated by frequencies of 0·017 and 0·1 Hz respectively. Values of ID50 (the concentration causing 50% inhibition of the twitches of the organ) were determined from log dose-response curves. The relative potencies were calculated as the ratio of the Met⁵-enkephalin-methylester ID50 to the ID50 for each of the other peptides.

Met⁵-enkephalin-methylester (180·0–1620·0 nM) reduced the twitches of the membrane in a dose dependent manner (Fig. 1). The effect is slow in onset and reaches a plateau within 10 min. After washing out the drug a slow recovery takes place. The inhibitory action of the peptide can be readily counteracted by naloxone (11 μM). The depression by Met⁵-enkephalin of responses to electrical stimulation appears to be due to an inhibition of noradrenaline release from nerve terminals innervating the membrane (Illés & Knoll, 1978; Knoll & Illés, submitted for publication).

Table 1 shows the ID50 values and relative activities of the enkephalin analogues in the two models used. In the ileum (Waterfield & others, 1977) and membrane (Knoll & Illés, submitted for publication) Met⁵-enkephalin, normorphine and morphine were reported to be equipotent in depressing electrically evoked contractions. We found Leu⁵-enkephalin was as active as Met⁵-enkephalin-methylester on the membrane in spite of having only about 1/5 of the potency on the muscle strip. These results agree with those of Waterfield & others (1977) who showed Leu⁵-enkephalin to have only 25% of the activity of Met⁵-enkephalin on the guinea-pig ileum, but to be marginally more potent on the mouse vas deferens. An explanation may be that in the muscle preparation the motor transmitter is acetylcholine but in the vas deferens and cat nictitating membrane it is noradrenaline, and the peptides suppress the release of the respective transmitters with differing effectiveness.

The replacement of the aromatic residues methionine or leucine by n-buthylimide or cyclohexylamide resulted in a substantial loss of activities. The ineffectiveness of Met⁵-enkephalin as an analgesic is supposed to be due to its rapid enzymatic destruction *in vivo* as suggested by Hambrook, Morgan & others (1976). Introduction of D-alanine at position 2 resulted in an enzyme resistant analogue, and the D-Ala²-enkephalin-amide produced a long lasting analgesic effect after injection into the periaquaductal grey matter (Pert, Pert & others, 1976). We found that substitution of one glycine by β-alanine abolished the activity of Met⁵-enkephalin. To protect the last peptide bond of enkephalin against proteolysis, the methionine moiety was replaced by proline-amide or proline-ethylamide, and the glycine at position 2 by D-alanine or D-methionine respectively. D-Met², Pro⁵-enkephalin-ethylamide proved to be a highly potent analgesic even

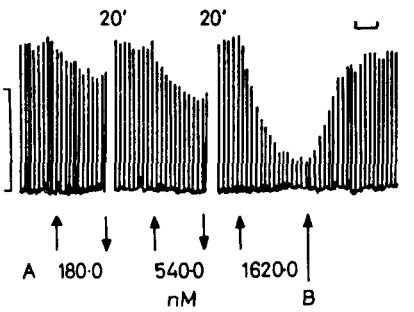

FIG. 1. The inhibitory effect of Met⁵-enkephalin-methylester (A) (180·0–1620·0 nM) on twitches of the medial smooth muscle of the cat nictitating membrane. Inhibition by the peptide (1620 nM) can be readily counteracted by naloxone (B; 11 μM). Stimulation with supramaximal field pulses of 1 ms impulse duration every 1 min. Vertical scale: 1 g; horizontal scale: 5 min.

* Correspondence.

Table 1. *Action of enkephalins and enkephalin analogues on the longitudinal muscle preparation of guinea-pig ileum, and on the medial smooth muscle of the cat nictitating membrane.*

Substances	Guinea-pig ileum longitudinal muscle		Cat nictitating membrane	
	ID50 (nM)	Relative activity	ID50 (nM)	Relative activity
N-Tyr-Gly-Gly-Phe-Met-OMe	440·0 ± 110·0 (4)	1·00	840·0 ± 190·0 (10)	1·00
H-Tyr-Gly-Gly-Ph-Leu-OH	2400·0 ± 220·0 (4)	0·18	890·0 ± 340·0 (5)	0·94
H-Tyr-Gly-Gly-Phe-NH-n-butyl	3100·0 ± 640·0 (4)	0·14	3500·0 ± 640·0 (3)	0·24
H-Tyr-Gly-Gly-Phe-NH-cyclohexyl	2000·0 ± 540·0 (4)	0·22	4100·0 ± 1100·0 (3)	0·20
H-Tyr-β-Ala-Phe-Met-OMe	>30 000·0 (3)	<0·015	>30 000·0 (3)	<0·028
H-Tyr-β-Ala-Gly-Phe-Met-OMe	>30 000·0 (3)	<0·015	>30 000·0 (3)	<0·028
H-Tyr-D-Ala-Gly-Phe-Pro-NH_2	56·0 ± 16·0 (7)	7·9	150·0 ± 30·0 (6)	5·6
H-Tyr-D-Met-Gly-Phe-Pro-NHEt	14·0 ± 3·0 (7)	31·4	90·0 ± 25·0 (6)	9·3

The longitudinal muscle preparation of the guinea-pig ileum and the medial smooth muscle of the nictitating membrane of the cat were stimulated with supramaximal field pulses of 1 ms duration and a frequency of 0·1 Hz and 0·017 Hz respectively. Number of experiments in parentheses.

after systemic administration (Székely, Rónai & others, 1977). D-Ala^2, Pro^5-enkephalin-amide and D-Met^2, Pro^5-enkephalin-ethylamide were also much more potent that Met^5-enkephalin-methylester in the *in vitro* systems used.

The shortening of the length of the peptide by substituting the glycine-glycine spacers by β-alanine yielded a compound which has no significant activity either on the ileum or the nictitating membrane, in good agreement with the findings of Morgan & others (1976). These authors reported Met^5-enkephalin to be the smallest peptide capable of interacting significantly with the opiate receptor.

In conclusion, a series of enkephalin analogues have been found to inhibit noradrenergic transmission in the cat nictitating membrane. With the exception of Leu^5-enkephalin, the rank order of potency of the peptides corresponded with that obtained on the guinea-pig ileum.

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