THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 10, Issue of March 7, pp. 8547–8555, 2003

Printed in U.S.A.

Novel Natural Peptide Substrates for Endopeptidase 24.15, Neurolysin, and Angiotensin-converting Enzyme*

Received for publication, November 25, 2002, and in revised form, December 12, 2002
Published, JBC Papers in Press, December 24, 2002, DOI 10.1074/jbc.M212030200

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Endopeptidase 24.15 (EC 3.4.24.15; ep24.15), neurolysin (EC 3.4.24.16; ep24.16), and angiotensin-converting enzyme (EC 3.4.15.1; ACE) are metallopeptidases involved in neuropeptide metabolism in vertebrates. Using catalytically inactive forms of ep24.15 and ep24.16, we have identified new peptide substrates for these enzymes. The enzymatic activity of ep24.15 and ep24.16 was inactivated by site-directed mutagenesis of amino acid residues within their conserved HEXXH motifs, without disturbing their secondary structure or peptide binding ability, as shown by circular dichroism and binding assays. Fifteen of the peptides isolated were sequenced by electrospray ionization tandem mass spectrometry and shared homology with fragments of intracellular proteins such as hemoglobin. Three of these peptides (PVNFKFLSH, VVYPWTQRY, and LVVYPWTQRY) were synthesized and shown to interact with ep24.15, ep24.16, and ACE, with K_i values ranging from 1.86 to 27.76 μM. The hemoglobin α-chain fragment PVNFKFLSH, which we have named *hemopressin*, produced dose-dependent hypotension in anesthetized rats, starting at 0.001 μg/kg. The hypotensive effect of the peptide was potentiated by enalapril only at the lowest peptide dose. These results suggest a role for hemopressin as a vasoactive substance *in vivo*. The identification of these putative intracellular substrates for ep24.15 and ep24.16 is an important step toward the elucidation of the role of these enzymes within cells.

Endopeptidase EC 3.4.24.15 (ep24.15; also referred to as thimet oligopeptidase) and endopeptidase EC 3.4.24.16 (ep24.16; also referred to as neurolysin) were initially detected in and purified from rat brain homogenates (1, 2). The cloned rat brain ep24.16 (3) showed 80% similarity and 63% identity with the previously cloned rat testis ep24.15 (4). Both peptidases share most of their natural substrates, including bradykinin, neurotensin, opioids, angiotensin I, and gonadotrophin-releasing hormone (5, 6). All of these natural substrates are hydrolyzed at the same peptide bond and at similar rates, except for neurotensin, which is hydrolyzed by ep24.15 and ep24.16 by cleavage of its Arg^8–Arg^9 and Pro^{10}–Tyr^{11} bonds, respectively (7).

Functional studies have suggested that ep24.15 and ep24.16 inactivate neuropeptides inside and outside the central nervous system. The central administration of *Z*-(Leu,Asp)Phe-$\psi(PO_2CH_2)$(Leu,Asp)Ala-Lys-Met, a fully specific endopeptidase ep24.15 inhibitor (8), prolongs the forepaw licking latency of mice in the hot plate test and following the injection of submaximally active doses of neurotensin (9). Likewise, the intracerebroventricular administration of Pro-Phe-$\psi(PO_2CH_2)$-Leu-Pro-NH_2, a selective ep24.16 inhibitor (10), significantly increases the neurotensin-induced antinociception of mice in the hot plate test (11).

Outside the central nervous system, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate, an inhibitor of both ep24.15 and ep24.16, potentiates bradykinin-induced hypotension, which suggests that one or both of these peptidases participate in the metabolism of bradykinin (12). In macrophages, CFP-AAF-pAb, a mixed inhibitor of ep24.15 and ep24.16 (7), reduces antigen presentation through the major histocompatibility complex class I (MHC-I)[1] but not through MHC-II (12). Conversely, liposome-mediated introduction of ep24.15 into macrophages stimulates the antigen presentation of MHC-I, but not that of MHC-II. The observation that ep24.15 can degrade or bind to several MHC-I antigenic peptides (13, 14), which are 8–11 amino acid fragments generated in the cytoplasm by proteasomes (15, 16), raises the possibility that ep24.15 and ep24.16 participate in the intracellular metabolism of peptides. The nature of such peptides is unknown.

Angiotensin I-converting enzyme (ACE; peptidyldipeptidase

* This work was supported in part by the São Paulo State Research Foundation Grants 96/1451-9, 99/01983-9, 00/04297-8, 00/11176-2, and 01/07544-9. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[b] Supported by a São Paulo State Research Foundation Ph.D, postdoctoral fellowship Grant 99/07738-6, and is a Ph.D. student in the Molecular Biology Program at the Paulista Medical School/UNIFESP, São Paulo.

[d] Supported by São Paulo State Research Foundation Ph.D and postdoctoral fellowship Grant 00/11524-0.

[g] Supported by São Paulo State Research Foundation Ph.D and postdoctoral fellowship Grant 00/01419-5.

[i] Supported by São Paulo State Research Foundation Ph.D and postdoctoral fellowship Grant 99/06541-4.

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[1] The abbreviations used are: MHC, major histocompatibility complex; ACE, angiotensin I-converting enzyme; HPLC, high performance liquid chromatography; QFS, quenched fluorescent substrate; BK, branykinin; Dyn, dynorphin A1–13; Aib, α-aminoisobutyric acid; NO, nitric oxide.

A) is a zinc metallopeptidase that cleaves the COOH-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II (17) and inactivates bradykinin by the sequential removal of two COOH-terminal dipeptides (18). In addition to these two main physiological substrates, which are involved in blood pressure regulation and water and salt metabolism, ACE also cleaves COOH-terminal dipeptides from various oligopeptides with a free COOH terminus. ACE has also been implicated in a range of physiological processes unrelated to blood pressure regulation, such as immunity, reproduction, and neuropeptide regulation, based on its localization and/or the *in vitro* cleavage of a range of biologically active peptides. The role of ACE in blood pressure control and water and salt metabolism has been defined mainly by the use of highly specific ACE inhibitors (19). These inhibitors are effective in the treatment of hypertension, congestive heart failure, and diabetic nephropathy (20–22). Moreover, ACE has recently been implicated in the hydrolysis *in vivo* of the tetrapeptide Ac-Ser-Asp-Lys-Pro, which modulates hematopoietic stem cell proliferation by preventing their recruitment into the S phase (23). The acute administration of captopril, an ACE inhibitor, produces a 7-fold increase in the plasma concentration of Ac-Ser-Asp-Lys-Pro in normal volunteers, thus demonstrating the importance of ACE in the metabolism of this substrate (24).

In this study, we show that ep24.15 and ep24.16, when catalytically inactivated by site-directed mutagenesis of amino acid residues within their HE*XX*H motifs, can be used to identify new endogenous peptides present in crude peptide extracts prepared from rat tissues. The ep24.15 or ep24.16 enzyme-bound peptides were isolated and many of them fully sequenced by electrospray ionization tandem mass spectrometry. Based on these sequences, synthetic peptides were prepared and shown to interact strongly with ep24.15, ep24.16, and ACE. One of the peptides identified here (PVNFKFLSH), derived from the α_1 chain of hemoglobin, was among the best natural substrates identified so far for these enzymes, and caused dose-dependent hypotension in rats. This peptide, which we have named *hemopressin*, may have a role in blood pressure regulation and in cardiovascular disease.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Protein Expression—A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce a specific point mutation in the wild type ep24.15 or ep24.16 cDNA cloned into the expression vector pGEX4t-2 (7). Oligonucleotide primers were synthesized with mismatches coding for alanine, based on prokaryotic codon usage rules to obviate the use of rare codons that could hinder subsequent protein expression. Point mutations were specified as H473A, E474A, H477A, and E502A for ep24.15, and H474A, E475A, H478A, and E503A for ep24.16. PCR was done in a 50-μl mixture using 50 ng of template plasmid DNA, 14 pmol of each primer, 10 nmol of dNTPs, and 2.5 units of *turbo Pfu* DNA polymerase (Stratagene) in 0.5× *Pfu* polymerase reaction buffer. The thermocycler was programmed for an initial denaturation at 95 °C for 1 min followed by 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 15 min, with a final incubation at 72 °C for 10 min. One microliter (20 units) of *Dpn*I (New England Biolabs) was added to the sample (50 μl) and incubated at 37 °C for 16 h. The sample was then denatured at 65 °C for 30 min. Two microliters of the final sample were used to transform competent *Escherichia coli* XL1-blue cells by electroporation. Putative positive colonies were confirmed by double-stranded template dideoxy sequencing (25). Expression and purification of the wild type or mutant proteins for biochemical characterization were done as described (7), with all enzymes stored at −80 °C for subsequent analysis.

SDS-PAGE—The homogeneity of the recombinant enzyme preparations was assessed by electrophoresis under reducing conditions in 8% polyacrylamide gels containing SDS-PAGE, as described previously (26). Protein bands were detected by staining the gels with Coomassie Brilliant Blue R-250 (Bio-Rad).

DNA Sequencing—DNA was sequenced using a multicapillary MegaBace1000 sequencer, according to the protocol supplied with the DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences).

Peptide Synthesis—Peptides were synthesized by the Resgen-Invitrogen Corporation using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry.

Peptide Extract from Rat Tissues—A crude peptide extract from rat brain or spleen was prepared as previously described (27). Briefly, male Wistar rats were killed and the brain and spleen were removed and rapidly frozen in liquid nitrogen prior to storage at −80 °C. Tissues from five rats were added to boiling 0.1 M acetic acid and homogenized (Polytron, Brinkmann). The tissue homogenates were boiled for 10 min, centrifuged at 50,000 × *g* for 30 min at 4 °C, and the supernatant was filtered through a Millipore centrifugal filter unit with a NMCO of 5,000. The filtrate was adjusted to pH 7.4 with 1 M Tris-HCl (pH 7.4) and then used in the experiments described below.

Enzyme-Peptide Binding Assay—Enzyme (1–5 nmol)-peptide complexes were produced by incubating a specific synthetic peptide or the peptide extract with catalytically inactive ep24.15 or ep24.16 in 200 μl of buffer (25 mM Tris-HCl, pH 7.5, containing 125 mM NaCl and 0.1% of bovine serum albumin) for 30 min at room temperature. At the end of this period, the reaction mixture was layered onto a dried Sephadex G-25 column (previously washed and equilibrated with Tris-buffered saline followed by centrifugation to remove the buffer) and centrifuged at 1000 × *g* for 2 min. The flow-through (~200 μl) was collected and the peptide content analyzed by high performance liquid chromatography (HPLC) using a Chromolith performance column (4.6 mm × 100 mm; Merck), with a linear gradient of 5–35% acetonitrile in 0.1% trifluoroacetic acid, for 20 min, and at a flow rate of 1 ml/min, as previously described (7). Control experiments were done by: (i) adding an excess of dynorphin A_{1-13} (30 μM) to the reaction mixture as a specific competitive inhibitor for ep24.15 and ep24.16, (ii) performing the assay in the presence of wild type active ep24.15 and ep24.16, and (iii) preforming the assay in the absence of ep24.15 and ep24.16 (reaction mixture containing only 0.1% of bovine serum albumin).

Peptide Sequencing by ESI-MS/MS—Peptides were sequenced by positive nano-electrospray ionization (nano-ESI+) using peptide-containing aliquots collected during HPLC. Typical conditions were a capillary voltage of 1 kV, a cone voltage of 30 V, and a desolvation gas temperature of 100 °C. The protonated peptides were subjected to collision-induced dissociation with argon in the 15–45 eV collision energy range. All of the mass spectrometry experiments were done with a Q-TOF mass spectrometer (Micromass, UK) in Qq-orthogonal time-of-flight configuration. Peptide sequences were determined manually from the ESI-MS/MS product ion mass spectra with the help of the PepSeq software (Micromass).

Determination of the Peptide Bonds Cleaved—The peptide bonds cleaved were identified by isolating the fragments by HPLC followed by ESI-MS/MS mass spectrometry sequencing, as described above.

Peptide Sequence Homologies—To identify the putative protein precursors of the peptides sequenced by ESI-MS/MS, a protein data base (www.ncbi.nlm.nih.gov/blast) was searched for short, nearly exact matches (rodentia origin), as previously described (28). When the perfect match for a given peptide was not found in a large protein sequence, more than one putative protein precursor containing part of the identified peptide was listed.

Enzyme Activity Assay and Determination of Kinetic Parameters—The enzymatic activity of wild type and mutant ep24.15 and ep24.16 was determined in duplicate in a continuous assay using the quenched fluorescent substrate (QFS) (7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-dLys-(2,4-dinitrophenyl), as previously described (29, 30). ACE (Sigma) enzymatic activity was measured similarly using the internally quenched fluorescent peptide Abz-FRK(2,4-dinitrophenol)P-OH (31). The relative inhibition constants (K_i) of the new synthesized peptides were determined in parallel with well known substrates or competitive inhibitors as a reference. The following equations were used to calculate the K_i values: $K_i = K_{i,app}/(1 + [S]/K_m)$, where [S] = molar concentration of the substrate, K_m = Michaelis-Menten constant, and $K_{i,app}$ = apparent inhibition constant, assuming [S] = K_m (10 μM) (26). The $K_{i,app}$ was calculated using the equation, $V_o/V_i = 1 + [I]/K_{i,app}$, where V_o = velocity of hydrolysis without the inhibitor, V_i = velocity of hydrolysis in the presence of the inhibitor, and [I] = molar inhibitor concentration. In a plot of $(V_o/V_i) - 1$ *versus* [I], the slope is $1/K_{i,app}$. To determine the K_i values, five solutions with synthetic peptide concentrations ranging from 0.1 to 100 μM were used to construct the graph $(V_o/V_i) - 1$ *versus* [I]. The relative hydrolysis ratio was determined using peptides at a concentration of 100 μM, under zero-order kinetics, with less than 10% of the substrate consumed by the end of the incubation period, which

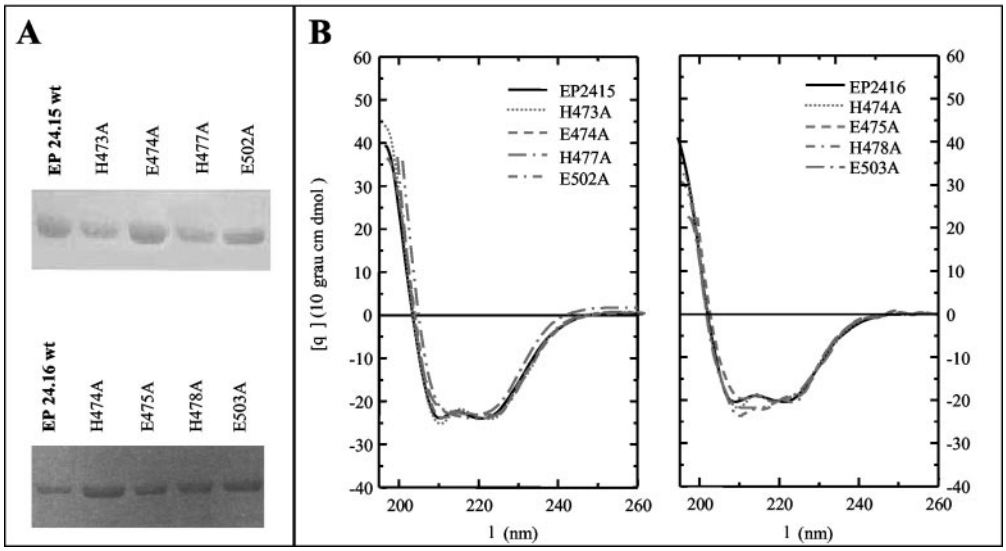

FIG. 1. **SDS-PAGE and circular dichroism spectra of wild type and mutated ep24.15 and ep24.16.** *A*, SDS-PAGE electrophoresis followed by Coomassie Blue staining showing the homogeneity of the wild type and mutated ep24.15 and ep24.16 enzymes (15 μg each) obtained after a single-step affinity chromatography on a Sepharose-glutathione *S*-transferase column. *B*, CD spectra were obtained using a Jasco model 720 spectrometer at 0.5-nm intervals in the wavelength range of 190 to 260 nm. The settings used were a resolution of 0.5 nm, a response time of 0.5 s, a scan speed of 10 or 20 nm/min (4 or 5 scans), a cell path length of 0.01 or 0.02 cm, and a temperature of 20–22 °C. The samples were prepared in 10 mM Tris-HCl (pH 7.4).

varied from 30 min to 2 h. The enzyme concentration varied from 5 to 50 ng/assay. All assays were done in triplicate.

Circular Dichroism (CD)—The secondary structure of selected mutants displaying a substantial decrease in catalytic and inhibitor binding capacity was examined by CD spectroscopy using a Jasco 720 spectropolarimeter. The instrument calibration was verified using an aqueous solution of d_{10}-camphorsulfonic acid, and the CD spectra were collected in the wavelength range of 190 to 260 nm at 0.5-nm intervals, with a resolution of 0.5 nm, a response time of 0.5 s, a scan speed of 10 or 20 nm/min for 4 or 5 scans, a cell path length of 0.01 or 0.02 cm, and a temperature of 20–22 °C. Samples were prepared in 10 mM Tris-HCl (pH 7.4). Secondary structure estimation of the proteins was done using the SELCON3 algorithm (27).

Protein Concentration—For the CD experiments, protein concentrations were determined as described by Gill and von Hippel (33). For all other purposes, protein concentrations were determined by the Bradford assay (34) using bovine serum albumin as standard.

Action of Selected Peptides on Blood Pressure in Anesthetized Rats—To examine the action of peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL on blood pressure, male Wistar rats (~200 g) were anesthetized with sodium pentobarbital (>60 mg/kg, intraperitoneal; Hypnol™, Cristália, Itapira, SP, Brazil) and placed under a heating lamp to maintain body temperature. The trachea was cannulated to facilitate breathing, and the left carotid artery and left femoral vein were cannulated with polyethylene tubing for the measurement of arterial blood pressure and drug/peptide administration, respectively. The cannulas were kept patent with heparinized 0.9% (w/v) saline. The arterial pressure was recorded continuously via a pressure transducer (Abbott, Chicago, IL) coupled to a computer-controlled data acquisition system (Transonics Systems, Inc., Ithaca, NY). The experiments were initiated after at least 15 min for stabilization. Bradykinin (BK), angiotensin II, and peptides (PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL) were dissolved and administered in 0.9% saline. For PVNFKFLSH, the doses tested ranged from 0.001 to 10 μg/kg, whereas for LVVYPWTQRY and FDLTADWPL, only two doses (10 and 100 μg/kg) were examined. The order of dose administration was randomized in all experiments. The responsiveness of the preparations was assessed by administering a single dose of BK (3 μg/kg) and angiotensin II (3 μg/kg) before peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL, and then at the end of the experiment to assess whether there was any alteration in the response to these two agonists. In separate experiments, enalapril (2 mg/kg, intravenously) was given 10–15 min before administration of the lowest doses (0.001, 0.01, and 0.1 μg/kg) of PVNFKFLSH to assess the influence of ACE inhibition on the action of this peptide. In all cases, bolus intravenous injections (100 μl) of peptides were washed in a further 100 μl of saline. The animal protocols and procedures described here were done in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the general principles for the care and use of animals established by the Brazilian College for Animal Experimentation (COBEA).

Statistical Analysis—The blood pressure changes were expressed as the mean ± S.E. of the peak changes in mean arterial blood pressure (in mm Hg) relative to the values recorded immediately prior to peptide administration. Differences between doses and treatments were compared using Student's t test or analysis of variance followed by the Tukey test, as appropriate. A value of $p < 0.05$ indicated significance.

RESULTS

Site-directed mutagenesis of the cDNA encoding rat testis ep24.15 and pig liver ep24.16 was used to prepare mutants in which the histidine and glutamic acid residues of the HE*XX*H motif conserved within an active site α-helix were genetically substituted. Two additional glutamate residues carboxyl to the HE*XX*H motif, Glu^{502} in ep24.15 and Glu^{503} in ep24.16, were also mutated. The wild-type ep24.15 and ep24.16 have previously been expressed in DH5α *E. coli* in a catalytically active form that resembles the proteins isolated from mammalian tissue (7). Isopropyl-1-thio-β-D-galactopyranoside induction of transformed DH5α *E. coli* triggers a time-dependent overexpression of specific proteins, the apparent molecular weight of which corresponds to the calculated mass of ep24.15 or ep24.16 fused with glutathione *S*-transferase; the maximal production of the fusion proteins similarly reached a plateau by 4 h (data not shown). Proteolytic removal of glutathione *S*-transferase and subsequent purification of the recombinant proteins allowed the recovery of apparently homogenous peptidases based on SDS-PAGE analysis (Fig. 1*A*). The production yield (~0.5 mg/liter of culture) was similar for all expressed proteins, suggesting that mutation of the above mentioned amino acid residues did not affect the relative levels of ep24.15 or ep24.16 expression in DH5α *E. coli*.

To ensure that the mutated ep24.15 and ep24.16 had not lost enzymatic activity as a result of gross structural alterations during mutagenesis and subsequent protein expression, the secondary structures of these enzymes were compared with those of the catalytically active wild-type proteins. The CD spectra suggested that both ep24.15 and ep24.16 had a typical α-helix secondary structure (Fig. 1*B*) that was not significantly modified by any of the mutations that inactivated the catalytic

TABLE I
Deconvolution of the CD spectra shown on Fig. 1B

Secondary structure estimation of the proteins was performed using data in the wavelength range of 190–260 nm using CONTIN algorithm (32). Protein was quantified according to the method described by Gill and von Hippel (33). The values are the mean ± S.E. of three determinations.

	Total α-helix	Total strand	Turns	Unordered
EP24.15 wt[a]	0.42 ± 0.05	0.16 ± 0.04	0.16 ± 0.03	0.27 ± 0.05
H473A	0.40 ± 0.05	0.20 ± 0.04	0.15 ± 0.03	0.26 ± 0.05
E474A	0.41 ± 0.05	0.20 ± 0.04	0.15 ± 0.03	0.26 ± 0.05
H477A	0.39 ± 0.05	0.16 ± 0.04	0.20 ± 0.03	0.26 ± 0.05
E502A	0.41 ± 0.05	0.16 ± 0.04	0.17 ± 0.03	0.25 ± 0.05
EP24.16 wt	0.47 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.26 ± 0.02
H474A	0.44 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.27 ± 0.02
E475A	0.47 ± 0.02	0.12 ± 0.01	0.13 ± 0.01	0.28 ± 0.02
H478A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.27 ± 0.02
E503A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.28 ± 0.02
E510A	0.44 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.27 ± 0.02

[a] wt = wild type

TABLE II
Quantitative measurements of the peak area of dynorphin $A_{1–13}$ (Dyn) and BK peptides separated by gel filtration, in the absence or presence of inactive ep24.15 (E474A) or ep24.16 (E475A)

The average of the two peaks areas obtained after gel filtration of the peptides in the absence of the inactive enzymes was taken as control (zero). Observe the proportional increment of the peptide peaks eluted in the presence of different concentrations of the inactive enzymes.

	Peak area		Average of first and second run	Resulting area relative to control experiments
	First run	Second run		
Dyn	20,605	19,657	20,131	Zero (control)
Dyn + E474A, 1 nM	51,399	48,460	49,929	29,798
Dyn + E474A, 5 nM	105,235	101,016	103,125	82,994
Dyn + E475A, 1 nM	59,854	59,880	59,867	39,736
Dyn + E475A, 5 nM	72,578	68,549	70,563	50,432
Bk	108,765	114,469	111,615	Zero (control)
Bk + E474A, 1 nM	132,327	137,971	135,149	23,534
Bk + E474A, 5 nM	148,450	157,923	153,186	41,571
Bk + E475A, 1 nM	150,060	153,752	151,906	40,291
Bk + E475A, 5 nM	164,417	161,976	163,196	51,581

activity (Table I). The effects of individual mutations on the catalytic activity of ep24.15 and ep24.16 were assessed using a QFS. As expected, complete ablation of enzymatic activity was observed when point mutations were made for H473A, E474A, H477A, and E502A in ep24.15, or H474A, E475A, H478A, and E503A in ep24.16.

Following the structural characterization of the wild type and mutant ep24.15 and ep24.16, we examined whether the inactive enzymes would bind to bioactive peptides such as dynorphin $A_{1–13}$ and bradykinin. Initial binding assays done with ^{125}I-dynorphin $A_{1–13}$ suggested a similar ability of all inactive enzymes to bind this peptide (data not shown). Therefore, the E474A and E475A mutants were selected for further experiments, as this specific glutamic acid is believed to be involved directly in substrate catalysis, whereas the other residues are involved in zinc ion coordination (19). To further characterize the ability of the E474A and E475A mutants to bind bioactive peptides, such as bradykinin and dynorphin $A_{1–13}$, enzyme-peptide complexes were allowed to form in solution and the excess of unbound peptide was removed by gel filtration. The resulting complexes were analyzed by HPLC. The results from these assays (Table II) supported the idea that catalytically inactive ep24.15 (E474A) and ep24.16 (E475A) retained the ability to bind bioactive peptides such as bradykinin and dynorphin $A_{1–13}$.

To assess the usefulness of these mutants for isolating new substrates for ep24.15 and ep24.16, the E474A and E475A mutants were incubated with a rat brain peptide extract and the enzyme-peptide complexes were separated from the excess of free peptides by gel filtration and then subjected to HPLC. The presence of either E474A or E475A in the incubation with peptide extract was critical for obtaining increasing amounts of specific peptide peaks, as shown in the HPLC chromatograms (Fig. 2). In control experiments without the inactive enzymes to complex and arrest the peptides, only small peaks were seen (Fig. 2). For the moment, it is unclear whether these smaller peaks represent lower amounts of peptides bound to the inactive enzymes. Equivalent experiments done with active ep24.15 or ep24.16 produced chromatograms without a significant increase in the peptide peaks, when compared with those obtained using the inactive enzymes (data not shown). Hence, catalytic inactivation before incubation with the crude peptide extracts was important to recover larger amounts of the putative natural substrates of these enzymes. To determine whether binding of the peptides by the inactive enzymes involved a specific interaction, dynorphin $A_{1–13}$ (30 μM), a potent competitive inhibitor of both ep24.15 and ep24.16, was added to the peptide extracts. Dynorphin $A_{1–13}$ clearly reduced the number of peptide peaks observed in the chromatograms (Fig. 2, *A* and *B*). Thus, it is reasonable to conclude that the above procedures were appropriate for identifying peptides that interact specifically with ep24.15 and ep24.16.

To identify the peptides that bound to inactive ep24.15 and ep24.16, the peptide peaks were collected manually during HPLC (Fig. 2*C*) and were further analyzed by nano-ESI-MS/MS. A representative deconvoluted ESI-MS/MS product-ion mass spectrum, which allowed the complete sequencing of peptide PVNFKFLSH, is shown in Fig. 3. The isotopic cluster separation of 0.5 mass/charge ratio (*m/z*) units of the precursor ion and its *m/z* reveals that doubly charged, doubly protonated peptide molecules of mass 1088.92 (M + H) were formed by ESI ionization and mass selected for MS/MS sequencing. The se-

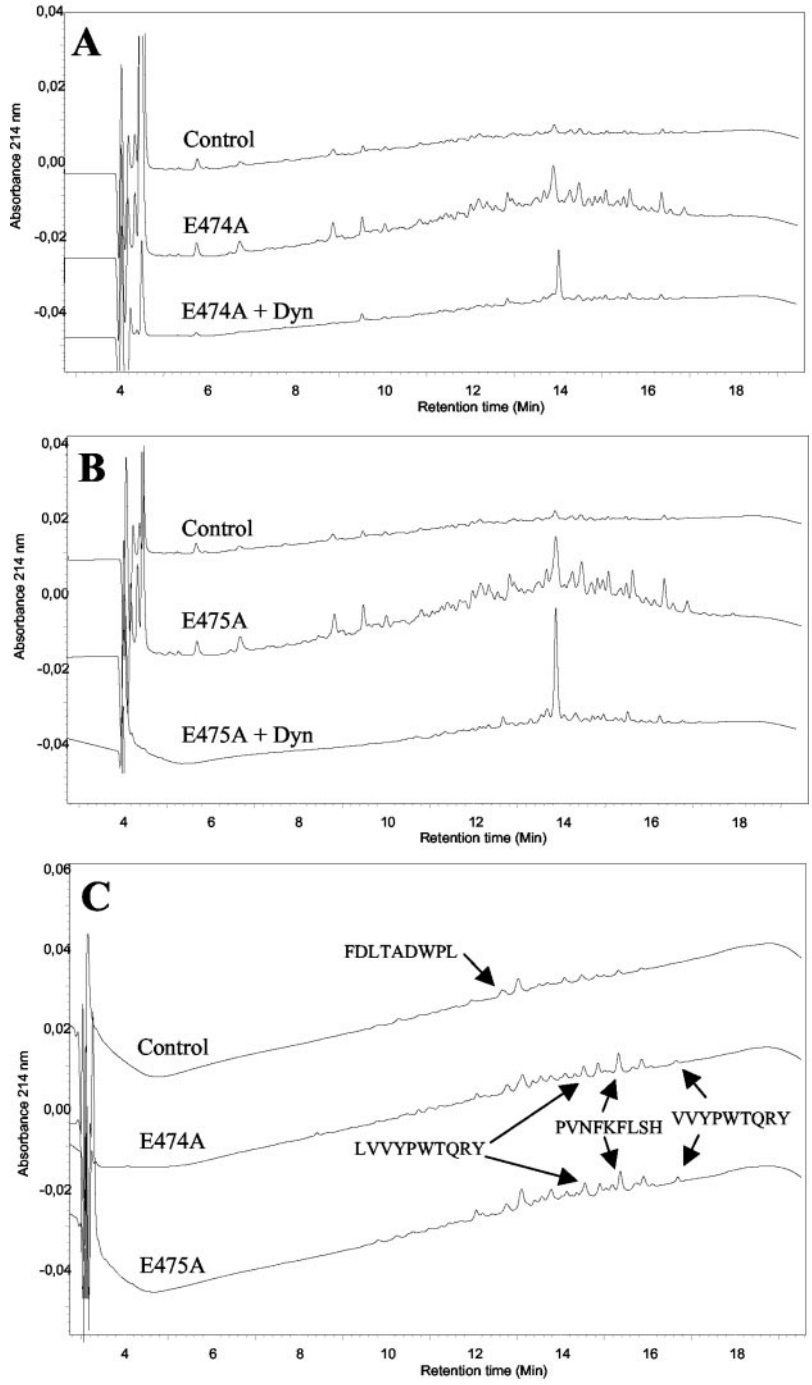

FIG. 2. **HPLC chromatograms showing the rat brain peptides arrested by inactive ep24.15 and ep24.16.** Following incubation of the rat brain peptide extract with inactive ep24.15 (E474A) or ep24.16 (E475A), the enzyme-peptide complexes were eluted through a Sephadex G-25 column and analyzed by HPLC, as detailed under "Experimental Procedures." Addition of the competitive inhibitor dynorphin A_{1-13} (*Dyn*; 30 μM) significantly reduced the amount of rat brain peptides arrested by inactive E474A (*A*) and E475A (*B*). The HPLC peptide peaks for E474A or E475A were collected manually and sequenced by ESI-MS/MS (*C*). Control experiments were done in the absence of inactive peptidases or in the presence of dynorphin A_{1-13} (30 μM) as a competitive peptide during incubation with the rat brain peptide extract (*A* and *B*).

quence of this peptide, and that of several other peptides sequenced from similar ESI-MS/MS spectra, was found to match specific sequences in various proteins (Table III).

To confirm whether the sequenced peptides were in fact ep24.15 and/or ep24.16 substrates, and to further validate this new method, four of the 15 peptides identified were chemically synthesized. One of these four peptides (FDLTADWPL) was selected for synthesis because it did not appear to be arrested by ep24.15 or ep24.16, as shown in the HPLC chromatograms (Fig. 2*C*). The other three peptides (LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH) were selected for synthesis because they were efficiently arrested by ep24.15 and ep24.16 (Fig. 2*C*). The constant of inhibition (K_i values) and relative hydrolyzes ratio for these peptides were determined in parallel with known bioactive peptides such as bradykinin, angiotensin I and II, and dynorphin A_{1-13} (Table IV).

The three peptides selected on the basis of a specific interaction with the inactive enzymes prevented the hydrolysis of QFS by ep24.15 or ep24.16 in a competitive enzyme assay, in contrast to the fourth peptide (FDLATDWPL), which did not inhibit the enzymes (Table IV). To estimate the affinity of these peptides for ep24.15 and ep24.16, the relative constant of inhibition (K_i) was determined. Peptide FDLTADWPL had a K_i above 100 μM, and was not efficiently degraded by these enzymes, even after prolonged incubations. On the other hand, peptides that specifically interacted with the inactive ep24.15 and ep24.16 enzymes had K_i values in the micromolar range (Table IV). Peptide PVNFKFLSH had the highest affinity for both enzymes, with a K_i eight times lower for ep24.16 (3.43 μM) than for ep24.15 (27.8 μM). Despite these differences, this peptide was a better substrate for ep24.15 and ep24.16 than bradykinin. Peptides LVVYPWTQRY and VVYPWTQRY, which

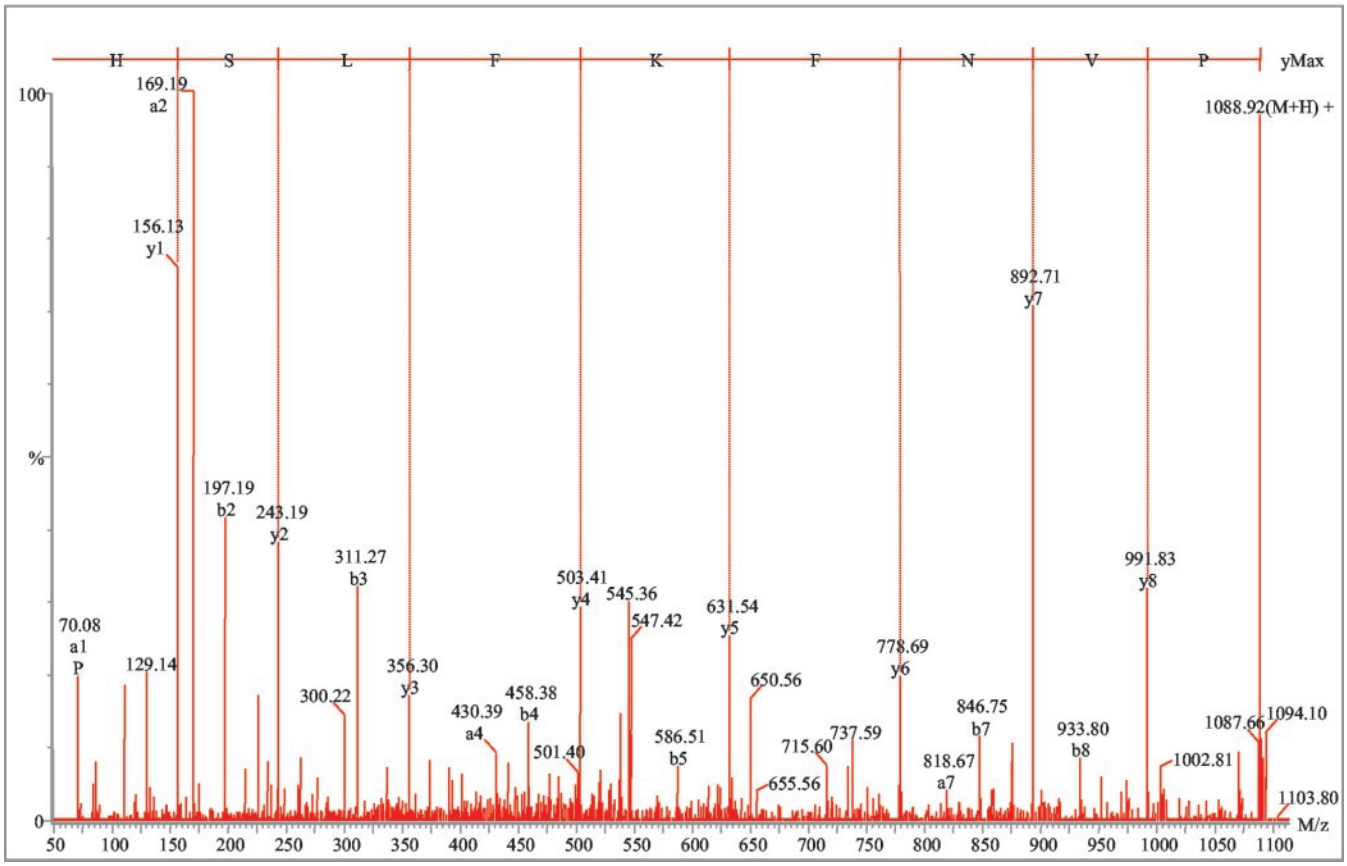

FIG. 3. **Representative peptide sequencing by ESI-MS/MS.** Product ion mass spectra for the mass-selected protonated peptide PVNFK-FLSH acquired using the high-resolution orthogonal time-of-flight mass analyzer after 26 eV collision-induced dissociation with argon. Note that all of the Y-type (Y1–Y8) ionic fragments were formed and clearly detected, which allowed complete sequencing of the peptide.

TABLE III

Analyses of the sequenced peptides by homology with proteins in the data base

The protein data base (www.ncbi.nlm.nih.gov/blast) was searched for short, nearly exact matches on *rodentia* organisms; (b) = brain; (s) = spleen.

Peptide sequenced	Putative precursor protein	Biological activity
VVYPWTQRY (b)	Hemoglobin β chain	Increased after ischemia (61)
LVVYPWTQRY (b)	Hemoglobin β chain	Specific binding to opioid receptor, guinea pig ileum contraction (61)
PVNFKFLSH (b)	Hemoglobin α chain	Unknown
FDLTADWPL (b)	Endogenous retrovirus HERV-K10 putative Protease; protein similar to F-box protein FBW7	Unknown
WSTVLTDVN (b)	Hypothetical protein XP_153553; olfactory receptor MOR13–2;	Unknown
VNMVPVGWASR (b)	Tripeptidyl-peptidase I precursor (TPP-I); zinc finger protein AT-BP2	Unknown
VYPWT (b)	Hemoglobin β chain	Unknown
HHPGDFTPAMHASLDK (s)	Hemoglobin α chain	Unknown
HPGDFTPAMHASLDK (s)	Hemoglobin α chain	Unknown
GDFTPAMHASLDK	Hemoglobin α chain	Unknown
NRTAE (s)	Protein-tyrosine phosphatase γ; SWI/SNF related, matrix-associated, actin-dependent regulator of chromatin, subfamily α-like 1 (*Homo sapiens*); cytokine-like nuclear factor n-pac (*H. sapiens*)	Unknown
KVNPDDVGGEALGRL (s)	Hemoglobin β II	Unknown
LNNPDDRWSKNA (s)	Hypothetical protein XP_070487 TRG γ-chain J-C (*H. sapiens*)	Unknown
TPGTDFWLHASLD (s)	Putative voltage-gated calcium channel γ-4 subunit (*H. sapiens*)	Unknown
QFWLHASL (s)	Oxidase (cytochrome *c*) assembly 1-like (*H. sapiens*)	Unknown

differed by a single NH_2-terminal amino acid, had similar K_i values for both ep24.15 and ep24.16. However, the relative hydrolysis ratio of VVYPWTQRY was five times higher for ep24.15 compared with ep24.16. In contrast, the peptide with the NH_2-terminal leucine, LVVYPWTQRY, was degraded at least five times faster by ep24.16 than by ep24.15 (Table IV), suggesting that a large nonpolar amino acid residue at the NH_2-terminal position could be of importance for defining specific substrates or inhibitors for ep24.15 or ep24.16.

The cleavage products of the peptides LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH, digested by either ep24.15 or ep24.16, were identified by ESI-MS/MS sequencing. Con-

TABLE IV
Side-by-side evaluation of the constant of inhibition (K_i values) and relative hydrolyses ratio of bradykinin and dynorphin A_{1-13} and the newly identified rat brain peptides for the ep24.15, ep24.16, and ACE

	K_i values (μM)			*Relative hydrolyze ratio (%)		
	EP24.15	EP24.16	ACE	EP24.15	EP24.16	ACE
Bradykinin	5.36	8.11	1.74	100*	100*	100*
Dynorphin A_{1-13}	0.04	0.22	>100	<0.01	<0.01	8.49
Angiotensin I	4.29	5.35	25.88	33.5	38.7	172.26
Angiotensin II	8.12	7.95	>100	6.99	20.88	<0.01
PVNFKFLSH	27.76	3.43	1.866	140	152.24	1146.19
VVYPWTQRY	10.02	7.04	10.63	10.62	1.9	138.89
LVVYPWTQRY	2.56	2.01	6.488	0.72	4.7	100.65
FDLTADWPL	>100	>100	>100	<0.01	<0.01	<0.01

TABLE V
Cleavage sites for ep24.15 and ep24.16 in selected peptides
The positions are hydrolyzed by EP24.15 (↑) and EP24.16 (↓).

Peptides	Cleavage sites
PVNFKFLSH	PVNF↑↓K↑↓F↑↓LSH
VVYPWTQRY	VVYPW↑↓T↑↓Q↑↓RY
LVVYPWTQRY	LVVYP↑↓W↑↓T↑↓Q↑↓RY

trary to previously described natural substrates for ep24.15 and ep24.16, hydrolysis of the peptides LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH involved at least three peptide bonds (Table V). These results agreed with data obtained using several synthetic peptides (36, 37), suggesting that these enzymes could also simultaneously hydrolyze more than one peptide bond in natural substrates.

Peptidases are not peptide-specific (38), and ep24.15 and ep24.16 share a series of substrates with ACE (7). Because the physiological function for ACE in the cardiovascular system is well known (20, 21), we examined the kinetic parameters of the peptides identified in this study. The ep24.15 and ep24.16 substrates LVVYPWTQRY, VVYPWTQRY, and PVNFKFLSH also interacted with ACE, with K_i values ranging from 1.7 μM up to 26 μM (Table IV). ACE hydrolyzed the peptide PVNFKFLSH more efficiently than it did angiotensin I or bradykinin (Table IV).

The effects of three of the peptides identified here (FDLTADWPL, LVVYPWTQRY, and PVNFKFLSH) were examined on the blood pressure of anesthetized rats. The intravenous injection of PVNFKFLSH produced immediate hypotension, the extent of which varied according to the dose (Fig. 4*A*). The rapid fall in blood pressure elicited by PVNFKFLSH was similar to that seen with bradykinin, but required a lower dose (0.01 *versus* 3 μg/kg for bradykinin). LVVYPWTQRY also produced hypotension at ≥10 μg/kg, whereas FDLTADWPL produced a slight effect only at 100 μg/kg. Based on this effect on blood pressure, PVNFKFLSH was named *hemopressin*. Whereas enalapril significantly enhanced the hypotensive response to bradykinin (decrease in mean arterial blood pressure: −14.9 + 4.2 mm Hg *versus* −28.2 + 2.4 mm Hg before and after enalapril, respectively; $n = 5$ each, $p < 0.05$), this ACE inhibitor had a significant effect only on the lowest dose of PVNFKFLSH. The responses to bradykinin were potentiated after the administration of PVNFKFLSH while those to angiotensin II were unaffected; LVVYPWTQRY had no such effect on the responses to these two agonists (Fig. 4*B*).

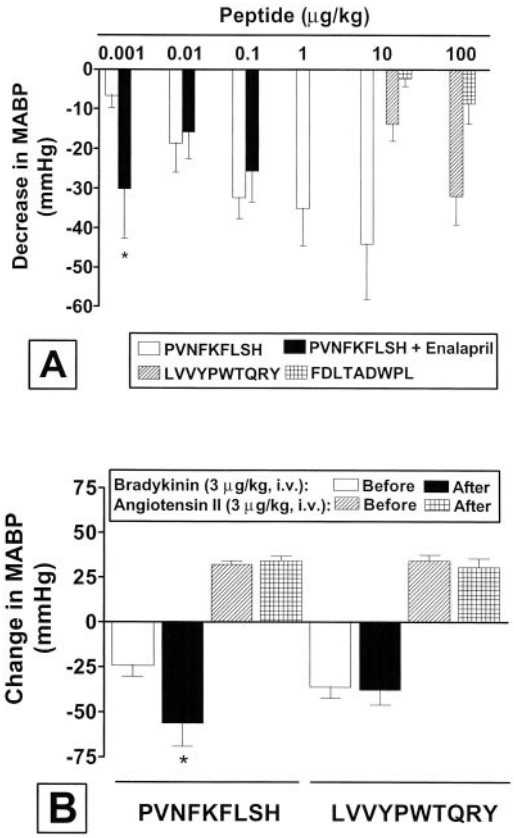

FIG. 4. **Hypotension produced by peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL in pentobarbital-anesthetized male Wistar rats.** *Panel A* shows the responses to various doses of the three peptides. Note that peptide FDLTADWPL caused little hypotension at the doses tested. The responses to PVNFKFLSH in the presence of enalapril are also shown. *Panel B* shows the potentiation of the responses to BK, but not to angiotensin II, in rats treated with PVNFKFLSH. Note that LVVYPWTQRY produced no potentiation. The columns in *panels A* and *B* represent the mean ± S.E. of the change in mean arterial blood pressure in three to nine rats. The mean arterial blood pressure (*MABP*) of the three groups of rats after stabilization but before peptide administration were not significantly different (138 + 4, 133 + 5, and 142 + 4 mm Hg for rats given peptides PVNFKFLSH, LVVYPWTQRY, and FDLTADWPL, respectively; analysis of variance followed by Tukey test). See "Experimental Procedures" for further experimental details. *, $p < 0.05$ compared with response without enalapril.

DISCUSSION

A major finding of the present study was the identification of a new peptide substrate for ep24.15, ep24.16, and ACE that causes hypotension. This peptide (named *hemopressin*) is a fragment of the α-chain of hemoglobin. This is the first report to identify intracellular protein fragments as natural substrates for endopeptidase 24.15 (ep24.15) and neurolysin (ep24.16). These findings suggest a role for ep24.15 and ep24.16 in intracellular peptide metabolism.

The substitution of amino acids His^{474}, Glu^{475}, His^{477}, or Glu^{502} abolishes the enzymatic activity of ep24.15 (39). The importance of the corresponding residues in ep24.16 for catalysis has not yet been experimentally demonstrated, but may be predicted from the recently determined tertiary structure (35). Indeed, substitution by alanine of the corresponding residues

on ep24.16 (H474A, E475A, H478A, and E503A) completely abolished the enzymatic activity. Because all of the proteins examined were expressed to an equivalent level, it is unlikely that the outcome observed with any of the substitutions resulted from improper protein folding. Analysis of the secondary structures by circular dichroism confirmed the similarity of the mutated proteins to the wild type, and indicated that inappropriate protein folding did not cause the loss of enzymatic activity. In addition, deconvolution of the circular dichroism data supported the previous assumption of secondary structure homology between these two oligopeptidases. In an attempt to identify new natural substrates for ep24.15 and ep24.16, catalytically inactive forms of these enzymes were used to identify peptides present in rat brain and spleen extracts.

All of the peptides isolated and sequenced here were within the size range previously reported for natural and synthetic substrates of ep24.15 and ep24.16 (36, 37, 53–55). Using a series of peptides structurally related to bradykinin, Oliveira *et al.* (53) showed that 5 amino acids was the minimum substrate size for ep24.15. Similar results were obtained for ep24.15 and ep24.16 using synthetic fluorescent substrates (37). The smallest peptide isolated here also contained 5 amino acids, which corroborated previous findings (37, 53). On the other hand, orphanin, a neuropeptide containing 17 amino acids, is the largest natural substrate described so far for ep24.15 (54). Using fluorescent substrates in a detailed, systematic analysis of the influence of substrate size on ep24.15 and ep24.16 catalysis, Oliveira *et al.* (37) confirmed that 17 amino acids was indeed the largest substrate size for both enzymes. The largest peptides identified here contained 16 amino acids, which also agrees with these earlier studies (37, 53–55).

There is increasing evidence that ep24.15 and ep24.16 may play a major role in the intracellular metabolism of peptides, probably at a stage beyond the proteasome (13–15, 40). As shown here, we have identified several putative intracellular substrates for ep24.15 and ep24.16. Because the substrates for ep24.15 and ep24.16 must be peptides containing 5–17 amino acids, there is a need for a proteolytic system able to generate such small peptides from larger proteins. The 20 S proteasome, a multicatalytic proteinase complex, is the main intracellular extralysosomal proteolytic system involved in ubiquitin-dependent and -independent intracellular proteolysis (41). In degrading cytosolic, mitochondrial, and nuclear proteins (42, 43), the proteasome generates peptides from 3 to 22 residues in size (16, 44, 45). The peptides generated by the proteasome are therefore within the optimum size range for substrates of ep24.15 and ep24.16 (14, 37). Of the new ep24.15 and ep24.16 substrates identified here, at least seven are hemoglobin fragments. Interestingly, ep24.15 is present in large amounts in human erythrocytes, where hemoglobin also occurs in large quantities (46). Short hemoglobin fragments have been shown to be generated directly by the proteolytic action of the proteasome (47, 48). Whereas additional studies will be necessary to clarify the putative enzymes involved in the generation of hemopressin *in vivo*, it seems reasonable to suggest that ep24.15 and ep24.16 may function in the later steps of intracellular protein degradation. The mechanisms whereby the peptides isolated here escaped degradation is unknown.

In addition to their well known receptor-mediated functions, some peptides also play a role in intracellular processes. For example, calmodulin-dependent protein kinase II is a multifunctional protein kinase with an important role in controlling a variety of cellular functions in the central nervous system (49). A 13-amino acid peptide (KKALRRQEAVDAL), known as autocamtide-2-related inhibitory peptide, is a highly specific inhibitor of calmodulin-dependent protein kinase II (50). In *Bacillus subtilis*, the RapA and RapB proteins are aspartylphosphate phosphatases that specifically dephosphorylate the Spo0F~P intermediate response regulator of the phosphorelay signal transduction system for sporulation (51). The RapA phosphatase activity on Spo0F~P is inhibited *in vivo* by a pentapeptide generated from the *phrA* gene, which displaces Spo0F~P from a preformed complex with RapA (51). The c-Jun NH_2-terminal kinase, a member of the stress-activated group of mitogen-activated protein kinases, is inhibited by a cell-permeable peptide that decreases intracellular c-Jun NH_2-terminal kinase signaling and confers long-term protection to pancreatic β-cells against interleukin-1β-induced apoptosis (52). Thus, by acting on the intracellular metabolism of peptides, ep24.15 and ep24.16 could contribute to the maintenance of cellular homeostasis.

Of the endogenous globin fragments identified in the present study, three (LVVYPWTQRY, VVYPWTQRY, and the fragment VYPWT) are apparently related to a family of peptides known a hemorphins, which are derived from the degradation of the β-chain of human hemoglobin and show morphine-like activity based on their ability to inhibit the contractions of electrically stimulated guinea pig ileum (Refs. 56 and 57 and references therein). LVVYPWTQRY and VVYPWTQRY are identical to human LVV-hemorphin-7 (57, 58) and VV-hemorphin-7 (59), respectively, except for their terminal amino acid residue, which is tyrosine instead of arginine and may reflect the rodent origin of our peptides.

Peptide PVNFKFLSH produced potent hypotension in anesthetized rats. This peptide is derived from the α_1-chain of rodent hemoglobin and shares no sequence identity with the hemorphins of the β-chain of human hemoglobin. Other peptides derived from the α-chain identified here included HHPGDFTPAMHASLDK and two truncated fragments of this peptide, but their effect on blood pressure was not examined. The mechanism by which PVNFKFLSH produces hypotension is still unclear but could involve a variety of pathways, including ion channel activation or blockade, the stimulation of nitric oxide (NO) formation through as yet unidentified receptors, the release of vasodilator peptides such as atrial natriuretic factor, or the inhibition of endogenous peptidase activity which could lead to an increase in circulating levels of hypotensive peptides.

In experiments not described here, we have observed that PVNFKFLSH does not contract or relax vascular (aorta) or nonvascular (guinea pig ileum) smooth muscle preparations. This finding is similar to the inability of hemorphins to contract isolated endothelium-denuded aortic strips from rats (60), and suggests that PVNFKFLSH probably does not have a direct action on vascular smooth muscle. The involvement of NO in the observed hypotension merits investigation, although Moisan *et al.* (57) observed that the blockade of NO production by L-N^{ω}-nitro-L-arginine methyl ester did not influence the hypertensive response to hemorphins.

Exogenous and endogenous peptides may be metabolized by a variety of peptidases, including the three enzymes studied here. To examine the influence of ACE on the hypotensive responses to PVNFKFLSH, rats were treated with enalapril to block this enzyme. Although the treatment was effective in potentiating BK-induced hypotension, it had little effect on the responses to PVNFKFLSH, except at the lowest dose of the peptide. This finding will have to be explored further in light of the role of ACE, and other peptidases such as the ep24.15 and ep24.16, in the metabolism of PVNFKFLSH. Experiments to address this aspect using specific inhibitors are in progress in our laboratory.

The ability of PVNFKFLSH to potentiate the hypotension to BK without affecting the hypertension to angiotensin II is

interesting, although it is still unclear whether this response is selective for BK or applies to vasodilatory peptides in general. This action of PVNFKFLSH could involve the sensitization of intracellular pathways to subsequent stimulation by BK or could involve the specific inhibition of a peptidase(s), possibly ACE, that degrades BK. The inhibition of ACE by PVNFKFLSH could influence the metabolism of other peptide substrates by this peptidase. The hypotensive action of PVNFKFLSH may involve therefore peptidase- and nonpeptidase-mediated pathways. Finally, the observation that FDLTADWPL, derived from a nonhemoglobin molecule(s), caused little hypotension compared with the varied effects observed for fragments from the α (PVNFKFLSH) and β (hemorphins and LVVYPWTQRY) chains of hemoglobin confirms data in the literature indicating that the degradation of hemoglobin is an important source of bioactive peptides, and could provide a lead for investigating the biological activities of the other peptides identified in this study.

In summary, we have demonstrated the feasibility of using catalytically inactive forms of ep24.15 and ep24.16 to identify new bioactive peptide substrates for these enzymes. However, the methodology should be applicable to other enzyme systems. One of the new substrates identified (*hemopressin*) is a fragment of the hemoglobin α-chain and reduces blood pressure in anesthetized rats. Further functional analyses will be necessary to evaluate the pharmacological and physiological relevance of hemopressin and of the other peptides identified in this study.

Acknowledgments—We thank Dr. Marc J. Glucksman for help, Dr. James L. Roberts for providing the wild type ep24.15 cDNA, Drs. Akira Kato and Shigerisa Hirose for providing the wild type ep24.16 cDNA, Dr. I. A. Smith for providing the QFS, Drs. Vitor Oliveira and Maurício C. Araujo for help with the kinetic experiments, Dr. Heitor Moreno, Jr. (Department of Pharmacology, UNICAMP) for allowing the use of his blood pressure monitoring system, and Dr. Shirley Schreier for allowing us to use her Jasco 720 spectropolarimeter. Dr. Lloyd D. Fricker provided suggestions and criticisms on the manuscript and Dr. Antonio C. M. Camargo gave important advice on various aspects of this study.

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