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BEHAVIORAL ACTIVITY OF MET-ENKEPHALIN AND $ACTH_{4-10}$ AND OTHER PEPTIDES CONTAINING A PHENYLALANINE AND METHIONINE RESIDUE

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SUMMARY

Met-enkephalin, injected i.p. 5 minutes before training, decreased acquisition of a step-up active avoidance response in rats. $ACTH_{4-10}$ exerted a similar effect if given i.p. within 15 minutes of training. $ACTH_{4-10}$ and Met-enkephalin share a phenylalanine and a methionine residue. The possibility was studied that these amino acid residues are a structural requirement for this effect. Accordingly, some linear and cyclic peptides sharing the sequence Phe-Met were synthesized and tested. The cyclic peptide cyclo(-Phe-Met-ε-Ahx-) decreased acquisition but with a longer time course of efficacy than for $ACTH_{4-10}$ or Met-enkephalin and also after oral administration.

Avoidance tests are frequently used to assess learning capacities of animals. In active avoidance tasks, animals acquire a response, such as jumping or running, to avoid the delivery of an aversive stimulus. In passive or inhibitory avoidance tasks, animals learn to refrain from a response to avoid punishment. Enkephalins have been found to affect acquisition of avoidance responses in rats. Thus, i.p. administration of Met-enkephalin, Leu-enkephalin or [D-Ala^2,D-Leu^5] enkephalin shortly before training, decreased acquisition of an active avoidance step-through (1) or step-up (2) response. [D-Ala^2,D-Leu^5]-enkephalin facilitated acquisition of a passive avoidance response (3). These opposite effects suggest that enkephalins did not affect some general memory process in these tests. Rather, it seems likely that these peptides increased a behavioral process or state, such as arousal or fear, that impeded the acquisition of an

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active avoidance response, but complied with the acquisition of a passive avoidance response.

$ACTH_{4-10}$ does not affect the rate of acquisition of a pole-jump active avoidance (4) response in intact rats and normalizes the impaired acquisition of a shuttle-box active avoidance response in hypophysectomized rats (5). $ACTH_{4-10}$ and Met-enkephalin both delay extinction of the pole-jump response in intact rats (6,7). This similarity of response in the latter test has been attributed to the proximity of the phenylalanine and methionine residues, which are intra-chain neighbours in Met-enkephalin and suggested to be extra-chain neighbours in $ACTH_{4-10}$, when the latter peptide assumes an α-helix conformation at the receptor site (7).

In the present study we examined the possible role of the phenylalanine and methionine residues in the effect of peptides on acquisition of the step-up active avoidance response. The effects of Met-enkephalin and $ACTH_{4-10}$ were compared with those of some linear and cyclic peptides containing the sequence Phe-Met. Since these peptides may differ in resistance to enzymatic breakdown, we studied variations in treatment-test interval and compared i.p. and oral routes of administration.

MATERIALS AND METHODS

Male Wistar rats from TNO-Zeist, The Netherlands, weighing approximately 200 g, were used. They were housed 10 per cage of 50x35x20 cm, with ad libitum access to water and standard food pellets. Testing was begun after one week of habituation to the experimental chamber. A rat was taken from its home cage, weighed and injected i.p. with 5 ml/kg vehicle or peptide solution, and placed in a small holding cage. Five minutes later, the animal was transferred to a Plexiglass box (55x55x35 cm) with a grid floor, consisting of 0.35 cm wide stainless steel rods, spaced 1.4 cm apart. A Plexiglass platform (19x 19x4 cm) was located in the middle of the box. The rat was placed on the grid, in the right corner parallel to the far-end wall. Ten seconds later, a scrambled 0.3 mA shock, produced by a 500 V a.c. source through a variable resistance, was delivered to the feet of the rat. The rat could escape by jumping onto the platform, ending the trial. The rat was transferred to a small cage (26x20x14 cm) for an intertrial interval of 10 seconds. If the rat did not escape within 10 seconds, it was taken from the grid for the duration of the intertrial interval. After the intertrial interval had elapsed, the rat was placed back on the grid and a new trial was started. The rat could now make an avoidance response by jumping onto the platform before the onset of shock, or escape from the activated shock, or fail to avoid or escape before the end of the trial. A total of 10 trials was run, with 10 sec intertrial intervals. The number of avoidance responses was

taken as a measure of acquisition performance and analyzed with the Student's t test.

The following peptides were used[2]: Met-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH); $ACTH_{4-10}$ (H-Met-Glu-His-Phe-Arg-Trp-Gly-OH); cyclo(-Phe-Met-ε-Ahx-); H-Phe-Met-ε-Ahx-OH; cyclo(-Phe-Met-); cyclo(-Phe-D-Met-ε-Ahx-); cyclo(-D-Phe-Met-ε-Ahx-) and cyclo(-Phe-Met(O_2)-ε-Ahx-).

The linear peptides Met-enkephalin, $ACTH_{4-10}$ and H-Phe-Met-ε-Ahx were dissolved in 0.9% saline. For i.p. administration, the cyclic peptides were dissolved in 2 ml of dimethylformamide; this solution was diluted with at least 13 ml of distilled water. For oral administration, cyclo(-Phe-Met-ε-Ahx-) was either dissolved in pure dimethylformamide or given in a lecithin emulsion (1.5 mg of vegetable lecithin for 1 mg of peptide in a mixture of glycerol and water). Control groups of rats were treated with the vehicle in each experiment.

Peptides were synthesized by classical methods in solution. The diketopiperazine cyclo(-Phe-Met-) was obtained via the linear dipeptide methylester; the other cyclic peptides were prepared by treatment of the corresponding open-chain analogs with diphenyl phosphoryl azide (8).

RESULTS

I.p. administration of $ACTH_{4-10}$ decreased acquisition of the step-up active avoidance response. Effective doses were 10 and 100 μg/kg (Table 1). In a repeat study, 100 μg/kg of $ACTH_{4-10}$ decreased acquisition by 40.7% relative to the control group ($P < 0.05$, one-tail) when training was started 5 minutes after injection, but did not significantly affect acquisition when given 15 minutes before training (reduction of acquisition with 18.5%, $t=0.89$). The direction of effect and the steep time course of efficacy for $ACTH_{4-10}$ are similar to previous findings with Met- and Leu-enkephalin (1,2). Results from an experiment confirming the efficacy of Met-enkephalin are also shown in Table 1.

The cyclic peptide, cyclo(-Phe-Met-ε-Ahx), also reduced active avoidance responding but its time course of efficacy was more protracted than for $ACTH_{4-10}$. Cyclo(-Phe-Met-ε-Ahx-), at a dose level of 100 μg/kg i.p., decreased acquisition relative to control performance when given 5 (-58.1%, $P < 0.01$), 15 (-79.1%, $P < 0.001$), or 30 minutes (-41.9%, $P < 0.05$) before training. The linear sequence, H-Phe-Met-ε-Ahx-OH, was slightly active at a dose level of 100 μg/kg, when injected i.p. 5 minutes before training, but to a lesser extent than the cyclic peptide (Table 1). Similarly, the analogs cyclo(-Phe-Met-) and cyclo(-Phe-D-Met-ε-Ahx-) showed diminshed but significant

[2]Nomenclature: ε-Ahx = ε-aminohexanoic acid or 6-aminocaproic acid.

TABLE 1

Effect of peptides, given i.p. to rats 5 minutes before training, on the acquisition of an active avoidance response.

Treatment (μg/kg)	Per cent difference from control[1]	t[2]
Met-enkephalin		
1	-58.9^{x}	2.08
10	-85.1^{xx}	3.25
$ACTH_{4-10}$		
1	-25.0	1.20
10	-52.3^{xx}	2.91
100	-43.2^{x}	2.44
cyclo(-Phe-Met-ε-Ahx-)		
1	+13.6	0.94
10	-20.5	1.05
100	-63.6^{xxx}	3.94
H-Phe-Met-ε-Ahx-OH		
1	- 8.0	0.73
10	- 9.0	0.87
100	-24.0^{+}	2.03
cyclo(-Phe-Met-)		
1	-24.5	1.27
10	-24.5	1.09
100	-49.1^{xx}	3.12
cyclo(-Phe-D-Met-ε-Ahx-)		
1	-30.5^{+}	1.90
10	-15.3	1.08
100	-37.3^{x}	2.54
cyclo(-D-Phe-Met-ε-Ahx-)		
1	+ 2.6	0.12
10	-18.4	0.76
100	-29.0	1.09
cyclo(-Phe-Met(O_2)-ε-Ahx-)		
1	- 2.8	0.11
10	- 8.3	0.34
100	+ 2.8	0.09

[1] In each experiment a control group was included. The value used to calculate the data presented was the mean number of avoidances. The range of this value for control groups in the various experiments was 3.8 ± 0.7 -5.9 ± 0.6 avoidances ($\pm$S.E.M.). Number of rats per group was 10, except for the Met-enkephalin study (n = 8) and the H-Phe-Met-ε-Ahx-OH study (n = 20). $^{+}P < 0.1$; $^{x}P < 0.05$; $^{xx}P < 0.01$; $^{xxx}P < 0.01$, relative to corresponding control group.

[2] Value of Student's t-test.

activity 5 minutes after i.p. administration. The analogs cyclo(-D-Phe-Met-ε-Ahx-) and cyclo(-Phe-Met(O_2)-ε-Ahx-) did not produce a significant effect at the doses tested.

The presumably high resistance to enzymatic degradation of cyclo(-Phe-Met-ε-Ahx-) raised the question whether this peptide would be orally active. In a first study, we dissolved this peptide in pure dimethylformamide and found it to be active when administered 1 hour before acquisition at a dose level of 10 or 50 mg/kg. It should be noted, however, that the linear peptide, H-Phe-Met-ε-Ahx-OH, was also orally active. Oral administration of 100 mg/kg of $ACTH_{4-10}$ was ineffective (Table 2). Control rats that had been treated with the dimetylformamide vehicle had normal avoidance scores. Nevertheless, in other experiments we have seen that i.p. injected pure dimethylformamide is toxic, and we were concerned that in the oral cyclo(-Phe-Met-ε-Ahx-) study some toxic effect of the vehicle might have played a role in the observed findings. Therefore, we decided to repeat this study, using a lecithin emulsion as vehicle. Again, treatment with cyclo(-Phe-Met-ε-Ahx-) (1 or 10 mg/kg), 1 hour before acquisition, reduced the number of avoidance scores (Table 2).

DISCUSSION

Met-enkephalin and $ACTH_{4-10}$ both decreased acquisition of the step-up active avoidance response, when injected i.p. shortly before training. This similarity in behavioral activities may be due to a structural feature common to both peptides, i.e. the presence of a phenylalanine and methionine residue. This suggestion is reminiscent of an earlier proposal (7) that the Phe and Met residues are important for structure-activity relationships for the effects of Met-enkephalin and $ACTH_{4-10}$ on extinction of the pole-jump response. However, the pole-jump extinction and step-up acquisition tests are clearly different as behavioral assays of peptide activities. While the time course of efficacy of Met-enkephalin and $ACTH_{4-10}$ is at least 1 hour after s.c. administration of peptide in the pole-jump test (7), this time course is shorter than 15 minutes in the step-up test. Also, oxidation of the thio-ether group of the methionine residue in $ACTH_{4-10}$ (9) or Met-enkephalin (7) resulted in potentiated activity in the pole-jump test but in loss of activity in the step-up test. The peptide H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH, which is a thousand times as active

TABLE 2

Effects of peptides, given orally to rats 1 hour before training, on the acquisition of an active avoidance response.

Treatment (mg/kg)	Per cent difference from control[1]	t[2]
cyclo(-Phe-Met-ε-Ahx-) in dimethylformamide		
10	-38.2^{x}	2.11
50	-55.6^{xxx}	4.24
cyclo(-Phe-Met-ε-Ahx-) in lecithin emulsion		
1	-25.3^{x}	2.52
10	-22.5^{+}	1.66
H-Phe-Met-ε-Ahx-OH in saline		
0.1	-11.1	1.04
1	-19.1^{+}	2.09
10	-39.7^{x}	2.69
$ACTH_{4-10}$ in saline		
100	-20.7	1.57

[1]Range of control values: 4.7±0.6-6.5±0.4 avoidances (± S.E.M.). Number of rats per group: 9-11. $^{+}P < 0.1$; $^{x}P < 0.05$; $^{xxx}P < 0.001$, relative to corresponding control groups.

[2]Value of Student's t-test.

as $ACTH_{4-10}$ in the pole-jump test (9), was inactive in the step-up test over a wide range of doses (Rigter, unpublished findings). Finally, inversion of configuration of the phenylalanine residue in the cyclic peptide, i.e. cyclo(-D-Phe-Met-ε-Ahx-), produced a 3-10 fold increase in potency in the pole-jump test (Greven, van Nispen and de Wied, unpublished findings), but a loss of activity in the step-up test. Therefore, the manner in which these peptides exert behavioral effects in the two tests may be different.

The linear and cyclic Phe-Met-ε-Ahx sequence, but not $ACTH_{4-10}$, were orally active in the step-up test. This may be due to increased metabolic stability or improved transport properties of the peptides. It is probable that the Phe-Met-ε-Ahx peptides are much more lipophylic than $ACTH_{4-10}$. Although

ring-closure of the Phe-Met-ε-Ahx sequence enhances behavioral potency when these peptides were given i.p., it did not further improve potency for oral administration.

The finding that cyclo(-Phe-Met-) was somewhat less active than cyclo-(-Phe-Met-ε-Ahx-) upon i.p. administration may be due to the more constrained steric conformation of the former peptide.

In conclusion, the step-up active avoidance test is clearly sensitive to short-term effects of peptides and can be used for structure-activity studies. The test is distinctly different from the pole-jump extinction test, although the presence of a phenylalanine and methionine residue is an important feature of peptides for behavioral effects in both tests.

REFERENCES

1. Rigter, H., Hannan, T.J., Messing, R.B., Martinez Jr., J.L., Vasquez, B.J., Jensen, R.A., Veliquette, J., and McGaugh, J.L. (1980) Life Sci. 26, 337-345.
2. Rigter, H., and Martinez Jr., J.L. (1980) Soc. Neurosci. Abstr. 6 (in press).
3. Rigter, H., Jensen, R.A., Martinez Jr., J.L., Messing, R.B., Vasquez, B.J., Liang, K.C., and McGaugh, J.L. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 3729-3732.
4. De Wied, D. and Gispen, W.H. (1977) In: H. Gainer (Ed.) Peptides in Neurobiology, pp. 397-448, Plenum Press, New York.
5. De Wied, D. (1969) In: W.F. Ganong and L. Martini (Eds.) Frontiers in Neuroendocrinology - 1969, pp. 97-140, Oxford University Press, Oxford.
6. Van Wimersma Greidanus, Tj.B. and de Wied, D. (1971) Neuroendocrinology 7, 291-301.
7. Greven, H.M. and De Wied, D. (1980) In: D. de Wied and P.A. van Keep (Eds.), Hormones and the Brain, MTP, Falcon House, in press.
8. Van Nispen, J.W., and Greven, H.M., to be published.
9. Greven, H.M., and De Wied, D. (1973) Progr. Brain Res. 39, 429-442.