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INHIBITION OF γ-ENDORPHIN GENERATING ENDOPEPTIDASE ACTIVITY OF RAT BRAIN BY PEPTIDES: STRUCTURE ACTIVITY RELATIONSHIP

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γ-Endorphin generating endopeptidase (γEGE) activity is an enzyme activity which converts β-endorphin into γ-endorphin and β-endorphin-(18-31). The inhibitory potency on γEGE activity of neuropeptides and analogues or fragments of neuropeptides was tested. Dynorphin-(1-13) (IC_{50}: 0.14 μM), human β-endorphin-(1-31) (IC_{50}: 15.5 μM), porcine ACTH-(1-39) (IC_{50}: 6.3 μM), and substance P (IC_{50}: 26 μM) had an inhibitory activity on γEGE activity. β-Endorphin-(18-31) (IC_{50}: 0.35 μM) but not γ-endorphin potently inhibited γEGE activity. The IC_{50} of poly $(Lys)_{40-60}$ was 0.8 μM. It is concluded that 1) γEGE activity is strongly inhibited by its product β-endorphin-(18-31), 2) the enzyme is strongly inhibited by peptides with an aromatic amino acid at the NH_2-terminal and/or basic amino acids in the COOH-terminal of the peptide chain.

γE is a biologically active peptide and occurs in the brain, pituitary gland, testis, and ileum (1-5). γE is generated by enzymatic cleavage of the Leu^{17}-Phe^{18} bond of βE-(1-31) (6,7). This enzyme activity has been termed "γ-endorphin generating endopeptidase" (γEGE) activity (8). A ^{14}C-labeled synthetic analogue of βE-(15-19) was used as substrate to study properties of γEGE activity. γEGE activity is a soluble basic thiol endopeptidase with an apparent molecular mass of 200 kDa (9). The enzyme is rather homogeneously distributed over the brain and pituitary gland (8). In the periphery γEGE activity is predominantly found in the uterus, ovary, and the germinal cells of the testis (10). In this study the substrate specificity of γEGE activity was investigated by the determination of the potency of neuropeptides to inhibit γEGE activity

Abbreviations: γEGE, γ-endorphin generating endopeptidase; Ac-, acetyl-; Z-, benzyloxycarbonyl-; NS, non sulfated; hβE, human β-endorphin; γE, γ-endorphin.

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on the synthetic substrate. The structural requirements for this inhibition were further specified by testing analogues and fragments of h E-(1-31) and ACTH-(1-39).

MATERIALS AND METHODS

Peptides. CCK-8 NS was donated by Dr. Gillessen (Hoffman-La Roche, Basel, Switzerland), substance P by Dr. J. Stewart (University of Colorado, Denver, USA), and βE-(1-21) by Dr. N. Ling (The Salk Institute, San Diego, USA). ACTH-(1-39) isolated from porcine, hβE-(1-31), analogues and fragments of hβE-(1-31) and ACTH were synthesized by Drs H.M. Greven, and J.W. van Nispen. hβE-(19-31) and hβE-(20-31) were prepared from hβE-(18-31), and βE-(1-18) from hβE-(1-31) by incubation with a subcellular fraction from rat brain. [Ac-Phe^{18},Lys(Ac)^{19,24,28,29}]hβE-(18-31) was obtained after acetylation of hβE-(18-31) with acetic anhydride. Spermine was obtained from Sigma Chemical Co. (St. Louis, USA), and polylysine from Serva (Heidelberg, FRG).

Cytosolic fraction. The cytosolic fraction from rat (Wistar, TNO Zeist, The Netherlands) forebrains was prepared by homogenization of 12 rat forebrains in 1 volume brain and 9 volumes of 100 mM Tris-HCl, pH 8.5, in a teflon glass homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min. Debris was discarded and the supernatant was centrifuged at 100,000 x g for 60 min. The supernatant, representing the cytosolic fraction, was diluted with 100 mM Tris-HCl, pH 8.5, to the appropriate protein concentration for the assay.

Assay for γEGE activity. The assay for γEGE activity is based on the cleavage of the Leu-Phe bond in the ^{14}C-labeled substrate Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$, which is an analogue of βE-(15-19). The assay was performed as previously described (9,11). Briefly, γEGE activity was determined with a substrate concentration of 0.86 μM (10,000 dpm) and a protein concentration of 0.25 mg cytosolic protein/ml in 50 mM Tris-HCl, pH 8.5. Incubations were performed in 50 μl at 37°C for 30 min. The inhibitory potency of peptides was determined by including these peptides in the incubation medium in varying concentrations. The incubation was stopped by the addition of 50 μl 2 M acetic acid and subsequent boiling for 10 min. The ^{14}C -labeled products were separated from the intact substrate by hydrophobic chromatography on Amberlite XAD-2 polystyrene beads. Products and the intact substrate were collected separately and radioactivity was determined in both fractions by liquid scintillation counting. Inhibitory potencies of peptides were determined by addition of 10^{-7} to 10^{-3} M of the peptide to the incubation. IC_{50}'s were determined from inhibition curves. The IC_{50}'s of the peptides could appropriately be measured, since the concentration of the substrate used in the assay was 0.86 μM, which was far below the Km (35 μM). Consequently the initial but not the maximal velocity is measured in the assay.

RESULTS

The kinetic parameters, Km and Vmax, of rat brain cytosolic γEGE activity on the substrate Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$ were calculated to be 35 μM and 200 nmol x mg protein^{-1} x h^{-1} respectively (Fig. 1). The conversion of the substrate was competitively inhibited by hβE-(1-31) (Fig. 1). A number of neuropeptides was tested for their inhibitory potency on γEGE activity. Dynorphin-(1-13) was the strongest inhibitor (IC_{50}: 0.14 uM) (Fig. 2). ACTH-(1-39) (IC_{50}: 6.3 μM), hβE-(1-31) (IC_{50}: 15.5 μM), and substance P (IC_{50}: 26 μM) had a 45- to

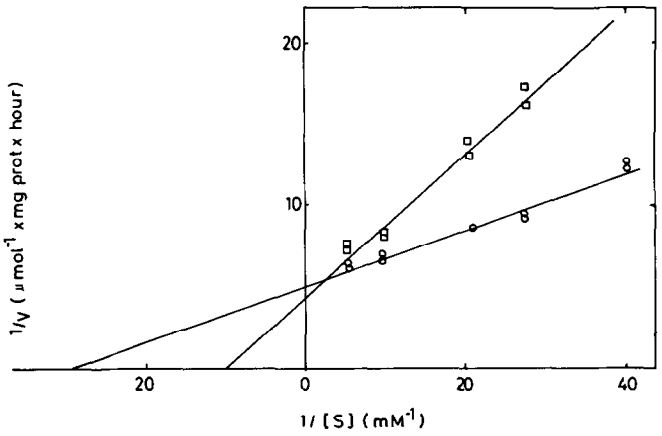

Fig. 1. Lineweaver Burk plot of the cleavage of Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$ by cytosolic γEGE activity from rat brain and the inhibition by hβE-(1-31). Cytosolic fraction (0.25 mg protein/ml) was incubated with the substrate Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$ (0.86 µM) in 50 mM Tris-HCl, pH 8.5, at 37°C for 30 min in absence (○) or presence (□) of 12 µM hβE.

140-fold lower inhibiting potency than dynorphin-(1-13) (Table 1). The IC_{50}'s of CCK-8 NS, Arg-vasopressin, and oxytocin were >150 µM (Table 1). The structural requirements for the inhibition of γEGE activity were further investigated by comparing the inhibitory potency of neuropeptide analogues and fragments. The COOH-terminal peptide hβE-(18-31) had the lowest IC_{50} (0.35 µM) of all tested βE fragments (Table 2). The NH_2-terminal peptide βE-(1-17) (γE) had an IC_{50} of >100 µM. The inhibitory potency of hβE-(18-31) was reduced in hβE-(19-31); its IC_{50} was 3.2 µM. βE-(20-31) had an IC_{50} of 5.0 µM (rat) or 7.0 µM (human). When the free α-NH_2-terminus of hβE-(18-31) and

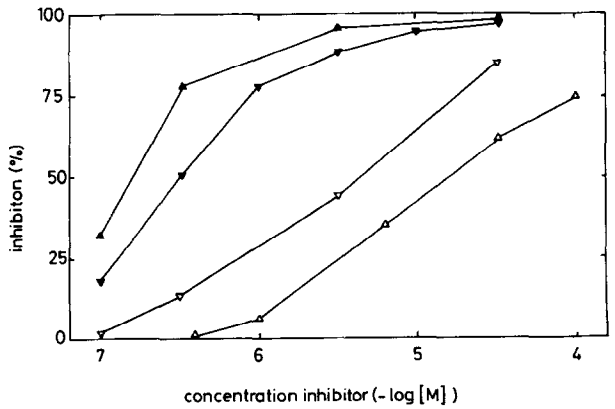

Fig. 2. Inhibition of the γEGE activity by dynorphin-(1-13) (▲), hβE-(18-31) (▼), hβE-(19-31) (▽), and hβE-(1-31) (△).

TABLE I
Inhibition of YEGE activity
by endogenous peptides

peptide	IC_{50} (μM)
dynorphin-(1-13)	0.14
porcine ACTH-(1-39)	6.3
hβE-(1-31)	15.5
substance P	26
CCK-8 NS	>100
Arg-vasopressin	>150
oxytocin	>150

the ε-NH_2-group of the Lys residues were acetylated with acetic anhydride to give [Ac-Phe^{18},$Lys(Ac)^{19,24,28,29}$]-hβE-(18-31), the IC_{50} was 2.1 μM. The NH_2-terminal tetrapeptide of hβE-(18-31), βE-(18-21), had only a weak inhibitory potency (IC_{50}: 42.2 μM). The oligopeptides with resemblance to the NH_2-terminus of hβE-(18-31): Phe-D-Lys, Phe-D-Lys-Phe, D-Lys-Phe, His-Phe-D-Lys, and D-Phe-Lys-Phe and the amino acids Phe and Asn had IC_{50}'s of >100 μM. All ACTH fragments had an IC_{50} of >1 μM. The analogue [D-Lys^8,Phe^9]ACTH-(7-24) was the most potent inhibitor in this group (IC_{50}:

TABLE II
Inhibition of YEGE activity by analogues and fragments of β-endorphin

peptide	IC_{50} (μM)
hβE-(18-31)	0.35
βE-(6-21)	1.5
[Ac-Phe^{18},$Lys(Ac)^{19,24,28,29}$]βE-(18-31)	2.1
βE-(18-21) + hβE-(20-31)	2.9
hβE-(19-31)	3.2
rat βE-(20-31)	5.0
hβE-(20-31)	7.0
βE-(1- 5)	9.5
[17-leucinol]βE-(2-17)	19.0
hβE-(1-31)	15.5
βE-(1-19)	25.0
βE-(2-19)	25.0
βE-(1-21)	25.0
βE-(18-21)	42.2
[Ac-Phe^{15},$Lys^{19}(CH_3)_2$-$NHCH_3$]βE-(15-19)	87.0
βE-(1-17)	>100.0
βE-(1-16)	>100.0
βE-(1-18)	>100.0
[Ac-Phe^{18},$Lys(Ac)^{19}$-$NHCH_3$]βE-(18-21)	>100.0
[Ac-Phe^{18}]βE-(18-23)	>100.0
[Leu^5]βE-(1- 5)	>100.0
[Z-Val^{15},Lys^{19}-$NHCH_3$]βE-(15-19)	>130.0
[Ac-Val^{15},Lys^{19}-$NHCH_3$]βE-(15-19)	>200.0

TABLE III
Inhibition of γEGE activity by ACTH fragments or analogues of fragments

peptide	IC_{50} (μM)
[D-Lys^{8},Phe^{9}]ACTH-(7-24)	2.6
[Lys^{16}-NH_2]ACTH-(1-16)	4.2
ACTH-(1-39)	6.3
[D-Ser^{1},Lys^{17},Lys^{18}]ACTH-(1-19)	9.1
ACTH-(5-18)	13.3
ACTH-(11-24)	6.0
[Lys^{16}-NH_2]ACTH-(7-16)	15.3
γ1-MSH	15.8
[Lys^{16}-NH_2]ACTH-(5-16)	17.2
[Lys^{16}-NH_2]ACTH-(4-16)	17.4
[Ac-Ser^{1},Val^{13}-NH_2]ACTH-(1-13)	25.3
ACTH-(25-39)	44.7
[D-Lys^{2},D-Phe^{7}]ACTH-(1-10)	63.1
[Met(O$_2$)^{4},D-Lys^{8},D-Phe^{9}]ACTH-(4- 9)	>100.0

TABLE IV
Inhibition of γEGE activity by (poly) Lys and spermine

chain length of Lys	IC_{50} (μM)
40-60	0.8
5	1.6
4	3.3
1	>100.0
spermine	>100.0

2.6 μM; Table 3). $(Lys)_{40-60}$ inhibited γEGE activity effectively (IC_{50}: 0.8 μM). $(Lys)_5$ had a 2-fold higher IC_{50} (1.6 μM). The IC_{50} of Lys was >100 μM (Table 4).

DISCUSSION

Several neuropeptides inhibited the action of γEGE activity from rat brain cytosolic fraction on the substrate Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$. This indicates that the affinity of the γEGE activity is not restricted to the Leu^{17}-Phe^{18} bond of hβE-(1-31). Whether and at what bond neuropeptides are cleaved by γEGE activity can only be determined when a purified γEGE preparation is available. βΞ and βE-(18-31) are the two products generated from βE-(1-31) by γEGE activity. γEGE activity is 60-fold stronger inhibited by hβE-(18-31) than by hβΞ-(1-31), but γE (βE-(1-17)) had a weak inhibitory potency. Thus βE-(18-31) may be involved in the regulatory process of γEGE activity in vivo. Dynorphin-(1-13), hβE-(18-31), $(Lys)_{40-60}$, and [D-Lys^{8},Phe^{9}]ACTH-(7-24) are very potent inhibitors of γEGE activity. Their IC_{50}'s are between 0.14 and 2.6 μM. These peptides are more potent inhibitors than the most potent inhibitor previously encountered in the classification of γEGE activity: p-chloromercuribenzoate, which had an IC_{50} of 2.8 μM under identical conditions (9). The actual IC_{50} of these peptides may even be lower since they are subject to degradation during incubution with the cytosolic fraction. The cytosolic fraction contains many

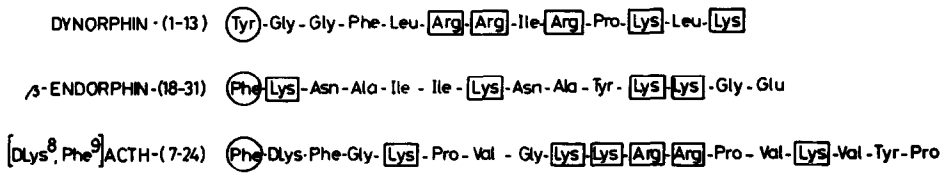

Fig. 3. Comparison of the primary structures of dynorphin-(1-13), hβE-(18-31), and [D-Lys^8,Phe^9]ACTH-(7-24). □, basic amino acids; ○, NH_2-terminal aromatic amino acids.

different proteolytic activities. These proteolytic enzymes also might account for some of the differences in IC_{50}'s since some peptides might be more susceptible to proteases than others are. Especially peptides with D-amino acids are more resistent to proteolytic enzymes. Conditions used in this study do not allow to extrapolate Ki-values from the emperical IC_{50} values. Ac-VTLFK([^{14}C]CH_3)$_2$-$NHCH_3$ is not converted by peptidases different from γEGE activity as previously demonstrated (11). The peptides dynorphin-(1-13), hβE-(18-31), and [D-Lys^8,Phe^9]ACTH-(7-24) have common structural features which may underly their high inhibitory potency (Fig. 3). There is an aromatic residue (Tyr or Phe) at the NH_2terminus. The peptides contain clusters of basic amino acids in the COOH-terminal portion. The two most potent inhibitors dynorphin-(1-13), and hβE-(18-31), have about the same chain length (13 or 14 amino acids). The presence of an aromatic amino acid with a free NH_2-terminus seems to be important, since hβE-(19-31) is much less potent than hβE-(18-31) in inhibiting γEGE activity.

It is concluded that: 1) γ EGE activity is inhibited in vitro by several naturally occurring peptides, which may indicate that γEGE activity is inhibited by many peptides in vivo, 2) the strong inhibition of γEGE activity by its product hβE-(18-31) points to product inhibition on γEGE activity, 3) peptides which potently inhibit γEGE activity have one or more of the following properties: A) a cluster of basic amino acids in the COOH-terminal portion of the peptide, B) a chain length of about 13 or 14 amino acids, C) an aromatic amino acid as NH_2terminus. These criteria may serve as a guideline for the development of potent inhibitors for γEGE activity.

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