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BREAKDOWN OF SMALL ENKEPHALIN DERIVATIVES AND ADRENAL PEPTIDE E BY HUMAN PLASMA

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Abstract—To provide information concerning the fate of opioid peptides introduced into the circulation of man, we have investigated the breakdown of the following peptides when incubated in human plasma: [Met]enkephalin, [Met]enkephalyl-Arg^6-Phe^7, [Met]enkephalyl-Arg^6-Gly^7-Leu^8, [Met]enkephalyl-Arg^6-Arg^7-Val^8-NH_2 and the complex opioid, peptide E. We used a radioimmunoassay recognizing the amino-terminus of opioid peptides (assay for total opioid peptide-like immunoreactivity). The three small enkephalin derivatives were broken down considerably faster than the enkephalins themselves. The rate of loss of immunoreactivity was considerably reduced by bacitracin. When peptide E was incubated in human plasma, a relatively sustained level of opioid peptide-like immunoreactivity was seen. This was shown to be due, not only to the relatively slow aminopeptidase attack of the larger peptide, but also to the generation during breakdown of peptide E of [Leu]enkephalin and [Met]-enkephalin.

[Met]enkephalin and other products of the pro-enkephalin gene have been described in both human adrenal and phaeochromocytoma [1–8] where they are located principally in chromaffin granules [6, 7]. Studies on adrenal medulla tissue and cell preparation from several species indicate that these peptides are released from chromaffin cells along with the catecholamines (see for review ref. [9]). Further, [Met]enkephalin and related sequences are found in the circulation [10, 11]. Apart from the two pentapeptide enkephalins, the peptides [Met]enkephalyl-Arg^6-Gly^7-Leu^8, [Met]enkephalyl-Arg^6-Phe^7, [Met]-enkephalyl-Arg^6-Arg^7-Val^8-NH_2 and adrenal peptide E are opioid active products of the pro-enkephalin precursor which are present in human adrenal [6–8]. Adrenal peptide E is opioid by virtue of the enkephalin sequence at its amino-terminus, but also contains a [Leu]enkephalin sequence at its carboxy-terminus [12]. Nothing is known about the fate of these peptides in the circulation. This paper describes some studies on the breakdown of these opioid peptides when incubated with human plasma.

METHODS

For the studies reported here, plasma was prepared by collection of blood from volunteers into EDTA-containing tubes (14 mg/10 ml plasma) on ice which were centrifuged at 3000 *g* for 20 min at 4°. Some experiments were also done with plasma prepared by adding 1.1 ml of 3.8% sodium citrate per 10 ml of blood; results were not significantly different from those with EDTA plasma and are not reported here. Peptides used in this study were purchased from Peninsula Laboratories, California, U.S.A. Peptides were incubated in plasma volumes of, in most cases, 500 μl to a final concentration of 0.5–500 pmol/ml introduced to the plasma in 20 μl of H_2O. Following rapid mixture, incubation was in a shaking water bath at 37°. In all experiments reported here, each incubation was carried out in duplicate. The reaction was stopped by removing the tubes to ice and immediately transferring aliquots, in duplicate, of each incubate to tubes on ice containing an equal volume or more of 50% acetic acid. These acidified samples were then dried under reduced pressure prior to acetylation and radioimmunoassay.

The assay for total opioid-like immunoreactivity was essentially as described earlier [7, 13]; it is dependent upon alpha-amino acetylation of the sample, followed by radioimmunoassay with an antiserum which recognizes the acetylated enkephalins with or without carboxy-terminal extensions. All endogenous mammalian peptides with opioid activity have one of the enkephalin pentapeptide sequences as their amino-terminus.

Briefly, the dried extract was taken up in 100 μl mM sodium phosphate buffer, pH 6.0, to which was added with vigorous mixing 20 μl of acetic anhydride (16%) in dimethylsulphoxide. After a few minutes this was dried under reduced pressure, taken into 20 μl of 20% ammonium hydroxide and dried again prior to dissolving in 100 μl of assay buffer (0.1 mM sodium phosphate, 50 mM NaCl, 0.1% Triton-X-100, 0.1% gelatin, 0.02% thimerosal, pH 6.0). The assay mixture was made up with 50 μl of antiserum at a final dilution of 1 : 15,000 and 50 μl of [^{125}I]alpha-*N*-acetyl [Met]enkephalin (containing 8–10,000 cpm) in assay buffer. The antiserum was a kind gift from Dr. E. Weber, Stanford Medical School, California, U.S.A. Separation of bound from free after an overnight incubation at 4° was by a second antibody procedure. The standard curve was constructed with alpha-*N*-acetyl [Met]enkephalin.

Using this procedure, the assay was highly specific for opioid peptides, cross-reacting 100% with opioid peptides derived from proenkephalin, including [Met]enkephalin, [Leu]enkephalin, [Met]enkephalyl-Arg^6, [Met]enkephalyl-Arg^6-Phe^7,

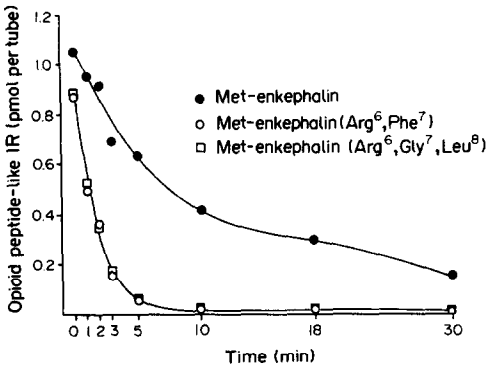
[Met]enkephalyl-Arg^6-Gly^7-Leu^8, [Met]enkephalyl-Arg^6-Arg^7-Val^8-NH_2, BAM-12P, BAM-20P, adrenal peptide E, but not cross-reacting with the non-opioid sequence Arg-Tyr-Gly-Gly-Phe-Met, as described previously [7].

Reversed-phase HPLC used a 0.5 × 25 cm column of Hypersil 5 ODS (Shandon) supplied and packed by HPLC Technology Limited, Macclesfield, U.K., with Applied Chromatography Systems solvent delivery system with a flow rate of 1.5 ml/min. Elution was with an acetonitrile gradient in 50 mM sodium phosphate (pH 2.7) with 5% methanol. One-minute fractions were collected, aliquots of which were dried under reduced pressure prior to assay.

For the assay of [^{3}H]tyrosine release from [^{3}H]-tyrosyl [Leu]enkephalin (Amersham International, U.K.), the enkephalin, with bacitracin when appropriate, was added in 20 μl to 180 μl of plasma to give either 500 pM or 500 nM enkephalin containing about 0.25 × 10^4 cpm per tube. Incubation at 37° was stopped by adding 800 μl of cold 25° acetic acid, and the incubate was applied directly to a pre-wetted Sep-Pak. The Sep-Pak was washed with 1 ml of H_2O and the tritium in the combined effluent estimated. It had earlier been shown that this is essentially all [^{3}H]tyrosine (by thin layer chromatography).

RESULTS

Under the conditions used in the present experiments, the extraction of plasma, with or without prior incubation at 37°, did not result in activity in the assay for total opioid peptide-like immunoreactivity. However, addition of [Met]enkephalin or its C-terminally extended derivatives to the plasma prior to assay did produce the expected immunoreactivity. Following incubation of the plasma containing added peptides, a decline in activity in the plasma extracts was seen with time, as illustrated in Fig. 1. In each case degradation of the peptides is noticeable at 1 min of incubation. The rate of breakdown of [Met]enkephalyl-Arg^6-Phe^7 and [Met]enkephalyl-Arg^6-Gly^7-Leu^8 is considerably greater than that of [Met]enkephalin itself. The difference between the breakdown of [Met]enkephalin and that of its heptapeptide and octapeptide derivatives can be seen in the pooled data from several experiments presented in Fig. 1, and in Fig. 3b where the breakdown of [Met]enkephalyl-Arg^6-Arg^7-Val^8-NH_2 is also shown to be rapid compared to [Met]enkephalin.



Half lives (mean ± S.E.)

Met-enkephalin	8.15 ± 1.35 (N=6)
Met-enkephalin (Arg^6,Phe^7)	2.54 ± 0.64 (N=5)
Met-enkephalin(Arg^6,Gly^7,Leu^8)	1.22 ± 0.2 (N=5)

Fig. 1. Loss of opioid peptide-like immunoreactivity with time, on incubation of [Met]enkephalin, [Met]enkephalyl-Arg^6-Phe^7 and [Met]enkephalyl-Arg^6-Gly^7-Leu^8 in human plasma. Means of duplicate incubations of 1 pmol of peptide with 500 μl of plasma. The time taken to reach 50% of initial immunoreactivity with several such experiments is indicated below the figure.

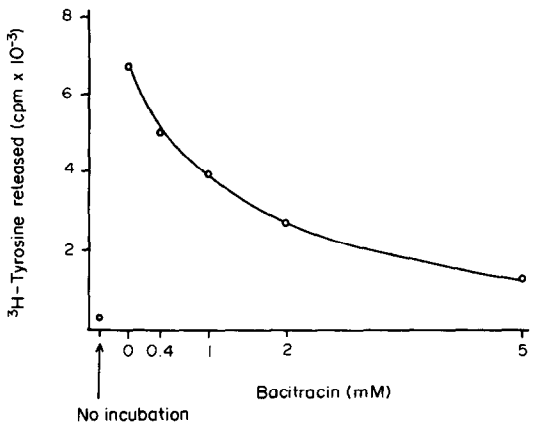


Fig. 2. Generation of [^{3}H]tyrosine from [^{3}H]tyrosyl [Leu]-enkephalin on incubation for 20 min in human plasma; effect of incubation with different bacitracin concentrations. Figures plotted are means of duplicate determinations.

The rate of loss of immunoreactivity on incubation of enkephalin in plasma was compared to the rate of generation of [^{3}H]tyrosine on incubation of [^{3}H]-tyrosyl [Leu]enkephalin with plasma. The two methods of estimation of enkephalin breakdown gave essentially the same result; the rate of generation of [^{3}H]tyrosine was the same whether the concentration of peptide was 500 pM or 500 nM (data not shown).

To investigate whether the breakdown in each case was due to aminopeptidase attack we examined the effect of incubation in the presence of bacitracin and bestatin. We found that a low concentration of bacitracin (50 μM, as used in many studies on release of small peptides) was ineffective, and so we determined the concentration of bacitracin required to inhibit the generation of [^{3}H]tyrosine on incubation of [^{3}H]tyrosyl [Leu]enkephalin with plasma (Fig. 2). The level required for substantial inhibition of this aminopeptidase attack was 2–5 mM. Subsequent experiments on the degradation of [Met]enkephalin, [Met]enkephalyl-Arg^6-Phe^7, [Met]enkephalyl-Arg^6-Gly^7-Leu^8 and [Met]enkephalyl-Arg^6-Arg^7-Val^8-NH_2 in plasma, as revealed by assay for total opioid peptide-like immunoreactivity, showed in each case a similar degree of inhibition of bacitracin at the 2–5 mM concentration. For example, in Fig. 3 the effect of incubation of [Met]enkephalin and [Met]-enkephalyl-Arg^6-Arg^7-Val^8-NH_2 with plasma, with and without 5 mM bacitracin, is compared. Studies

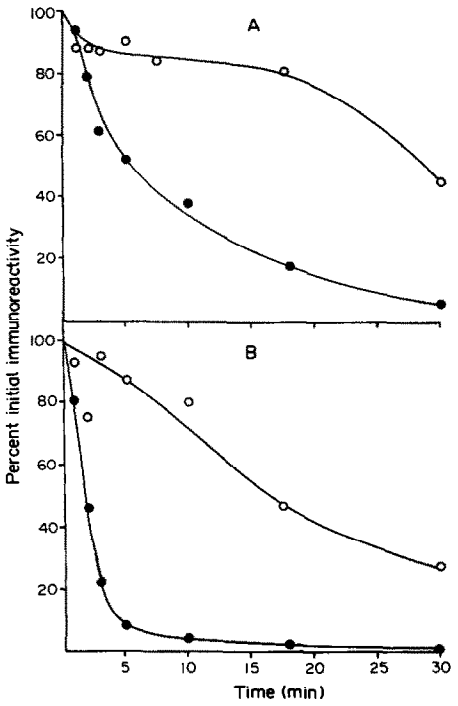


Fig. 3. Loss of opioid peptide-like immunoreactivity with time, on incubation of (A) [Met]enkephalin and (B) [Met]-enkephalyl-Arg^6-Arg^7-Val^8-NH_2 in human plasma with no inhibitor (closed circles) or 5 mm bacitracin (open circles). Figures are means of duplicate incubations.

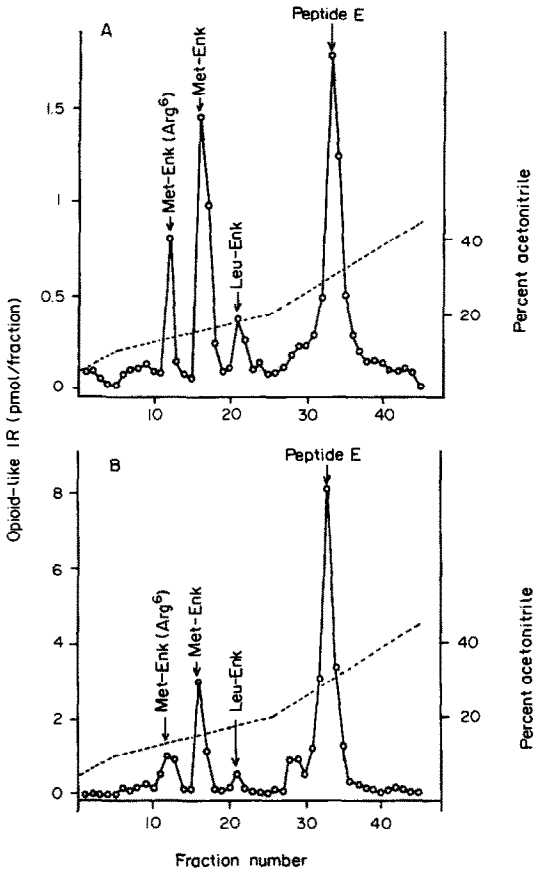


Fig. 5. Reverse-phase HPLC separation of the products of incubation of peptide E in human plasma for 18 min in the presence of (A) and absence of (B) of bacitracin (5 mM). The acetonitrile gradient (broken line) was 5–10% (5 min), 10–20% (20 min), 20–45% (20 min). Met-enk(Arg^6) = [Met]enkephalyl-Arg^6; Met-enk = [Met]enkephalin; Leu-enk = [Leu]enkephalin.

on aminopeptides attack using release of [^{3}H]tyrosine from [^{3}H]tyrosyl [Leu]enkephalin showed that bacitracin (5 mM) and bestatin (1 mM) had closely similar effects on the time course of this proteolysis, confirming that the bacitracin effect was due to aminopeptidase inhibition.

Incubation with peptide E (Fig. 4) gave a much slower rate of loss of total opioid peptide-like activity, with a half-life of 56 ± 12 min (±S.E.M., $N = 4$). Loss of activity after the first hour of incubation was minimal, almost reaching a plateau, the height of which was variable. In the presence of 2 mM bacitracin an initial increase in activity was followed by a fall to a plateau level which was similar to that reached following incubation in the absence of bacitracin. To investigate the nature of the increased activity in the tubes incubated with bacitracin, aliquots were taken at 18 min for analysis by reversed-phase HPLC. In the absence of bacitracin, 18 min incubation with plasma yielded four principle peaks of activity on subsequent HPLC (Fig. 5A), corresponding to the positions of peptide E, [Leu]-enkephalin, [Met]enkephalin and [Met]enkephalyl-Arg^6. The predominant peak corresponded to residual peptide E, while immunoreactivity eluting at the retention time for [Met]enkephalin accounted for a major proportion of the remaining activity. In the presence of bacitracin the overall elution pattern was the same (Fig. 5B), with each peak containing more activity than in the absence of bacitracin.

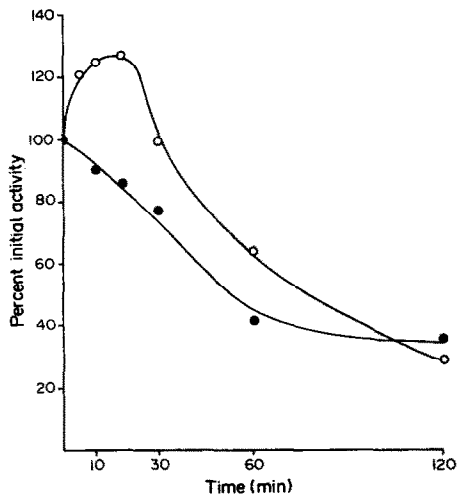


Fig. 4. Loss of opioid peptide-like immunoreactivity with time, on incubation of peptide E in human plasma, with (open circles) and without (closed circles) 5 mM bacitracin. Figures are means of duplicate incubations.

DISCUSSION

Numerous studies have investigated the degradation of enkephalins and related peptides by preparations from the brain, presumably with the intention of understanding the neuroregulatory function of these peptides (see, for example, refs 14–19). These studies illustrate the ubiquitous importance of aminopeptidase attack, with endopeptidase attack at the Gly^3–Phe^4 bond being significant under certain conditions. There has, however, been little information available as to the fate of these enkephalin-related peptides when introduced into the circulation, whether by endogenous release (from, for example, the adrenal medulla) or by administration. An early report shows a half-life for enkephalin of 2 min when incubated in rate plasma, with a reported slower rate of breakdown in human plasma [20] and suggests that the primary attack on the enkephalins in plasma is aminopeptidase.

The studies reported here utilized an assay cross-reacting with all these products of proenkephalin which have a free amino-terminal enkephalin sequence, enabling a wide variety of opioid peptides to be monitored. It should be noted that this cross-reactivity means that the assay of crude plasma extracts will not detect carboxypeptidase attack of enkephalin congeners until proteolysis reaches the pentapeptide enkephalin sequence itself. Thus, for example, proteolysis converting [Met]enkephalyl-Arg^6-Phe^7 to either [Met]enkephalin or [Met]enkephalyl-Arg^6 would not be detected. However, aminopeptidase attack of any of these peptides will abolish immunoreactivity.

Using this procedure our results show that the opioid peptide-like activity in plasma following introduction of [Met]enkephalin had a half-life of 8 min; when one of the small [Met]enkephalin derivatives was incubated in plasma instead, the half-life was about 2 min. The apparent half-life of [Met]enkephalin was surprisingly long; this was not a peculiarity of our method of measurement, since incubation of [^{3}H]tyrosyl [Leu]enkephalin in the place of unlabelled enkephalin (data not shown) gave a closely similar half-life, judged by generation of free [^{3}H] tyrosine. This observation also illustrated the possibility that the decline in immunoreactivity could be accounted for by aminopeptidase attack. The demonstration that both bacitracin and bestatin had a profound inhibitory effect on the breakdown of the enkephalins supports this possibility.

Adrenal peptide E is a twenty-five amino acid sequence [12] conserved in the proenkephalin of human and bovine tissue [21–23]. [Met]enkephalin forms the amino-terminus followed by double basic residues (Arg–Arg). [Leu]enkephalin forms the carboxyl-terminus preceded by double basic residues (Lys–Arg). On introduction into the human plasma the half-life of opioid peptide-like immunoreactivity was considerably greater than that generated by introduction of the smaller peptides, consistent with the observation that larger peptides are less susceptible to aminopeptidase attack than smaller peptides [24]. Half-life in plasma might then be related to endopeptidase attack generating smaller peptides which were rapidly destroyed by aminopeptidase. When adrenal peptide E was incubated in plasma under conditions in which aminopeptidase activity was inhibited by bacitracin, the observed transient increase in activity could only be accounted for by the exposure of the additional opioid sequence of the carboxy-terminal [Leu]enkephalin. We have shown here that both [Leu]enkephalin and [Met]-enkephalin were generated, with the digestion product [Met]-enkephalyl-Arg^6 during the incubation with human plasma both in the presence and absence of bacitracin. In the presence of this inhibitor, however, the levels of all these peptides were higher, as was the level of remaining adrenal peptide E. The latter observation suggests that peptide E, while being subjected to endopeptidase attack in plasma which generates the enkephalins, was also susceptible to a relatively slow rate of aminopeptidase attack. The observations reported here indicate that peptide E, if introduced into the circulation, may provide a sustained elevation of circulating opioid activity, and can act as a circulating precursor to the enkephalins.

The rapid breakdown of the small enkephalin congeners compared to that of [Met]enkephalin itself was unexpected. If the small congeners are released from chromaffin cells to have their physiological function locally in the adrenal medulla, as has been suggested [25] for [Met]enkephalin-Arg^6-Phe^7, then a rapid mechanism for their clearance from the circulation may be important.

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