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KININ AND ENKEPHALIN CONVERSION BY AN ENDOTHELIAL, PLASMA MEMBRANE CARBOXYPEPTIDASE

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Abstract—Utilizing both thin-layer chromatography and high pressure liquid chromatography, it was determined that a vascular plasma membrane preparation contains a carboxypeptidase capable of converting kinins (B_2 agonists) to des(Arg)kinins (B_1 agonists) by hydrolysis of C-terminal Arg. The plasma membrane carboxypeptidase also converted Leu^5-enkephalin-Arg^6 to Leu^5-enkephalin. Carboxypeptidase activity was significantly higher in cultured endothelial (1.47 ± 0.4 units/mg) than in cultured smooth muscle cells (0.16 ± 0.4 units/mg). Both the vascular and endothelial activities had neutral pH optima and were activated 4- to 5-fold by 0.1 mM $CoCl_2$. The carboxypeptidase N inhibitor MERGETPA (D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid) inhibited the plasma membrane bound carboxypeptidase with an I_{50} of 0.3 μM. Conversion was also inhibited by *o*-phenanthroline and EDTA, whereas inhibitors of aminopeptidases (bestatin, puromycin), endopeptidases (phosphoramidon), "enkephalinase" (ZINCOV) or enkephalin convertase (PCMS) were without effect. The affinity of the endothelial plasma membrane carboxypeptidase for bradykinin (K_m = 56.8 ± 4.7 μM) was higher than that for Leu^5-enkephalin-Arg^6 (K_m = 92.7 ± 10.1 μM), whereas the maximal rates of conversion (calculated per mg of endothelial plasma membrane protein) were similar (17.1 and 21.3 nmoles/min/mg respectively). These results demonstrate that a carboxypeptidase is present on the cell surface of vascular endothelium which can convert kinins and enkephalins in the micro-environments of vascular cell surface receptors.

Both plasma and vasculature contain enzymes that can form, convert, and/or inactivate vasoactive peptides. These enzymes include angiotensin I converting enzyme (ACE, EC 3.4.15.1) [1, 2] and aminopeptidase A (EC 3.4.11.7) [3]. In recent work, we have found that a plasma membrane fraction purified from porcine aorta or mesenteric artery is enriched not only in ACE, but also in enzymes immunologically indistiguishable from renal aminopeptidase M (AmM; EC 3.4.11.2) and dipeptidyl(amino)peptidase IV (DAP IV, EC 3.4.14.5) [4]. Vascular AmM and DAP IV can differentially hydrolyze a number of vasoactive peptides [5, 6] and, like ACE, circulating forms of both enzymes are present in plasma [4].

Plasma carboxypeptidase N (CPN: EC 3.4.17.3) hydrolyzes C-terminal basic amino acids from a variety of biologically active peptides including kinins, anaphylatoxins, fibrinopeptides and enkephalins [7–9]. Plasma CPN can convert the kappa agonists Lys^6- and Arg^6-enkephalin into their pentapeptide forms [9] which are reportedly more specific for delta receptors [10]. Similarly, plasma CPN carboxyl-terminal hydrolysis of kinins (B_2 agonists) will produce des(Arg)kinins which have potent effects on B_1 receptors [11].

Although little is known regarding vascular CPN-like activities, vascular tissues do hydrolyze synthetic carboxypeptidase substrates [9], and an enzyme immunologically related to plasma CPN has been identified on pulmonary endothelium [12]. These reports are consistent with our own observations that purified vascular plasma membrane [5, 6] hydrolyzes the C-terminus of both bradykinin and Arg^6-enkephalin. Considering the potential for local modulation of both the form and concentration of the above peptides, the present studies investigated vascular conversion of both kinins and enkephalins.

MATERIALS AND METHODS

Materials. Kallidin, bradykinin, Leu^5-enkephalin-Arg^6, leucyl-4-amino-4-methyl-coumarin, phenanthroline, bestatin, amastatin, phosphoramidon [*N*-(α - rhamnopyranosyl - oxyhydroxyphosphinyl - L - leucyl-L-tryptophan)], *p*-chloromercuriphenyl sulfonic acid (PCMS) and dithiothreitol (DTT) were obtained from the Sigma Chemical Co. (St. Louis, MO). The ACE substrate ([^{3}H]benzoyl-Phe-Ala-Pro) and inhibitor (captopril) were obtained from Ventrex (Portland, ME) and Squibb (Princeton, NJ) respectively. The plasma CPN inhibitor MERGETPA (D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid) and the "enkephalinase" inhibitor ZINCOV [2-(*N*-hydroxycarboxamido)-4-methylpentanoyl-L-Gly amide] were obtained from Calbiochem-Behring (San Diego, CA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum were obtained from Gibco (Grand Island, NY) and Hyclone (Logan, UT) respectively. Amino acid standards, *o*-phthalaldehyde crystals and *o*-phthalaldehyde reagent solution (OPA) were from the Pierce Chemical Co. (Rockford, IL). The MN

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300 Uniplates used for TLC were from Analtech, Inc. (Newark, DE).

Plasma membrane preparation. Vessels were obtained from freshly slaughtered hogs, cleaned in 0.9% (w/v) saline, and subfractionated into low speed pellets (P), and cytosolic (C), microsomal (M) and plasma membrane (PM)-enriched fractions as previously described [4, 5]. Enrichments of 5'-nucleotidase (EC 3.1.3.5), ACE, AmM and DAP IV in the plasma membrane fraction were comparable (7- to 14-fold) to those obtained in previous preparations [4–6]. Suspensions of plasma membrane were pooled, aliquoted, and frozen until assayed (1–8 weeks). Storage over these time periods had no significant effect on enzyme activity.

Cultured cells. Primary cultures of endothelium were obtained by gently removing endothelial cells from the intimal surface of fresh hog aorta with a scalpel blade. Scrapings were placed in a Corning cell culture centrifuge tube (15 ml) containing DMEM and spun at 500 *g* (20 min) to separate intact cells from debris. Cells were collected as a pellet in DMEM, resuspended in growth medium [DMEM: 10% (v/v) fetal calf serum: 1% (v/v) penicillin-streptomycin] and incubated in 5% CO_2 at 37°. After 1 hr, medium containing non-adherent cells was removed and replaced with 5 ml of fresh growth medium. Cells adherent to the bottom of the flask were allowed to grow to near confluency (7–10 days) with changes in growth medium every 2 days [13, 14]. Cultures were then either processed for characterization or passed in a 1 to 6 split by scraping the cells and following procedures identical to those used for primary cultures.

Smooth muscle cells were obtained from the same aorta using explants of the medial layer. Explants (1–2 cm^2) were incubated in growth medium until migration of juvenile muscle cells was seen by phase contrast microscopy (10–14 days). Juvenile cells typically evidenced a spindle shape with long, thin cytoplasmic processes extending from one cell to another, and oval or sausage-shaped nuclei containing two or more dense nucleoli [15]. After 4–6 weeks in culture, cells reached a density of approximately three million cells/T-75 flask.

For enzyme assay, preparations of cell membranes were isolated from cultured endothelium and smooth muscle. After repeated washing in phosphate-buffered saline to remove culture medium, cells were detached and collected by mechanical scraping with a rubber spatula. Cell suspensions were homogenized by sonication (3 × 10 sec) using a Fisher Sonic Dismembrator set at 33% maximal power. Homogenates were then centrifuged at 1000 *g* (10 min) and 10,000 *g* (10 min) to remove unbroken cells and cellular debris. The supernatant fraction was centrifuged at 100,000 *g* (60 min) to obtain the microsomal fraction. Alternatively, endothelial homogenate was subfractionated to the plasma membrane-enriched fraction as described above.

Enzyme assays. ACE and AmM were assayed using [^{3}H]-benzoyl-Phe-Ala-Pro and leucyl-7-amino-4-methyl-coumarin as substrates respectively [16, 17]. Enzyme specific activities are expressed as units/mg protein where 1 unit equals the hydrolysis of 1 nmole substrate per min (vascular carboxypeptidase), 1% substrate hydrolyzed per min (ACE, Ref. 16) or change in relative fluorescence per min (AmM). Relative specific activity (RSA) is calculated as (mean specific activity in the subcellular fraction)/(mean specific activity in the homogenate). Protein was determined with the protein assay kit of BioRad (Richmond, CA) using bovine serum albumin as a standard [18].

Peptide metabolism: TLC. Vascular carboxypeptidase activity was determined as the rate of MERGETPA-sensitive C-terminal Arg hydrolysis from bradykinin and Leu^5-enkephalin-Arg^6. Qualitative assays were carried out by TLC on MN 300 cellulose plates. The standard incubation consisted of mixing the peptide (500 μM final concentration) in 40 μl buffer (100 mM Tris/HCl, pH 7.0) with 10 μl of a dilution of the vascular fraction. At sequential time intervals, 5-μl aliquots were spotted on the plate and immediately dried. Plates were developed in butanol–acetic acid–water (4:1:5), and the products were visualized by staining with 0.4% (w/v) ninhydrin in acetone according to Toennies and Kolb [19].

Peptide metabolism: HPLC. Quantitative analysis of kinin and enkephalin conversion (C-terminal Arg hydrolysis inhibited by MERGETPA) was carried out by incubating 5 μl of a dilution of the vascular fraction in 295 μl of 100 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM $CoCl_2$, 0.1 mM captopril (to inhibit kinin carboxyl-hydrolysis by ACE) and 0.1 mM amastatin (to inhibit amino-terminal enkephalin hydrolysis by AmM). For assays of crude cellular and subcellular fractions, 10 μM phosphoramidon was also added to prevent non-specific hydrolysis by endopeptidases. These inhibitors, which had no effect on C-terminal Arg hydrolysis by the vascular carboxypeptidase, were preincubated with enzyme and buffer for 20 min at 37° before addition of the peptide substrate (25 μM final concentration).

At sequential time intervals, 60-μl aliquots of the reaction mixture were immersed in a boiling water bath (6 min) to terminate the reaction, cooled on ice and centrifuged in a Brinkmann table top centrifuge (3 min); the supernatant fraction was collected for analysis. Control experiments established that recovery of all peptide and amino acid metabolites was greater than 95%. Reaction rates, calculated during the first 15% of the reaction, were linear with respect to both amount of enzyme used and the time of incubation.

For pH experiments, a 100 mM sodium acetate buffer was used over the pH range 4.0 to 6.0, and the above 100 mM phosphate buffer was used from pH 6.5 to 8.0. For K_m determinations, measurements of the initial velocity of hydrolysis were determined at six substrate concentrations (12.5 to 250 μM). Data were plotted according to the Lineweaver–Burk method (1/V vs 1/S) and fit to the best straight line [20].

A high performance liquid chromatograph (Waters Associates: Milford, MA), consisting of two model 6000A pumps, a model 730 Data Module, a model 721 System Controller, a model 710B WISP™ Autosampler and a model 420 Fluorescence Detector, was employed for the HPLC analysis. Standards and unknowns (10–60 μl) were automatically deri-

vatized with OPA solution (20 μl) 3 min prior to chromatography (Pre-column Derivatization Program, Waters 710B WISP™ Autosampler) [21] and subsequently separated on a reverse phase column (Waters, 5 μm, C_{18}-Radial-PAK™, 8 mm × 10 cm) at a constant flow rate of 2.5 ml/min utilizing a linear gradient from 100% Buffer A to 40% Buffer A/60% Buffer B. The column was subsequently washed with 100% Buffer B and then re-equilibrated in 100% Buffer A. Integration of sample peak areas and quantitation of metabolites against the most recent standard peak areas were automatically calculated by the data module. Standards were run every sixth injection. The OPA solution was made fresh daily by mixing 3.8 ml *o*-phthalaldehyde reagent solution, 0.1 ml methanol, 0.1 ml β-mercaptoethanol and 16.8 mg *o*-phthalaldehyde crystals. Buffer A was 100 mM sodium phosphate (pH 7.0) and Buffer B was a 50/50 (v/v) mixture of Buffer A and acetonitrile. Prior to use, both buffers were degassed by filtration through a Millipore AP Pre-Filter.

RESULTS

Kinin and enkephalin conversion: TLC. In preliminary experiments, hog aorta vascular plasma membrane was incubated with either bradykinin (Fig. 1a) or Leu^5-enkephalin-Arg^6 (Fig. 1b). After separation of metabolites by TLC, the progressive release of Arg from both peptides was seen clearly (Fig. 1; left side). This hydrolysis was not due to plasma membrane ACE since the concentration of captopril used in the incubation completely inhibited ACE. Further, the Arg released from bradykinin (Fig. 1a) was not due to hydrolysis of the N-terminal Arg^1-Pro^2 bond since no Arg was produced when des(Arg^9)bradykinin was used as substrate (not shown). This C-terminal hydrolysis of both bradykinin and Leu^5-enkephalin-Arg^6 was inhibited completely by the carboxypeptidase inhibitor MERGETPA (Fig. 1; right side).

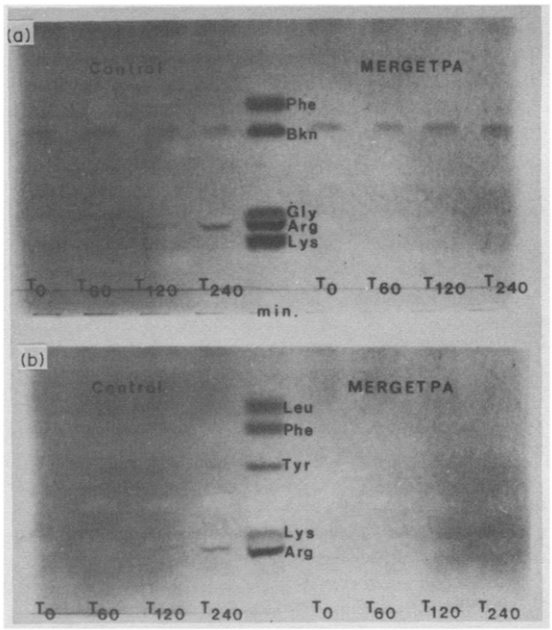

Fig. 1. Incubation of vascular plasma membrane with (a) bradykinin or (b) Leu^5-enkephalin-Arg^6 with or without 100 μM MERGETPA. All incubations contained 100 μM captopril and amastatin to inhibit plasma membrane ACE and AmM. Metabolites and amino acid standards were separated by thin-layer chromatography as described in Materials and Methods.

Kinin and enkephalin conversion: Subcellular localization. To quantitatively determine whether this MERGETPA-sensitive carboxypeptidase was localized to the plasma membrane fraction (rather than being artifactual contamination from another subcellular fraction), the rates of C-terminal hydrolysis of bradykinin (inhibited by MERGETPA) were determined in the low speed pellets (P), and cytosolic (C), microsomal (M) and plasma membrane (PM) fractions and compared to that in whole homogenate (H). For the sake of comparison, the subcellular distribution of plasma membrane AmM was also determined.

As reported previously [4] and shown in Fig. 2A, AmM was localized to the PM-enriched fraction (1.08 ± 0.33 units/mg; RSA = 12.8). This plasma

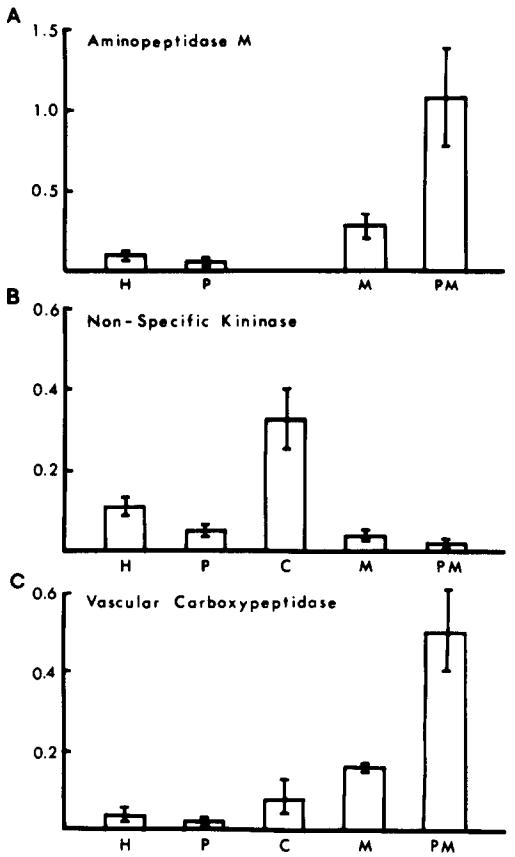

Fig. 2. Subcellular distribution of (A) aminopeptidase M, (B) non-specific kinase and (C) MERGETPA-sensitive carboxypeptidase in porcine vascular homogenate (H), low speed pellets (P), cytosolic (C), microsomal (M) and plasma membrane (PM) fractions. Bradykinin metabolism (B and C) was determined as described in Materials and Methods at a final substrate concentration of 25 μM. Specific activity is given on the ordinate as units/mg protein as defined in Materials and Methods.

membrane localization contrasted sharply with the distribution of non-specific kinin degradation (determined as kinin hydrolysis not inhibited by either captopril or MERGETPA). As shown in Fig. 2B, the majority of non-specific kinin degradation occurred in the cytosolic fraction (0.32 ± 0.07 nmole/min/mg; RSA = 2.9). This result was not surprising in view of the large number of peptidases known to be localized in the cytosol [22].

In contrast to the above, the distribution of MERGETPA-sensitive C-terminal bradykinin hydrolysis (Fig. 2C) was found to be essentially the same as that of AmM, with the highest specific activity associated with the PM-enriched fraction (0.50 ± 0.11 nmole/min/mg). This represented a 14.3-fold enrichment over that in starting homogenate (0.035 nmole/min/mg) and was comparable to the enrichment of AmM (RSA = 12.8). In separate experiments (not shown), the hydrolysis of MERGETPA-sensitive Leu^5-enkephalin-Arg^6 hydrolysis was similarly enriched (RSA = 16.3). Considering that little or no non-specific peptidase activity was associated with the plasma membrane fraction (0.02 nmole/min/mg; Fig. 2B), these data demonstrate that, in the presence of a converting enzyme inhibitor, bradykinin conversion by the MERGETPA-sensitive carboxypeptidase is the primary pathway of metabolism by the vascular plasma membrane.

Cellular localization. Since the plasma membrane fraction used in the above and previous [4–6] studies contains plasma membrane from both endothelium and smooth muscle, experiments were undertaken to determine the cellular distribution of the above carboxypeptidase. As shown in Fig. 3a, primary cultures of endothelium were characterized by cobblestone patterned cells growing in a densely packed monolayer. Individual cells were polygonal in shape containing prominent, large round nuclei surrounded by relatively little cytoplasm [23]. Examination of two cultures by transmission microscopy revealed ultrastructural features characteristic of cultured endothelium including Weibel–Palade bodies [24, 25], numerous mitochondria, abundant microfilaments in the peripheral cytoplasm [26] and micropinocytotic vesicles adjacent to the inner aspect of the outer membrane [27]. Also characteristic of cultured endothelial cells was the release of PGI_2 upon stimulation with bradykinin (not shown).

Cultured smooth muscle cells grew slowly. After 2–3 weeks in culture, cells exhibited characteristic features including a ribbon-like appearance: phase-dense cytoplasm and multi-layered arrays (Fig. 3b). After 4–5 weeks, marked traverse ridges of piled cells gave the entire culture a hill and valley appearance with hillock-like protrusions (nodules) characteristic of smooth muscle cells at this stage of growth [15]. Approximately 80% of the cells contracted spontaneously with changes in growth medium [28].

High speed microsomal fractions were prepared from pooled endothelial and smooth muscle cultures and assayed. As expected (Fig. 4), cultured endothelium contained significantly higher levels of ACE activity (355 ± 52 units/mg) than did cultured smooth muscle cells (34 ± 13 units/mg). Similarly, in the presence of the ACE inhibitor captopril, endothelium converted bradykinin to des(Arg^9)bradykinin by a MERGETPA-sensitive carboxypeptidase at a rate (1.47 ± 0.4) nmoles/min/mg) that was nearly 10-fold that of cultured smooth muscle cells (0.16 ± 0.04 nmole/min/mg). An endothelial localization for the vascular carboxypeptidase is further supported by the fact that the rate of kinin conversion by this endothelial microsome fraction (1.47 ± 0.4) was three times that of the plasma membrane (endothelial/smooth muscle) fraction prepared from hog aorta (Fig. 2C; 0.50 nmole/min/mg).

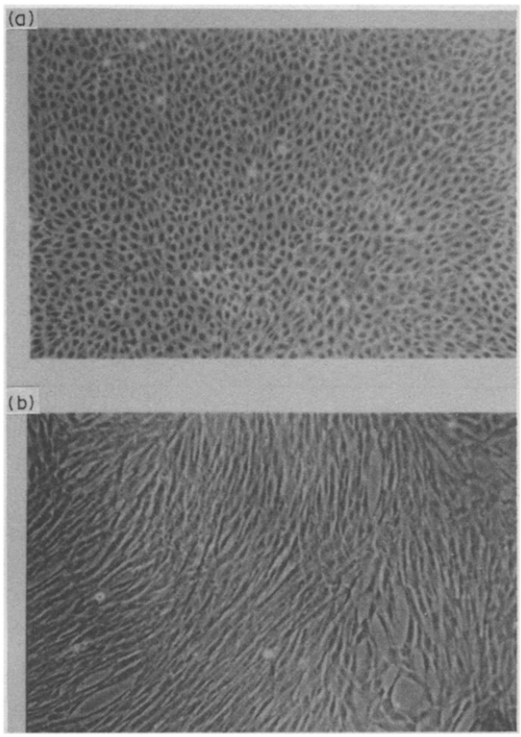

Fig. 3. Phase contrast photomicrographs of porcine (a) endothelial and (b) smooth muscle cells in culture.

For subsequent studies, two preparations of purified endothelial plasma membrane were prepared from ten T-75 flasks (three million cells/flask). Although the limited amount of material did not allow for extensive marker enzyme analysis, these endothelial plasma membranes were found to be enriched in ACE activity (2059 units/mg; RSA = 6.0; average of the two preparations) and contained significant levels of AmM. When incubated with kallidin (in the presence of captopril and amastatin to inhibit ACE and AmM respectively), significant conversion to Arg and des(Arg^{10})kallidin was detectable within 60 min (Fig. 5a). Confirming the absence of other kinin hydrolyzing activity, no other metabolites were produced even after extended incubation. Indeed, this pattern of metabolism was essentially identical to that seen for plasma CPN (Fig. 5b).

At a final substrate concentration of 250 μM, pooled endothelial plasma membrane converted bradykinin to des(Arg^9)bradykinin at a rate (15.9 nmoles/min/mg) comparable to that of Leu^5-enkephalin-Arg^6 conversion (17.6 nmoles/min/mg). Again consistent with an endothelial localization, these rates of conversion were approximately 13-fold

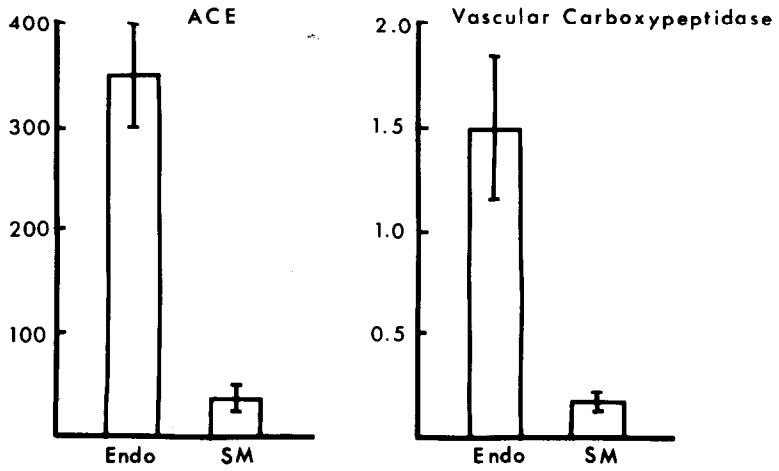

Fig. 4. Cellular distribution of angiotensin I converting enzyme (left panel) and MERGETPA-sensitive carboxypeptidase (right panel) in microsomal fractions of cultured endothelium and smooth muscle. Bradykinin conversion to des(Arg^9) bradykinin (right panel) was determined in the presence of 100 μM captopril as described in Materials and Methods at a final substrate concentration of 25 μM. Specific activity is given on the ordinate as units/mg protein as defined in Materials and Methods.

those found for pooled vascular (endothelial/smooth muscle) plasma membrane (1.29 and 1.40 nmoles/min/mg respectively). Nevertheless, despite the fact that the endothelial plasma membrane was significantly more active than plasma membrane prepared from intact vessels, all subsequent characterization studies were carried out with both membrane preparations to avoid possible artifacts that might be related to *in vitro* culture conditions.

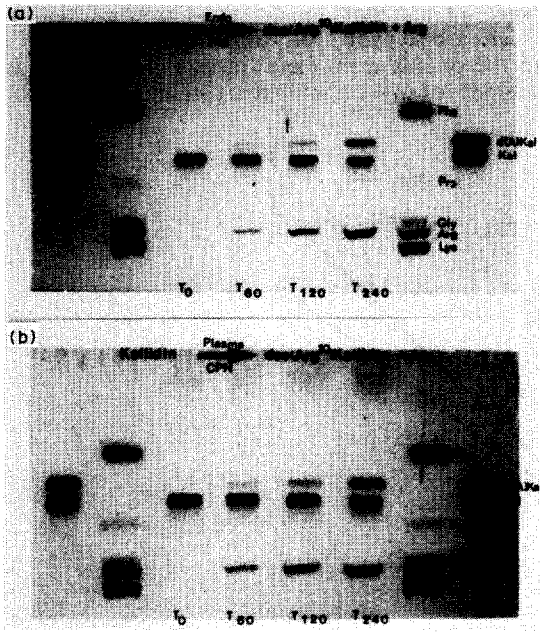

Fig. 5. Incubation of (a) endothelial plasma membrane or (b) plasma CPN with kallidin in the presence of 100 μM captopril and amastatin. Metabolites and amino acid standards were separated by thin-layer chromatography as described in Materials and Methods. Identified standards include kallidin (Kal) and des(Arg^{10}) kallidin [d(A)Kal).

Further, unless otherwise noted, all subsequent studies were carried out at a final substrate concentration of 250 μM.

Enzyme characterization. Kinin and enkephalin conversion by both the vascular and endothelial plasma membrane carboxypeptidases had neutral pH optima (pH 6.8 to 7.0). Addition of $CoCl_2$ to the incubation increased both kinin and enkephalin conversion. Maximal activation (10^{-4} M $CoCl_2$) of the vascular and endothelial carboxypeptidases was 499 ± 71% (N = 3) and 432 ± 35% (N = 3) respectively.

MERGETPA [29] inhibited both the vascular and endothelial plasma membrane carboxypeptidases dose-dependently, with I_{50} values at or near 0.3 μM (Fig. 6). Conversion was also inhibited by 1 mM *o*-phenanthroline and EDTA (≥90%) whereas aminopeptidase (bestatin, puromycin), neutral endopeptidase and "enkephalinase" (phosphoramidon; ZINCOV) [30] inhibitors had no significant effect (≤5% at 10 μM final concentration). In addition, unlike enkephalin covertase [31, 32], the vascular carboxypeptidase was not activated by dithiothreitol (1.5 mM) or inhibited by PCMS (15 μM).

To determine the affinity of the plasma membrane carboxypeptidase for bradykinin and Leu^5-enkephalin-Arg^6, comparative studies were carried out at various substrate concentrations. As plotted in Fig. 7 (individual experiments) and calculated in Table 1, the K_m values of the vascular plasma membrane carboxypeptidase were 46.8 ± 4.6 and 84.3 ± 11.4 μM for bradykinin and Leu^5-enkephalin-Arg^6 respectively. Comparable values were obtained for the endothelial plasma membrane carboxypeptidase (Table 1), with the maximal rates of hydrolysis (calculated per mg endothelial plasma membrane protein) being similar though slightly higher for enkephalin conversion (21.3 ± 2.2 nmoles/min/mg) than for bradykinin conversion (17.1 ± 1.4). Since the rates of N-terminal hydrolysis of enkephalins by aminopeptidase M have been reported to be affected

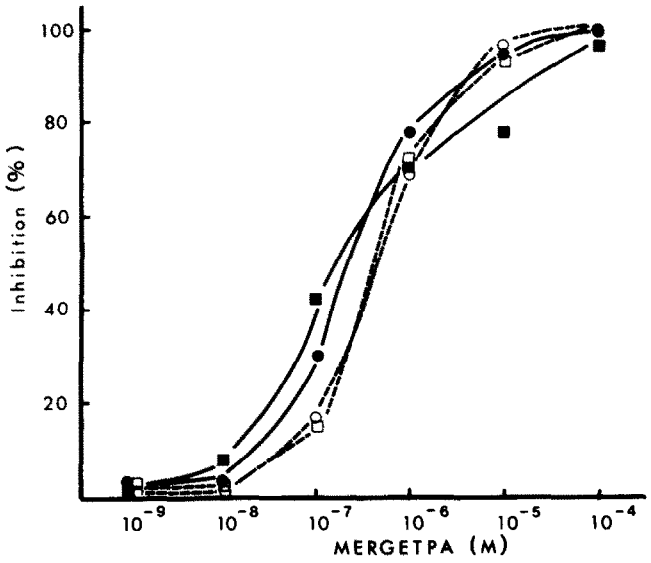

Fig. 6. Inhibition (%) by MERGETPA of vascular (solid lines) and endothelial (broken lines) plasma membrane carboxypeptidase conversion of bradykinin to des(Arg^9) bradykinin (●/○) and Leu^5-enkephalin-Arg^6 to Leu^5-enkephalin (■/□).

by peptide chain length [33], experiments were carried out to determine whether the maximal rates of C-terminal hydrolysis of Arg from Met-Lys-bradykinin and Lys-bradykinin (kallidin) were different from that of bradykinin. In single determinations (due to the limited aount of endothelial plasma membrane), maximal rates of conversion were comparable (not shown).

DISCUSSION

Although numerous studies have demonstrated that ACE degrades kinins and is present on the surface membrane of vascular endothelium [1–3], little is known about other vascular enzymes which could participate in kinin metabolism *in vivo*. The results of the present study have demonstrated that vasculature contains substantial non-specific kinin degrading activity in addition to a MERGETPA-sensitive carboxypeptidase. However, since the majority of non-specific activity was present within the cytosol, it is probably irrelevant to kinin metabolism under normal conditions. In contrast, the vascular carboxypeptidase was co-localized with ACE to the plasma membrane fraction. Further, in the presence of an ACE inhibitor, C-terminal conversion

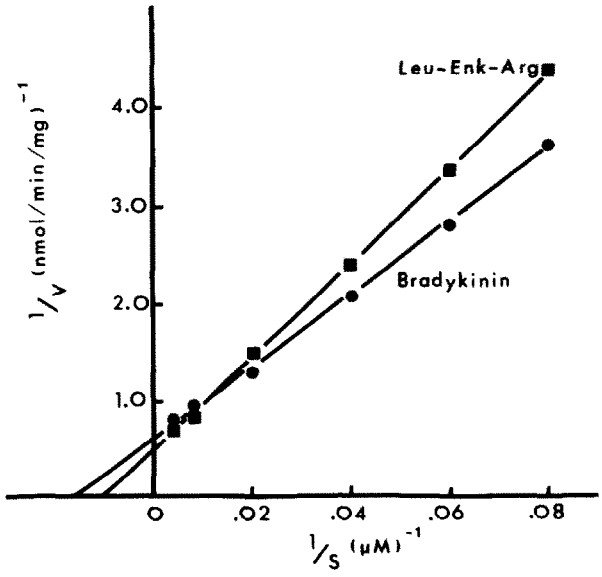

Fig. 7. Lineweaver–Burk plots of the rates of conversion of bradykinin (●) and Leu^5-enkephalin-Arg^6 (■) by vascular plasma membrane carboxypeptidase.

Table 1. Kinetics of C-terminal Arg hydrolysis from bradykinin and Leu[5]-enkephalin-Arg[6] by vascular and endothelial plasma membrane carboxypeptidase

Peptide	K_m (μM)	Maximal velocity (nmoles/min/mg)
Vascular plasma membrane		
Bradykinin	46.8 ± 4.6 (N = 8)	
Leu[5]-enkephalin-Arg[6]	84.3 ± 11.4 (N = 3)	
Endothelial plasma membrane		
Bradykinin	56.8 ± 4.7 (N = 3)	17.1 ± 1.4 (N = 3)
Leu[5]-enkephalin-Arg[6]	92.7 ± 10.1 (N = 3)	21.3 ± 2.2 (N = 3)

Assays were performed as described in Materials and Methods at six substrate concentrations (12.5 to 250 μM), and the above values were calculated according to Lineweaver–Burk [20] as depicted in Fig. 7. Maximal velocities are calculated as units of activity per mg plasma membrane protein. Values given are the means ± S.E.M.

of bradykinin to des(Arg[9])bradykinin was the only detectable pathway of kinin metabolism.

Characterization of the endothelial carboxypeptidase demonstrated that it has many properties in common with plasma CPN. The vascular enzyme hydrolyzed a basic C-terminal amino acid (Arg) from both kinins and enkephalin, had a neutral pH optima, was activated by $CoCl_2$ and was inhibited by MERGETPA, EDTA and *o*-phenanthroline. Conversely, inhibitors of aminopeptidase M [6], neutral endopeptidase, "enkephalinase" [30] and enkephalin convertase [30, 32] had no effect. The affinity of the porcine vascular enzyme for bradykinin (K_m = 46–57 μM) was higher than that for enkephalin. Human and porcine CPN are biochemically similar [34], and Skidgel *et al.* [9] have reported recently that the K_m of human plasma CPN for bradykinin is 25–45 μM. Although these values are quite comparable, they differ from that originally reported by Zacest *et al.* [35] (K_m = 0.4 μM). The reason for this difference is not clear, but may be related to the use in the latter study of low concentrations of radiolabeled peptide [9, 35].

Also similar to the results obtained for purified plasma CPN by Skidgel *et al.* [9], the vascular carboxypeptidase cleaved enkephalin somewhat more rapidly than bradykinin. Nevertheless, since the present study used only preliminary membrane purification procedures, the maximal rates of enzymatic activity obtained (21.3 and 17.1 units/mg respectively) must be considered only rough estimates. Further support for a close relationship between the vascular and plasma enzymes comes from our observation that the vascular enzyme is localized almost exclusively to cultured endothelium. This is consistent with the report of Ryan and Ryan [12] that pulmonary endothelium contains an enzyme which cross-reacts with antibody to plasma CPN.

Numerous peptidases (ACE, AmA, AmM, DAP IV) are present in both plasma and vasculature [3, 4, 36], and significant experimental evidence suggests a close association between the vascular and circulating forms of these enzymes [4, 36–38]. Plasma ACE levels are commonly used as an index of endogenous angiotensin I conversion. However, because of the large surface area of vascular endothelium, there is little doubt that it is the vascular enzyme which accounts for the majority of conversion *in vivo*. Further, such cell surface metabolism may have particular physiologic significance since it would occur in the micro-environment of the vascular cell surface receptors.

Although the present study includes no physiologic data, it is interesting to speculate on the possible physiologic importance of C-terminal hydrolysis of kinins and enkephalins by plasma CPN and the endothelial carboxypeptidase. Opioid peptides are present in the circulation and have significant cardiovascular effects [39]. Conversion of Arg[6]- and Lys[6]-enkephalin (kappa agonists) to their pentapeptide forms (delta agonists) may modulate such effects [10]. Conversion of kinins (B_2 agonists) to des(Arg)kinins (B_1 agonists) could alter the relative balance between B_1- and B_2-mediated effects, particularly in inflammatory states [11] or in patients receiving ACE inhibitors. Although full activation of kininogen would yield plasma kinin concentrations significantly below the K_m of the enzyme, local concentrations of kinins formed by vascular and other glandular kallikreins may be considerably higher.

Support for a role of carboxypeptidase in *in vivo* kinin metabolism comes from the work of Odya and co-workers [40] who have reported that high levels of des(Arg)kinins are present in normal human plasma. Further, Streeten *et al.* [41] have shown that low levels of plasma CPN are associated with elevated plasma kinin levels and physiologic symptoms consistent with the biologic action of kinins. However, under normal conditions, endothelial carboxypeptidase converstion of kinins may be more commonly relevant to local (rather than systemic) effects since Ryan *et al.* [42] have reported that MERGETPA, unlike ACE inhibitors, does not potentiate the biologic action of exogenously administered kinins.

In summary, the results of the present study demonstrate that a carboxypeptidase is present on the cell

surface of vascular endothelium which can convert kinins and enkephalins in the micro-environment of vascular cell surface receptors. Nevertheless, numerous studies will be required before the physiologic significance of the vascular enzyme can be determined. Such studies include determining its vascular and microvascular distribution, determining the biochemical characteristics of the purified enzyme, and utilizing specific inhibitors in *in vivo* studies. Further, although the results of the present study are confined to kinin and enkephalin metabolism, the possible relevance of vascular carboxypeptidase to the metabolism of other biologically active compounds must be considered. For instance, a physiologically significant role for carboxypeptidase-medaited degradation of anaphylatoxin C3a and C4a has been suggested by the work of Huey *et al.* [8]. MERGETPA caused a significant potentiation of anaphylatoxins *in vivo*, and these studies were the first to demonstrate that C3a anaphylatoxin can elicit a lethal response.

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