EFFECT OF BACITRACIN ON THE BIODEGRADATION OF β-ENDORPHIN

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β-endorphin was incubated with rat brain homogenate, and the amino acids released were measured by amino acid analysis. Phe, Leu, Tyr, and Lys were liberated in the greatest amount indicating that the cleavage of Leu^{77}-Phe^{78} and some Lys-X peptide bonds with endopeptidases followed by the removal of the terminal residues by exopeptidases are the main routes of β-endorphin degradation in the brain. Bacitracin considerably reduced the amino acid release from β-endorphin incubated with rat brain homogenate, and its action is suggested to be due to the inhibition of brain amino- and carboxypeptidases. Bacitracin also potentiated and prolonged the *in vivo* analgesic activity of β-endorphin.

It has been previously suggested that the outstanding *in vivo* biological activity of β-endorphin /LPH^{61-91}[+]/ /1,2/ as compared to those of its shorter NH_2-terminal fragments may be due, at least partly, to the relatively higher metabolic stability of the β-endorphin molecule /2/. More recent comparative studies on the degradation of Met-enkephalin /LPH^{61-65}/, β-endorphin and other β-LPH fragments of intermediate size by aminopeptidase M /3-5/ and different brain extracts /5,6/ have clearly shown that indeed a correlation exists between the susceptibility of these peptides to enzymic destruction and their *in vivo* analgesic potency. Enzyme resistance also appears to be a common feature of some newly developed enkephalin analogs with enhanced biological activity /7-9/. In addition, an antibiotic protease inhibitor, bacitracin /10/ has been found to potentiate the effect of enkephalins *in vitro* /11/.

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[+]Abbreviations: LPH, lipotropic hormone, all the position numbers used refer to the β-LPH sequence; EI-enzyme, endorphin inactivating enzyme.

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In view of the above data it has been of particular interest to study the effect of bacitracin on the biodegradation of β-endorphin and on its in vivo analgesic properties as well.

MATERIALS AND METHODS

Porcine β-endorphin was prepared as described previously /12/. Highly purified bacitracin A was obtained from Dr. A. Kótai /L. Eötvös University, Budapest/. For studies on the biodegradation of β-endorphin in brain homogenate Marks' approach was used /ref. 13 and refs therein/. Freshly removed rat brains were homogenized in 10 vol. of cold 1% saline /w/v/, and then exhaustively dialysed against distilled water at 0-4 °C. The protein content of the dialysed homogenate was 7.5 mg/ml as determined by Palladin's method /14/. In a routine experiment the incubation of β-endorphin /0.8 mg; 250 nmol/ was carried out in a mixture consisting of 0.9 ml of homogenate and 0.1 ml of 0.1 M Tris-HCl buffer of pH 7.5 for 60-120 min at 37°C. Incubations were carried out in the absence or presence of 5×10^{-4} M bacitracin in triplicate with controls not containing exogenous β-endorphin. Aliquots of 0.2 ml /containing 50 nmol substrate/ were taken at the indicated times, and the reaction was terminated by adding an equal volume of acetone and 10 µl of 1 M acetic acid to the samples. The mixtures were then centrifuged at 2000 g, and the supernatants were evaporated for amino acid analysis. Amino acid analysis was carried out in a JEOL /JLC-5AH/ automatic analyzer. The analgesic effect of the substances was tested in rats using the conventional tail-flick test as described previously /2/.

RESULTS AND DISCUSSION

Incubation of β-endorphin with rat brain homogenate led to a time-dependent liberation of all the constituent amino acids /Table I/. At 120 min, the amount of the released amino acids ranged from 47% /Phe/ to 22% /a sum of Ser, Gln and Asn/. The proportions of amino acids liberated generally agree with those reported by Marks et al. /6/, except for that of Met which was relatively low in our case /Table I/. The preferential release of Leu and Phe from β-endorphin indicates a selective and rapid cleavage at an internal site, presumably at the Leu^{77}-Phe^{78} peptide bond by an endopeptidase /designated as EI-enzyme by us; 3,15,16/ and the subsequent removal of the newly formed terminal residues /Leu and Phe/ by exopeptidases. The location of Phe^{64} within a relatively enzyme-resistant sequence portion of the molecule /see

Table I

Release of Amino Acids[+] from β-endorphin /LPH$^{61-91}$/ on its Incubation with Rat Brain Homogenate in the Absence or Presence of Bacitracin at 60 and 120 min

Amino acid[§]	60 min			120 min		
	Control	+Bacitracin	Inhibition by bacitracin /%/	Control	+Bacitracin	Inhibition by bacitracin /%/
Lys /69,79,84,88,89/	21	13	38	37	21	43
His /87/	18	11	39	32	18	44
Thr /66,72,76/	16	7	56	33	17	48
Ser /67,70/ Gln /71,79/ Asn /80, 85/	14	8	43	22	14	36
Glu /68/	16	12	25	31	26	16
Pro /73/	19	12	37	25	15	39
Gly /62,63,90/	11	5	54	24	13	46
Ala /81,86/	18	10	44	29	18	38
Val /75,83/	15	6	60	32	15	53
Met + MeSO /65/	+	+		24	16	33
Ile /82/	14	5	64	32	13	59
Leu /74,77/	28	13	53	46	18	61
Tyr /61/	29	22	24	44	30	32
Phe /64,78/	24	17	29	47	28	40

[+]Expressed in nmol % based on the total amount of the corresponding amino acid in the β-endorphin structure.

[§]Position numbers related to β-LPH sequence are indicated in parentheses; Ser, Gln, and Asn were not separated by the analyzer; the sum of Met and its artifact, MeSO is given.

the low values for $Gly^{62,63}$ and Met^{65} in Table I/ also suggests Phe^{78} to contribute primarily to the high proportion of Phe released. The relatively high yield of Lys in the incubation mixture points to the presence of trypsin-like enzyme/s/ as well in the brain homogenate. The preferential release of Tyr and the delayed appearance of Gly in the digest suggest that Tyr^{61} is split by aminopeptidase action as proposed previously /6,17/. Considering the resistance of β-endorphin to aminopeptidase M action /3-5/ however, it is reasonable to speculate that Tyr is released from smaller fragment/s/ rather than from β-endorphin itself. The closely similar proportions of Leu, Phe and Tyr liberated also indicate that the cleavage of Leu^{77}-Phe^{78} peptide bond may be the rate-limiting step of this complex degradation process.

From the above data and the known structure-activity relationships in β-endorphin /2/, the major routes of inactivation in the brain may be ascertained as /a/ cleavage of the Leu^{77}-Phe^{78} peptide bond by EI-enzyme /3,15,16/, /b/ cleavage of some further internal peptide bonds by trypsin-like enzyme/s/ and /c/ removal of Tyr^{61} from the newly formed fragments by aminopeptidase/s/.

As may be seen in Table I, bacitracin considerably reduced the release of all the amino acids from β-endorphin, though to a different extent. In view of our failure to inhibit EI-enzyme, trypsin and some trypsin-like enzyme/s/ of a pituitary homogenate by bacitracin /unpublished data/, the effect of this antibiotic on the biodegradation of β-endorphin may be due to the inhibition of exo-, rather than endopeptidases. The decreased Tyr release in the presence of bacitracin can be regarded as a direct evidence for the inhibition of brain aminopeptidase/s/. In accord with the above suggestion for the release mechanism of Phe /i.e. Phe originates primarily from Phe^{78} by aminopeptidase action following the cleavage of the Leu^{77}-Phe^{78} peptide bond/ the appearance of Phe is inhibited by bacitracin to a similar

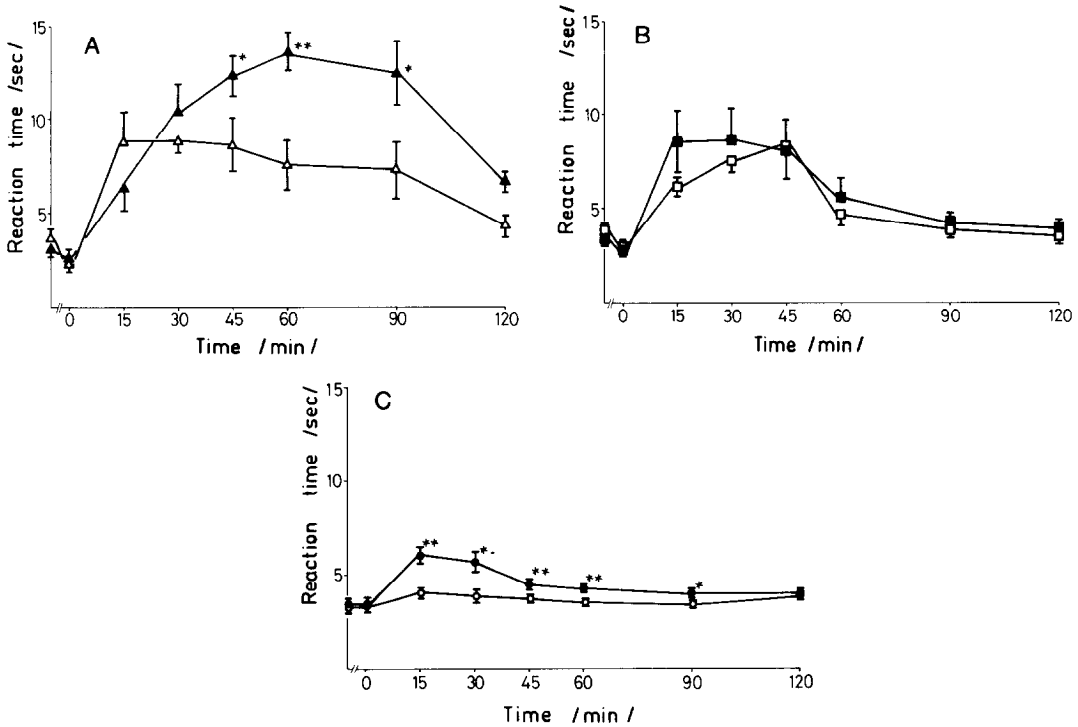

Figure 1. Time-response curves for the analgesic effect of β-endorphin /0.4 nmol/rat/, morphine /5 nmol/rat/ and bacitracin /30 µg/rat/ administered intracerebroventricularly to rats. On the ordinate the reaction time, on the abscissa the time elapsed after administration is plotted. Number of animals is 8 for each group. △ : β-endorphin, ▲ : β-endorphin + bacitracin for A; □ : morphine, ■ : morphine + bacitracin for B; ○ : saline, ● : bacitracin for C, respectively. *: $P < 0.05$, **: $P < 0,01$.

extent to that of Tyr^{61} /Table I/. Since the release of Leu /ascribed mainly to the liberation of Leu^{77} by sequential actions of EI-enzyme and carboxypeptidase/s/ on β-endorphin/ is much more reduced by the antibiotic than that of Tyr and Phe /Table I/, bacitracin may be assumed to be a stronger inhibitor for brain carboxypeptidase/s/ than for aminopeptidase/s/. Based on the similarly strong inhibition of the Ile and Val release one may even suspect that these residues are also liberated mostly by carboxypeptidase action. However, further studies on the inhibitory action of bacitracin on purified enzymes are required to clarify this point.

In the light of the above results it has been of interest to examine whether or not bacitracin /an inhibitor of brain exopeptidases/ alters the in vivo analgesic effect of β-endorphin. When a mixture of β-endorphin and bacitracin was injected intracerebroventricularly to rats, the analgesic effect of β-endorphin was potentiated and prolonged /Figure 1A/. In the same time the action of morphine was not influenced by bacitracin /Figure 1B/. Surprisingly, bacitracin itself caused a slight but statistically significant "analgesic effect" as shown in Figure 1C. Is this response an aspecific one or is it due to the protection of endogenous opioid peptide/s/ against enzymic destruction? Further studies are in progress to answer these questions.

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