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PRELIMINARY COMMUNICATIONS

INHIBITION OF THE PARTIALLY PURIFIED CANINE LUNG ANGIOTENSIN I CONVERTING ENZYME BY OPIOID PEPTIDES

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The angiotensin I converting enzyme (EC 3.4.15.1), also referred to as kininase II, activates angiotensin I by removing the C-terminal dipeptide his-leu to form angiotensin II, and inactivates bradykinin, again by removal of a C-terminal dipeptide, phe-arg (1). Erdos et al. (2) have demonstrated that the angiotensin I converting enzyme (ACE) from human umbilical cell cultures can also remove a C-terminal dipeptide from methionine enkephalin (met-enk) or leucine enkephalin (leu-enk), thus functioning as an enkephalinase; these authors have hypothesized that the opioid peptides may function as ACE inhibitors, thus metabolically linking the opioid peptides with the angiotensins and kinins. Benuck and Marks reported that ACE isolated from rabbit brain removed dipeptides from both met-enk and leu-enk; the hydrolysis of met-enk was competitively inhibited by the ACE inhibitor SQ14225 (3). Cheung et al. have recently reported the isolation of two distinct metallopeptidases from rat brain; both cleave C-terminal dipeptides from met-enk, leu-enk, angiotensin I, and the synthetic ACE substrate hip-his-leu. However, on the basis of differential activation by chloride ions and differential inhibition by the peptide SQ20881, these authors theorize that the brain contains both the classical ACE and a distinct, although very similar, enkephalinase activity (4).

Arregui and Iversen have demonstrated that the opioid precursor β-lipotropin (β-LPH), a 91 amino acid pituitary peptide containing the sequences of β-endorphin (β-LPH: 61-91) and met-enk (β-LPH: 61-65), can competitively inhibit an ACE purified from human brain, with a calculated K_i of 0.78 μM. These authors were unable to demonstrate significant inhibition of this enzyme preparation by β-endorphin, met-enk, or D-ala^2-met-enk at concentrations up to 40 μM (5).

We report that β-endorphin, met-enk, and leu-enk competitively inhibit the ACE from canine lung.

METHODS AND MATERIALS

Enzyme preparation. A particulate ACE fraction was prepared from canine lungs using the general methodology of Sander and Huggins (6). Fresh lungs were perfused free of blood

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with iced saline, dissected free of hilar structures, homogenized in 50 mM Tris-HCl buffer (pH 7.4), and centrifuged at 2300 g for 30 min. The resulting supernatant fraction was centrifuged at 50,000 g for 30 min, and the pellet washed twice with the Tris buffer. The final pellet was resuspended in the Tris-HCl buffer and used as the source of ACE. Substrate hydrolysis by this fraction was chloride ion dependent and completely inhibited by both bradykinin (33.3 μM) and EDTA (16.7 μM), indicating that the hip-his-leu hydrolysis was due solely to ACE activity.

ACE assay. (^{14}C)Hip-his-leu (sp. act. 2-4 mCi/mmole), obtained from the New England Nuclear Corp. (Boston, MA), was used as ACE substrate in all experiments; no unlabeled substrate was added. All reagents were made up in the assay buffer, which was 50 mM Tris-HCl (pH 7.5, 0.3 M NaCl). Each reaction mixture consisted of 10 μl of enzyme preparation (containing 48 μg protein), appropriate substrate and opioid concentrations, and buffer to a final volume of 300 μl. All components of the reaction mixture were preincubated at 37° for 30 min, and the reactions initiated by addition of substrate to the enzyme-inhibitor mixture. Reactions were run for 30 min at 37°, and then stopped by addition of 1.0 ml of 0.1 N HCl. One ml of ethyl acetate was added to each tube, and the tube was shaken on a vortex mixer for 5 sec and then centrifuged at 1000 g for 10 min. Five hundred μl of the ethyl acetate (upper) layer, containing the (^{14}C)hippuric acid, was then added directly to a vial containing 10 ml of scintillation fluid. Activity was expressed as nmoles (^{14}C)hip released·min^{-1}·(mg protein)$^{-1}$. β-Endorphin, met-enk, and leu-enk were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

The ACE from canine lung is inhibited by β-endorphin, met-enk, and leu-enk, as indicated in Table 1. β-Endorphin-induced inhibition is first evident at concentrations of 5 μM, and

Table 1. Inhibition of angiotensin I converting enzyme by opioid peptides*

Inhibitor	Concn (μM)	I/S Ratio	% Inhibition
Met-enk	33.3	0.36	12
	160	1.7	20
	333	3.6	30
Leu-enk	33.3	0.36	11
	160	1.7	23
	333	3.6	38
β-Endorphin	5.0	0.05	12
	9.7	0.10	28
	24	0.26	38
	48	0.52	57

*All reaction mixtures contained (^{14}C)hip-his-leu in a final concentration of 94 μM. The I/S ratio represents the molar ratio of inhibitor to substrate in that particular reaction. The % inhibition is expressed relative to control reactions containing no inhibitor. Each data point represents the mean of two experiments, with each experiment performed in duplicate.

inhibition by met-enk and leu-enk at 33.3 μM. In these experiments the substrate concentration was 94 μM. The kinetics of ACE inhibition by β-endorphin and met-enk are illustrated in Fig. 1a; these experiments were performed with final hip-his-leu concentrations ranging from 5.8 to 29.2 nmoles per reaction mixture (19.3 μM to 97.3 μM). Fig. 1b depicts a Lineweaver-Burk plot of ACE inhibition by two concentrations (33.3 μM and 66.7 μM) of leu-enk.

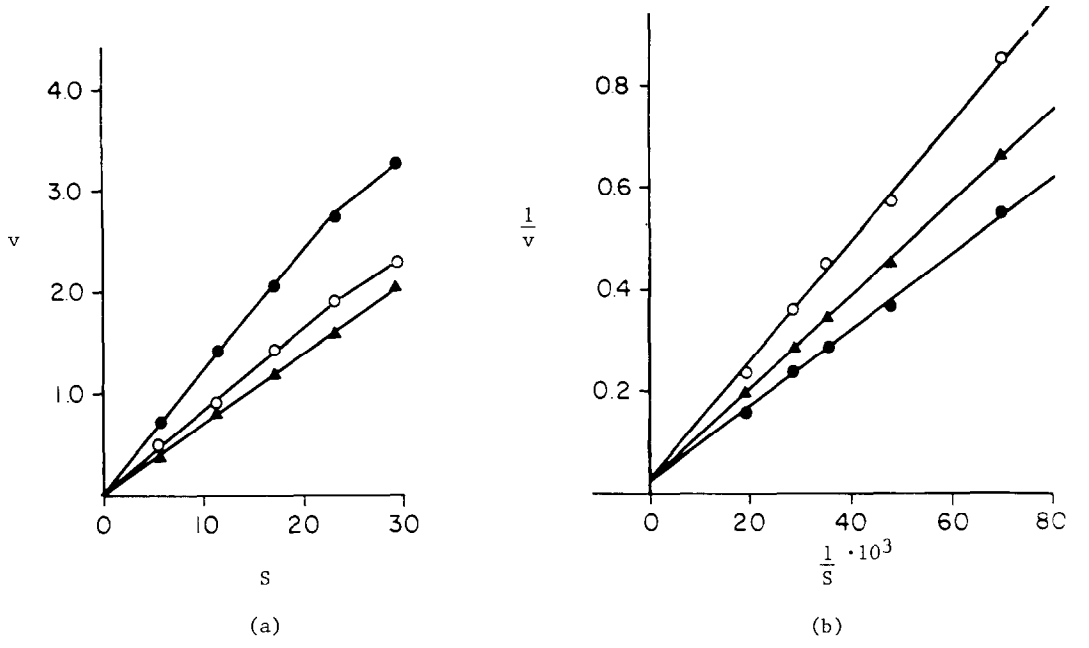

Fig. 1. (a) Rate of release of (^{14}C)hippuric acid by canine lung ACE as a function of the initial substrate (hip-his-leu) concentration, in the absence of inhibitor (●), with 66.7 μM met-enk (o), and with 24 μM β-endorphin (▲). S is expressed as total nmoles of substrate present per reaction mixture and v as nmoles·min^{-1}·(mg protein)$^{-1}$.
(b) Lineweaver-Burk plot of the hydrolysis of hip-his-leu by ACE in the absence (●) of leu-enk, and with 33.3 μM (▲) and 66.7 μM (o) leu-enk.

The inhibition pattern is indicative of competitive inhibition, with a calculated K_i for leu-enk of 146 μM. The data shown represent the results from a typical experiment. Each experiment was performed a minimum of two times, with virtually identical results. Met-enk and β-endorphin also inhibit ACE competitively (Lineweaver-Burk plots not shown), with a calculated K_i of 118 μM for met-enk and of 32 μM for β-endorphin.

The failure of Arregui and Iversen to demonstrate inhibition with these peptides may reflect the use of a much higher hip-his-leu concentration (1 mM) in the assay system which they utilized (7), as contrasted with the 94 μM concentration in our system. Thus, the inhibition is correspondingly more likely to be observed. Furthermore, the ACE which they used was isolated from human brain and may differ in activity and substrate specificity from the canine lung ACE.

Thus, β-endorphin, met-enk, and leu-enk competitively inhibit the ACE from canine lung;

this pattern is consistent with the fact that the enkephalins can function as ACE substrates, thus competing for the active site on the enzyme. β-Endorphin displays the greatest affinity for ACE, followed by leu-enk and met-enk. We did not use β-LPH in our experiments; however, based on the results of Arregui and Iversen, it presumably would display a higher affinity than β-endorphin. Leu-enk would appear to be a slightly better substrate than met-enk; this may reflect a greater structural similarity of leu-enk to the natural substrate angiotensin I, since both peptides have the leucine residue at the C-terminal position.

Although these opioids do competitively inhibit the ACE, they display considerably less affinity for the ACE than does the nonapeptide SQ20881, which has a reported K_i of 0.6 μM (8). Furthermore, the concentrations of these opioids used to inhibit the ACE are in excess of the concentrations of these peptides normally present in vivo (9,10). However, the hip-his-leu concentration (94 μM) was also far in excess of the physiological concentrations of the natural substrates angiotensin I, 8.48 to 67.8 pM (11), and bradykinin, 2.02 to 4.19 nM (12), present in human plasma. β-Endorphin has been shown to be released from the pituitary in parallel with ACTH following stress, with plasma levels rising as high as 2.5 nM in rats after adrenalectomy (13). Thus, concentrations of β-endorphin in the 2.5 nM range would provide I/S ratios in excess of the 0.52 I/S ratio which we have demonstrated to produce 57% inhibition of ACE activity. The intravenous administration of β-endorphin and the enkephalins has been demonstrated to alter both blood pressure and heart rate (14,15). Hence the possibility must be considered that, at least in certain physiological situations characterized by high endogenous opioid levels, β-endorphin may influence the metabolism of angiotensin I and bradykinin by interacting with the lung ACE, and in this way participate in cardiovascular control.

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