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Angiotensin-Converting Enzyme Inhibitors Potentiate the Analgesic Activity of [Met]-Enkephalin-Arg^6-Phe^7 by Inhibiting its Degradation in Mouse Brain

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SUMMARY

The proteolytic degradation of the enkephalin-containing heptapeptide Tyr-Gly-Gly-Phe-Met-Arg-Phe (YGGFMRF) was investigated by incubating the peptide with synaptic membranes from mouse whole brain and characterizing the formed products. The degradation products were derivatized with 4-dimethylaminoazobenzene-4′-isothiocyanate and then analyzed by high pressure liquid chromatography and by amino-terminal analysis. The incubation of YGGFMRF with synaptic membranes yielded YGGFM and RF as the degradation products. The angiotensin-converting enzyme (ACE) inhibitors, MK-422 and captopril, potently inhibited the formation of YGGFM and RF with IC_{50} values of 8 nM and 95 nM, respectively. The "enkephalinase A" inhibitor, thiorphan, weakly inhibited this dipeptidyl carboxypeptidase activity with an $IC_{50} > 1$ μM. YGGFMRF, MK-422, captopril, and thiorphan all produced a dose-dependent analgesic response in the mouse hot plate test when administered intracerebroventricularly. However, when subanalgesic doses of inhibitors were co-administered with a subanalgesic dose of YGGFMRF, only the ACE inhibitors, MK-422 and captopril, potentiated the analgesic response of the peptide. These data provide *in vitro* and *in vivo* evidence that ACE is the primary enzyme involved in the proteolytic degradation of YGGFMRF in the mouse brain.

INTRODUCTION

[Met]-Enkephalin-Arg^6-Phe^7 was originally purified from bovine adrenal medullary granules and striatum (1) and subsequently shown to have potent analgesic activity when administered ICV[1] to mice (2). It is now evident that [Met]-enkephalin-Arg^6-Phe^7 belongs to a family of enkephalin-containing peptides and is derived from the proenkephalin A precursor protein (3). [Met]-Enkephalin-Arg^6-Phe^7 has been found to coexist with the pentapeptide enkephalins in all tissues examined (4, 5) but has distinct properties as a neurotransmitter or neuromodulator. For example, [Met]-enkephalin-Arg^6-Phe^7 has potent analgesic activity (2), binds to κ-opiate receptors (6), and can be released from brain slices in a Ca^{2+}-dependent manner (7).

The pathway of degradation for [Met]-enkephalin-Arg^6-Phe^7 was first investigated by Yang *et al.* (8), who found that this heptapeptide is rapidly hydrolyzed by a dipeptidyl carboxypeptidase present in bovine striatal microsomes. This observation has been substantiated by Benuck *et al.* (9), who have demonstrated that [Met]-enkephalin-Arg^6-Phe^7 is converted primarily to Met-enkephalin and Arg-Phe by rat brain synaptic membranes. They have found that this degradative step can be inhibited by MK-421, a potent and selective inhibitor of ACE (EC 3.4.15.1). Recently, Chou *et al.* (10) have found that substance P will induce the release of [Met]-enkephalin-Arg^6-Phe^7 from the perfused rat spinal cord and that the recovery of this peptide from the perfusate is enhanced when the ACE inhibitor, captopril, is present. These data strongly suggest that ACE is a primary enzyme in the degradation of [Met]-enkephalin-Arg^6-Phe^7 in rat brain. However, these studies used only one ACE inhibitor at a given concentration in their analysis of [Met]-enkephalin-Arg^6-Phe^7 degradation. In order to characterize pharmacologically the ACE activity in mouse brain responsible for the degradation of [Met]-enkephalin-Arg^6-Phe^7, several inhibitors of varying structure were evaluated in a concentration-dependent manner for their inhibition of this degradative process. The mouse was used in this study because the analgesic activity of these inhibitors and the heptapeptide can be more readily assessed in this organism, thus allowing a

[1] ICV, intracerebroventricular; ACE, angiotensin-converting enzyme (EC 3.4.15.1); MK-421, the ethyl ester (prodrug form) of MK-422; DABITC, 4-dimethylaminoazobenzene-4′-isothiocyanate; HPLC, high pressure liquid chromatography; DABTH, 4-dimethylaminoazobenzene-4′-thiohydantoin; DABTC, 4-dimethylaminoazobenzene-4′-thiocarbamoyl; MK-422, *N*-(1-(*S*)-carboxy-3-phenyl-propyl)-L-alanyl-L-proline; YGGFMRF, Tyr-Gly-Gly-Phe-Met-Arg-Phe.

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systematic investigation of the role of brain ACE in the degradation of [Met]-enkephalin-Arg^6-Phe^7. [Met]-Enkephalin-Arg^6-Phe^7 was incubated with synaptic membranes prepared from whole mouse brain, and the primary products formed were [Met]-enkephalin and Arg-Phe. This proteolytic step was potently inhibited in a concentration-dependent manner by the ACE inhibitors, MK-422 and captopril, but not by thiorphan, an inhibitor of enkephalinase A (EC 3.4.24.11). [Met]-Enkephalin-Arg^6-Phe^7 as well as MK-422, captopril, and thiorphan caused concentration-dependent increases in the mean latency to jump in the mouse hot plate test when injected ICV. However, only inhibitors of ACE potentiated the analgesic effect of [Met]-enkephalin-Arg^6-Phe^7 when co-injected ICV at subthreshold doses. These data provide biochemical as well as *in vivo* evidence that ACE is an important degradative enzyme in the proteolytic processing of Met-enkephalin-Arg^6-Phe^7 in the mouse central nervous system.

MATERIALS AND METHODS

Male albino mice (Cr1:CF-1 strain) weighing approximately 20 g were purchased from Charles River (Kingston, NY) and housed nine per cage with food and water available *ad libitum*. Mice were maintained on a diurnal light-dark cycle with all testing done during daylight hours. Arg-Phe And Met-enkephalin were purchased from Sigma Chemical Co. (St. Louis, MO). YGGFMRF was purchased from Peninsula Laboratories, Inc. (Belmont, CA). DABITC was purchased from Fluka Chemical Corp. (Hauppauge, NY) and was recrystallized in hot acetone according to the method of Chang *et al.* (11). Trifluoroacetic acid, phenylisothiocyanate, and pyridine (all sequencing grade) were purchased from Pierce Chemical Co. (Rockford, IL). Micropolyamide sheets for thin layer chromatography were purchased from Schleicher and Schuell, Inc. (Keene, NH). Ethyl acetate, *n*-heptane, hexane, toluene, and acetonitrile (HPLC grade) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). MK-422 was a gift from Merck Institute for Therapeutic Research (West Point, PA). Captopril was a gift from Squibb Institute for Medical Research (Princeton, NJ). Thiorphan was synthesized by chemists at CIBA-GEIGY Corp. (Summit, NJ).

HPLC. HPLC was carried out on a Partisil 5 ODS-3 C18 reversed-phase column (250 × 4.6 mm inside diameter, Whatman Inc., Clifton, NJ) using Waters Associates Inc. (Milford, MA) equipment consisting of two pumps (model 6000A), an automatic sample processor (model 710B), a UV-visible absorbance detector (model 441), a data module (model M730), and a system controller (model 720). Chromatography was performed by gradient elution in 35 mM sodium acetate containing 0.5% triethylamine, pH 5.0 (solvent A), and acetonitrile containing 0.5% triethylamine (solvent B) using the following gradient elution program. A linear gradient (program 6) was initiated after injecting 40 μl of sample running from 30% B to 80% B in 30 min. The eluate was monitored at 436 nm at an AUFS setting of 0.02.

Determination of amino-terminal amino acid of newly formed peptides. DABTC-peptides formed by proteolytic degradation were dissolved in 100 μl of 50% trifluoroacetic acid and incubated at 50° for 45 min. The samples were dried under N_2 and redissolved in 50 μl of 80% ethanol. The released DABTH-derivatized amino acids were identified by two-dimensional thin layer chromatography using 2.5 × 2.5 cm polyamide sheets. Ten microliters of sample were applied to the sheet with a glass micropipette and developed by ascending solvent flow in acetic acid/water (1:2, v/v) for the first dimension followed by toluene/*n*-hexane/acetic acid, (2:1:1, v/v/v) for the second dimension. The sheet was dried after the second dimension and exposed to HCl vapors causing all yellow spots to turn red or blue. The position of the DABTH amino acids were identified as red spots relative to the blue synthetic marker DABTC-diethylamine according to the method of Chang *et al.* (11).

Preparation of mouse synaptic membranes. Membranes were prepared from whole brains of male albino mice according to the procedure of Jones and Matus (12). The fractionation of brain tissue by this procedure involved the production of a crude mitochondrial fraction by differential centrifugation followed by hypotonic lysis and subsequent separation by simultaneous sedimentation and flotation centrifugation. Membranes prepared by this procedure had morphological characteristics typical of synaptic membranes and were enriched in peptidase activity. Membranes were stored at −70° in 50 mM Tris-HCl, pH 7.0, with no loss of enzyme activity for 2–3 months. The protein concentration in the striatal synaptic membranes was determined by the method of Lowry *et al.* (13).

Incubations. [Met]-Enkephalin-Arg^6-Phe^7 was incubated (final concentration 5 × 10^{-5} M) with membranes (250 μg/ml) in a total volume of 50 μl containing 15 mM sodium phosphate buffer, pH 7.0. Incubations were carried out at 30° for 1 hr and stopped by the addition of 100 μl of DABITC solution (3.53 mg/ml in pyridine). The DABITC reaction was carried out at 70° for 50 min; then 5 μl of phenylisothiocyanate were added for an additional 10 min at 70°. The excess DABITC was extracted by the addition of 0.7 ml of heptane/ethyl acetate (2:1, v/v). The tubes were vortexed and centrifuged at 2000 rpm in a Beckman table top centrifuge for 2 min. The heptane-ethyl acetate layer (top) was removed and the aqueous layer was reextracted until the top layer was clear. The aqueous layer containing the DABTC-peptides was dried under N_2 and then redissolved in 400 μl of H_2O/acetonitrile (7:3, v/v); 125 μl were removed for HPLC and the remaining 275 μl were dried under N_2 and stored at −20° for subsequent amino-terminal amino acid analysis.

Analgesia. Analgesia testing was done with a hot plate analgesia meter (IITC Inc., model 35-D) with a surface temperature maintained at 55° ± 0.5°C. An open Plexiglas cylinder (25.5 cm high and 16 cm inner diameter) confined the mice to the central area of the hot plate. The latency to the first jump constituted the response measure, with a cut-off time of 240 sec. Ten mice were used per dose, and each mouse was individually tested at 15 min following drug administration. Drugs and peptides were dissolved in distilled water. In studies of concurrent ICV administration, the inhibitor solution was used as the vehicle for [Met]-enkephalin-Arg^6-Phe^7. ICV injections consisting of a total volume of 10 μl were performed according to the technique of Haley and McCormick (14). Statistical significance ($p \leq 0.05$) was determined by analysis of variance followed by Dunnett's test for multiple comparisons. Statistical significance ($p \leq 0.05$) of the interaction groups was determined by analysis of variance followed by multiple comparisons using Tukey's Studentized range (HSD) test.

RESULTS

Degradation of [Met]-enkephalin-Arg^6-Phe^7 by synaptic membranes and characterization of formed products. The incubation of [Met]-enkephalin-Arg^6-Phe^7 with mouse brain synaptic membranes for 1 hr produced two major products as analyzed by HPLC (Fig. 1). The products formed were derived from the heptapeptide since membranes incubated without heptapeptide showed no DABTC-peptide peaks (Fig. 1A). Moreover, area and peak height of the heptapeptide were reduced relative to the zero time incubation which showed only a single peak of DABTC-peptide (Fig. 1B). The two DABTC-peptide products shown in Fig. 1C were identified as Arg-Phe and Met-enkephalin relative to the retention times of DABTC-derivatized reference peptides run under identical conditions. Amino-terminal amino acid analysis of the DABTC-peptides revealed that only DABTH-Tyr and DABTH-Arg were formed after 1 hr of incubation. Only DABTH-Tyr was present in the zero time incuba-

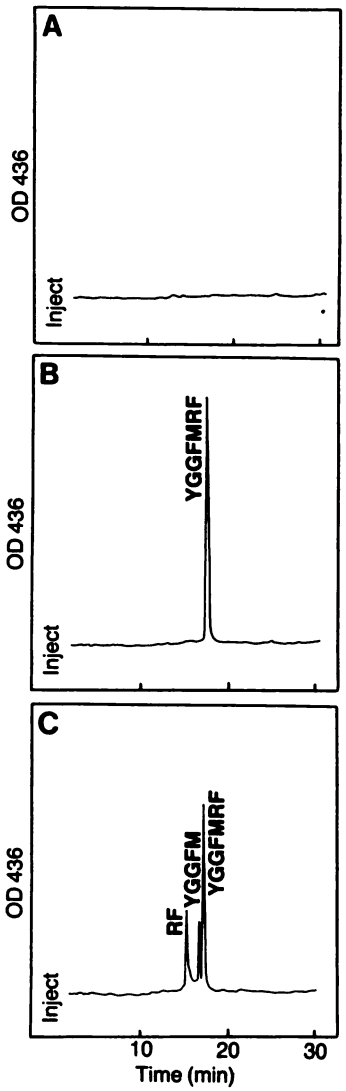

FIG. 1. *HPLC profiles of [Met]-enkephalin-Arg^6-Phe^7 digestion by synaptic membranes*

[Met]-enkephalin-Arg^6-Phe^7 (50 μM) was incubated with synaptic membranes for 60 min. The incubation was stopped by the addition of DABITC as described in "Materials and Methods," and aliquots were injected into a Partisil 5 ODS-3C18 reverse-phase column and eluted with a linear gradient of acetonitrile, containing 0.5% tetraethylamine, and 35 mM sodium acetate buffer, containing 0.5% tetraethylamine, pH 5.0, over 30 min. The eluate was monitored at 436 nm at an AUFS setting of 0.02. A, synaptic membranes were incubated in the absence of [Met]-enkephalin-Arg^6-Phe^7. B, synaptic membranes and [Met]-enkephalin-Arg^6-Phe^7 (50 μM) were incubated for zero time. C, synaptic membranes and [Met]-enkephalin-Arg^6-Phe^7 (50 μM) were incubated for 60 min.

tion. These results suggest that [Met]-enkephalin-Arg^6-Phe^7 is degraded by a dipeptidyl carboxypeptidase activity in mouse synaptic membranes. This dipeptidyl carboxypeptidase activity could not be stimulated by NaCl at concentrations up to 300 mM (data not shown).

Inhibition of [Met]-enkephalin-Arg^6-Phe^7 degradation by MK-422, captopril, and thiorphan. The degradation of [Met]-enkephalin-Arg^6-Phe^7 by synaptic membranes was potently inhibited by the ACE inhibitors MK-422 and captopril but not by the enkephalinase A inhibitor thiorphan. The formation of both products, [Met]-enkephalin and Arg-Phe, was equally inhibited by all drugs tested. Fig. 2 illustrates the concentration-dependent inhibition of product formation observed with these agents. MK-422 was the most potent inhibitor of [Met]-enkephalin-Arg^6-Phe^{7}degradation with an IC_{50} value of 8×10^{-9} M. This IC_{50} value is comparable to the results of Benuck *et al.* (9), who have reported an IC_{50} value of 10 nM with MK-421 (the prodrug form of MK-422) using rat synaptic membranes incubated with [Met]-enkephalin-Arg^6-Phe^7. The IC_{50} value for inhibition of purified ACE with MK-422 is 1.2 nM (15).

Captopril, another inhibitor of ACE, was also a potent inhibitor of [Met]-enkephalin-Arg^6-Phe^7 degradation with an IC_{50} value of 95 nM. This value agrees well with the reported IC_{50} value of 23 nM against purified ACE (16). The relative potency of these two ACE inhibitors is in good agreement with their potency for inhibiting [Met]-enkephalin-Arg^6-Phe^7 degradation. Thiorphan, an inhibitor of enkephalinase A, was a very weak inhibitor of [Met]-enkephalin-Arg^6-Phe^7 degradation ($IC_{50} > 10^{-6}$ M). The limited inhibition of [Met]-enkephalin-Arg^6-Phe^7 degradation by thiorphan is probably due to its weak ACE inhibitory activity (17). These results strongly indicate that ACE is the primary enzyme in mouse synaptic membranes responsible for the degradation of [Met]-enkephalin-Arg^6-Phe^7.

Analgesic activity of [Met]-enkephalin-Arg^6-Phe^7, MK-422, captopril, and thiorphan. [Met]-Enkephalin-Arg^6-Phe^7 showed a dose-dependent analgesic activity in the mouse hot plate test when injected ICV (Fig. 3). The range of active doses of heptapeptide shown in Fig. 3 was 4.3 to 68 nmol. This dose range is similar to that reported by Inturrisi *et al.* (2) and Hollt *et al.* (18) when they measured the analgesic activity of [Met]-enkephalin-Arg^6-Phe^7 in the mouse tail flick test after ICV injection of the peptide. These results demonstrate that [Met]-enkephalin-Arg^6-Phe^7 has analgesic activity in both types of analgesic tests in the same dose range. MK-422 had a dose-dependent analgesic activity in the range of 21.6 to 287 nmol when injected ICV (Fig. 3), but captopril required a higher dose of 1.4 μmol ICV to produce an analgesic response (Fig. 3). Thiorphan showed a dose-dependent analgesic activity (Fig. 3) with a potency comparable to that reported by Rocques *et al.* (19) using the mouse hot plate test at 55°.

To determine whether the analgesic activity of the ACE inhibitors, MK-422 and captopril, is due to the inhibition of [Met]-enkephalin-Arg^6-Phe^7 degradation, subanalgesic doses of peptide were co-administered with subanalgesic doses of inhibitor. A subanalgesic dose of 21.6 nmol of MK-422 significantly potentiated the analgesic activity of 1.1 nmol of [Met]-enkephalin-Arg^6-Phe^7 when co-administered ICV (Fig. 4). Captopril also potentiated the analgesic activity of the same dose of peptide when co-injected ICV at a dose of 463 nmol (Fig. 4). These results provide *in vivo* support for the *in vitro* evidence that ACE is the primary enzyme in brain for degrading [Met]-enkephalin-Arg^6-Phe^7. Thiorphan did not potentiate the analgesic activity of [Met]-enkephalin-Arg^6-Phe^7 (Fig. 4). Although thiorphan is a potent analgesic, it is a weak inhibitor of ACE and this is the

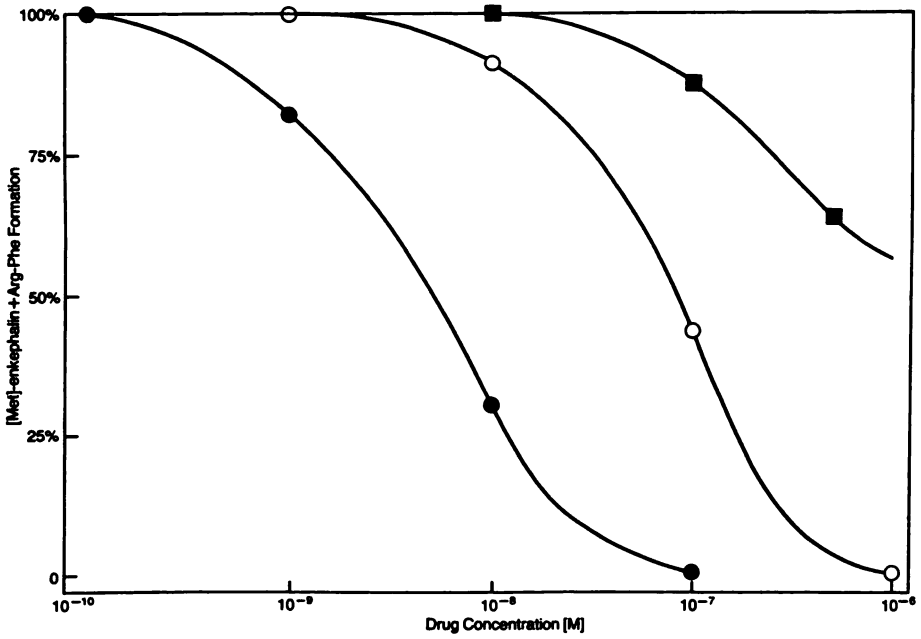

FIG. 2. *The inhibitory effects of MK-422, captopril, and thiorphan on [Met]-enkephalin-Arg^6-Phe^7 digestion by synaptic membranes*

[Met]-enkephalin-Arg^6-Phe^7 (50 μM) was incubated with synaptic membranes for 1 hr in the presence of increasing concentrations of the indicated drug. The incubations were stopped by the addition of DABITC solution and reacted as described in "Materials and Methods." The DABTC-peptides were analyzed by HPLC and the parallel decreases in YGGFM and RF formation were determined from the decrease in area under each peak. The results are expressed as the percentage of YGGFM plus RF formation in the absence of inhibitor. All drugs were dissolved in distilled H_2O just before dilution into the assay mix. (●), MK-422; (○), captopril; (■), thiorphan.

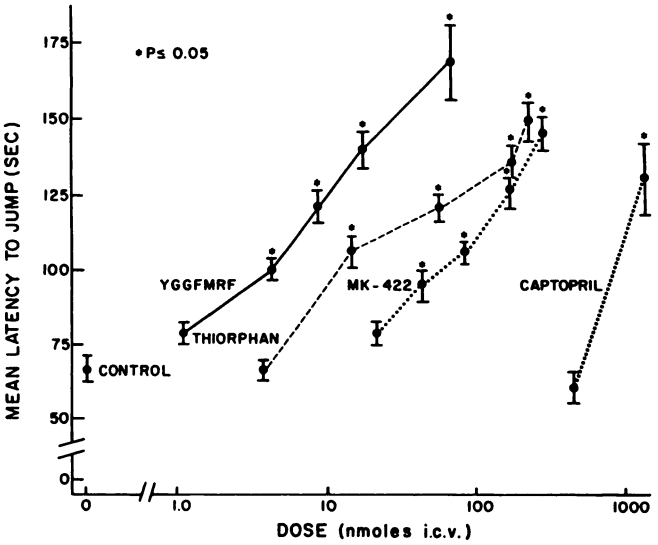

FIG. 3. *The analgesic effects of [Met]-enkephalin-Arg^6-Phe^7, MK-422, captopril, and thiorphan in the mouse hot plate test*

Groups of 10 mice were injected ICV with the indicated dose of drug or peptide in a volume of 10 μl. Fifteen minutes later, the mice were placed on a 55° hot plate and the latency to jump was recorded. All data points represent the mean latency to jump for the 10 mice in that group. The range indicated with each data point represents the standard error. *, significant at $p \leq 0.05$.

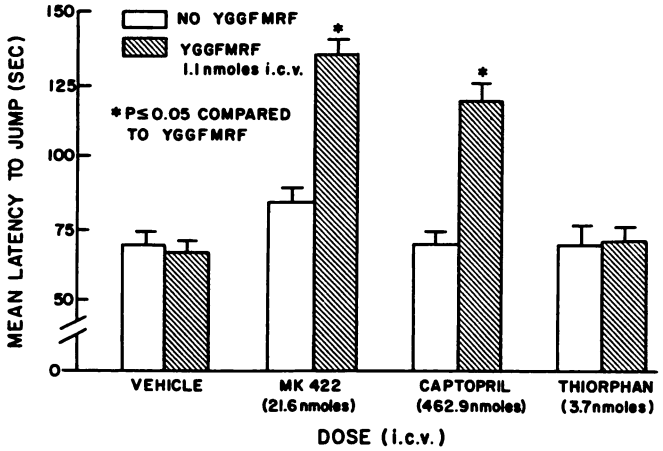

FIG. 4. *The potentiation of [Met]-enkephalin-Arg^6-Phe^7-induced analgesia by MK-422 and captopril*

Groups of 10 mice were given concomitant ICV injections of 1.1 nmol of [Met]-enkephalin-Arg^6-Phe^7 and the indicated drug at subanalgesic doses. Analgesia was measured as described in the legend to Fig. 3. The range indicated with each data point represents the standard error.

most likely explanation of its inability to potentiate [Met]-enkephalin-Arg^6-Phe^7.

DISCUSSION

This study systematically investigated the role of mouse brain ACE in the proteolytic degradation of [Met]-enkephalin-Arg^6-Phe^7. Although previous investigators have shown that [Met]-enkephalin-Arg^6-Phe^7 can be degraded by ACE in the striatum (8, 9), this study showed that synaptic membranes from mouse whole brain had one major degradative enzyme activity which cleaved the Arg-Phe dipeptide from the carboxyl terminus of [Met]-enkephalin-Arg^6-Phe^7. This enzyme activity could be potently inhibited in a dose-dependent manner by ACE inhibitors but not by an inhibitor of enkephalinase A. In the mouse hot plate test, [Met]-enkephalin-Arg^6-Phe^7, thiorphan, and both ACE inhibitors were found to produce a dose-dependent analgesic response when administered ICV. However, only the ACE inhibitors could potentiate the analgesic activity of [Met]-enkephalin-Arg^6-Phe^7. These data provide biochemical as well as *in vivo* pharmacological evidence that ACE is the primary enzyme in brain responsible for the proteolytic inactivation of [Met]-enkephalin-Arg^6-Phe^7.

Recently, Giraud *et al.* (5) have found that [Met]-enkephalin-Arg^6-Phe^7 is distributed heterogeneously

throughout the rat central nervous system, with the highest concentration in the striatum. Interestingly, Yang and Neff (20) have found that the striatum has the highest ACE activity in rat brain. Although a precise correlation of these data is not evident, there are no brain regions that contain [Met]-enkephalin-Arg^6-Phe^7 without also containing ACE activity. These investigators have also demonstrated that brain ACE activity can be stimulated 5-fold by the addition of NaCl, using Hip-His-Leu as a substrate. Cheung *et al.* (21) have shown that Cl^- stimulation of ACE activity is dependent on the substrate used in the assay. They have shown that ACE can be stimulated as much as 10-fold by 300 mM NaCl with substrates having His-Leu as the carboxyl-terminal dipeptide. Yet, the hydrolysis of other peptide substrates having Phe-Arg as the carboxyl-terminal dipeptide is optimally stimulated only 3-fold by 20 mM NaCl. Other peptide substrates such as bradykinin are cleaved by ACE but are inhibited by the presence of Cl^-. The lack of Cl^- stimulation observed in the present study with [Met]-enkephalin-Arg^6-Phe^7 as a substrate for brain ACE is also consistent with the finding of Benuck *et al.* (9) that [Met]-enkephalin-Arg^6-Phe^7 degradation by rat synaptic membranes cannot be stimulated by Cl^- but can be inhibited by MK-421.

An increasing number of reports have suggested that brain ACE is an important degradative enzyme for other neuropeptides. Norman and Chang (22) have shown that ACE is the primary enzyme in the degradation of [Met]-enkephalin-Arg^6-Gly^7-Leu^8 in rat striatum. The conversion of neurotensin 1-10 to neurotensin 1-8 by rat brain synaptic membranes has been shown by Checler *et al.* (23) to be potently inhibited by captopril. Yokosawa *et al.* (24) and Cascieri *et al.* (25) have both shown an unusual endopeptidase activity for ACE with substance P as a substrate. Although such *in vitro* evidence suggests that brain ACE may be a primary enzyme in the degradation of neuropeptides, more *in vivo* evidence is necessary to support these observations. In the study by Cascieri *et al.* (25), they have shown that substance P-induced salivation in rats can be potentiated by the co-administration of ACE inhibitors. This evidence supports the role of ACE as an important degradative enzyme for substance P in the periphery. In the present study, the potentiation of [Met]-enkephalin-Arg^6-Phe^7-induced analgesic activity by ACE inhibitors suggests that ACE plays a physiological role in the degradation of this peptide in the brain. This experimental approach has a precedent in the study by Rocques *et al.* (19) who have used thiorphan to potentiate the analgesic activity of (D-Ala^2-Met^5)-enkephalin in the tail-withdrawal test. This provided *in vivo* pharmacological evidence to support the role of enkephalinase A as an important enzyme in the catabolism of the pentapeptide enkephalins at the Gly^3-Phe^4 bond. Although enkephalinase A may also be able to cleave the Gly^3-Phe^4 bond of [Met]-enkephalin-Arg^6-Phe^7, the inability of thiorphan to potentiate the analgesic activity of [Met]-enkephalin-Arg^6-Phe^7 in the present study provides physiological evidence that enkephalin-containing peptides with carboxyl-terminal extensions follow different degradative pathways than the pentapeptide enkephalins.

Inhibitors of ACE are expected to become increasingly important as a first line therapy in the management of hypertension (16). The distribution of ACE inhibitors into various tissues, including the central nervous system, may be an important factor in the side effects manifested by these agents. For example, captopril has been shown to inhibit brain ACE when administered orally to rats (26). This result and the results of the present study, which demonstrate that ACE inhibitors can produce analgesia by preventing the degradation of an endogenous opioid peptide, may explain the mood-elevating properties of captopril in depressed patients (27). Such mood-elevating effects are difficult to characterize as favorable or unfavorable until more extensive clinical studies have been performed with ACE inhibitors. Studies are now in progress in this laboratory to determine whether ACE inhibitors of varying structural types will inhibit brain ACE activity after oral administration.

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