Biochemical Pharmacology, Vol. 37, No. 2, pp. 155–160, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ANGIOTENSIN METABOLISM BY CEREBRAL MICROVASCULAR AMINOPEPTIDASE A

HESTER H. BAUSBACK, LAURIE CHURCHILL and PATRICK E. WARD*
Department of Pharmacology, New York Medical College, Valhalla, NY 10595, U.S.A.

(*Received* 31 *December* 1986; *accepted* 3 *April* 1987)

Abstract—Porcine cerebral microvessels were isolated by differential sieving and centrifugation and were characterized by microscopic examination and marker enzyme enrichment (γ-glutamyltransferase; EC 2.3.2.2). Purified microvessels contained a membrane-bound enzyme immunologically indistinguishable from renal aminopeptidase A (AmA; EC 3.4.11.7). AmA hydrolyzed both α-glutamyl- and α-aspartyl-2-naphthylamide, and hydrolysis was competitively inhibited by angiotensin II. Microvessel AmA hydrolyzed the N-terminal Asp^1-Arg^2 bond of both angiotensin I and angiotensin II, whereas the angiotensin II antagonist saralasin [(Sar^1,Ala^8)angiotensin II] was resistant to N-terminal hydrolysis. Angiotensin metabolism was optimal at pH 8.5 and was inhibited by EDTA, *o*-phenanthroline and amastatin. Conversely, inhibitors of neutral endopeptidase (phosphoramidon), post-proline cleaving enzyme (Z-Pro-Prolinal), carboxypeptidase N [D-L-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA)] and angiotensin I converting enzyme (captopril) had no effect. The K_m values of angiotensin I, angiotensin II and (Asn^1,Val^5)angiotensin II for microvessel AmA were 40.1 ± 8.2, 35.3 ± 4.3 and 156 ± 22 μM respectively. Cerebral microvascular aminopeptidase A may play a role *in vivo* in modulating angiotensin-mediated local cerebral blood flow, and in preventing circulating angiotensins from crossing the blood–brain barrier.

Angiotensin I is produced by renin (EC 3.4.99.19) and is converted to angiotensin II by angiotensin I converting enzyme (ACE; EC 3.4.15.1) [1]. In addition to affecting systemic blood pressure, angiotensin II may also be generated locally to modulate blood flow in particular vascular beds. Renin has been identified in cultured endothelial and smooth muscle cells [2, 3], and ACE is present on vascular endothelium.

Both renin and ACE have also been identified in cerebral microvessels [4, 5], and angiotensin II is a potent constrictor of cerebral vessels [6]. If circulating and/or locally released angiotensin II modulates cerebral blood flow, the capacity of the cerebral vasculature to degrade angiotensin II could have important physiologic significance. However, although both ACE and aminopeptidase M (AmM; EC 3.4.11.2) are present in cerebral microvessels [7, 8], neither enzyme metabolizes angiotensin II [9]. Thus, the present studies were conducted to examine the degradation of angiotensin II by purified porcine cerebral microvessels.

MATERIALS AND METHODS

Materials. Angiotensin I and II, (Asn^1,Val^5)-angiotensin II and saralasin [(Sar^1,Ala^8)angiotensin II], α-glutamyl- and α-aspartyl-2-naphthylamide, α-glutamyl-4-methoxy-2-naphthylamide (α-Glu-MNA), *o*-phenanthroline, amastatin, phosphoramidon [*N*-(α-rhamno-pyranosyl-oxyhydroxyphosphinyl-L-leucyl-L-tryptophan)] and papain were obtained from the Sigma Chemical Co. (St. Louis, MO). The ACE inhibitor (captopril) and the carboxypeptidase N (EC 3.4.17.3) inhibitor MERGETPA (D-L-mercaptomethyl-3-guanidinoethylthiopropanoic acid) were obtained from Squibb (Princeton, NJ) and Calbiochem-Behring (San Diego, CA) respectively. The post-proline cleaving enzyme (EC 3.4.21.26) inhibitor Z-Pro-prolinal was provided by Dr. Sherwin Wilk (Mount Sinai School of Medicine, New York, NY). Antibody to porcine aminopeptidase A (AmA; EC 3.4.11.7), obtained as previously described [10, 11], precipitates AmA but does not cross-react with other renal peptidases including ACE, AmM, dipeptidyl(amino)peptidase IV (EC 3.4.14.5), neutral endopeptidase (EC 3.4.24.11) and carboxypeptidase P (EC 3.4.12.__). The nylon sieves used for microvessel isolation were from Tetko, Inc. (Elmsford, NY). The MN 300 Uniplates used for TLC were from Analtech, Inc. (Newark, DE), and the amino acid standards, *o*-phthalaldehyde crystals and reagent solution (OPA) used for HPLC were from the Pierce Chemical Co. (Rockford, IL).

Cerebral microvessel isolation. Whole brains, obtained both frozen (Pel-Freeze, Rogers, AR) and from freshly slaughtered hogs, were immersed in cold Earle's buffer (pH 7.4) containing 0.1% (w/v) bovine serum albumin. Microvessels were prepared as previously described [8] according to Brendel *et al.* [12] as modified by Selivonchick and Roots [13]. Cortical grey matter was minced and homogenized (30 sec) in 3 vol. of buffer using a Waring blender. The extract was then homogenized (Dounce, four strokes), poured through 1000 and 300 micron mesh nylon sieves, and re-homogenized. Vessels were collected on a 44-micron mesh sieve, centrifuged (4000 *g*, 10 min) and resuspended (0.25 M sucrose). After centrifugation (21,000 *g*, 45 min) on a discontinuous sucrose gradient

* Author to whom reprint requests should be sent.

(1.0 and 1.5 M), the pellet was freeze-powdered, sonicated and pelleted (20,000 *g*, 15 min). Activity was measured either directly or after solubilization with 5% (v/v) Triton X-100. Microvessel purity was determined by phase constrast microscopy and assay of γ-glutamyltransferase (γ-GT; EC 2.3.2.2) [8].

Immunoelectrophoresis. Rocket and fused-rocket immunoelectrophoresis of detergent/papain-solubilized microvessel membrane against antisera to porcine renal brush border AmA were carried out in 1% (w/v) agarose gels as described previously [10, 11], and the AmA precipitin line was visualized histochemically using α-Glu-MNA.

Enzyme assays. Aminopeptidase A activity was assayed as the rate of hydrolysis of α-glutamyl- and α-aspartyl-2-naphthylamide [8]. For competitive substrate experiments, angiotensin II was added simultaneously with α-glutamyl-2-naphthylamide. Specific activities are expressed as units/mg protein where one unit equals the hydrolysis of one nmole substrate per min. Protein was determined according to Bradford [14] using bovine serum albumin as a standard.

Angiotensin metabolism. Qualitative analysis of angiotensin metabolism was carried out by TLC on MN 300 cellulose plates [9]. Quantitative analysis was performed by HPLC as described previously [8, 15]. Microvessel AmA was determined as the rate of N-terminal Asp released from angiotensin I and II in the presence of 1.25 mM $CaCl_2$. The standard incubation consisted of mixing the peptide in 295 μl of 100 mM sodium phosphate buffer (pH 8.3) with 5 μl of a dilution of microvessel protein (37°). At sequential time intervals, 60-μl aliquots were immersed in a boiling water bath (5 min) to terminate the reaction, cooled on ice, centrifuged in a Brinkmann table top centrifuge (3 min), and the supernatant fractions were collected for analysis.

For determination of pH dependence, 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. pH studies were also conducted with the Britton and Robinson type [16] universal buffer (pH 5.5 to 10.5). For inhibition studies, inhibitors were preincubated with enzyme and buffer for 20 min at 37°. For K_m determinations, measurements of the initial velocity of hydrolysis were determined over a range of substrate concentrations (12.5 to 250 μM). Data were plotted as $1/V$ vs $1/S$ and fit to the best straight line [17].

A high performance liquid chromatograph system (Waters Associates, Milford, MA) consisting of two model 6000A pumps, a model 730 Data Module, a model 721 System controller, a model 712B WISP™ Autosampler and a model 420 Fluorescence Detector were employed for the HPLC analysis. Standards and unknowns (10–60 μl) were automatically derivatized with OPA solution (20 μl) 3 min prior to chromatography (Precolumn Derivatization Program—Waters 710B WISP™ Autosampler) and subsequently separated on a reverse phase column (Waters, 10 micron, C_{18}-Radial-PAK™, 8 mm × 10 cm) at a constant flow rate of 5.0 ml/min utilizing a linear gradient from 100% Buffer A to 40% Buffer A/60% Buffer B. Integration of sample peak areas and quantitation of metabolites against the last-run standards were automatically calculated by the data module. Standards were run every sixth injection.

The OPA solution was made freshly daily by mixing 3.8 ml *o*-phthalaldehyde reagents solution, 0.1 ml methanol, 0.1 ml β-mercaptoethanol and 16.8 mg *o*-phthalaldehyde crystals. Buffer A was 10 mM sodium phosphate (pH 7.0) and Buffer B was a 50/50 (v/v) mixture of Buffer A and acetonitrile.

RESULTS

Isolated microvessels. As previously found [8], phase contrast microscopy of isolated cerebral microvessel preparations revealed intact vessels devoid of non-vascular contamination (Fig. 1). Vessels (7–70 micron diameter) exhibited extensive dichot-

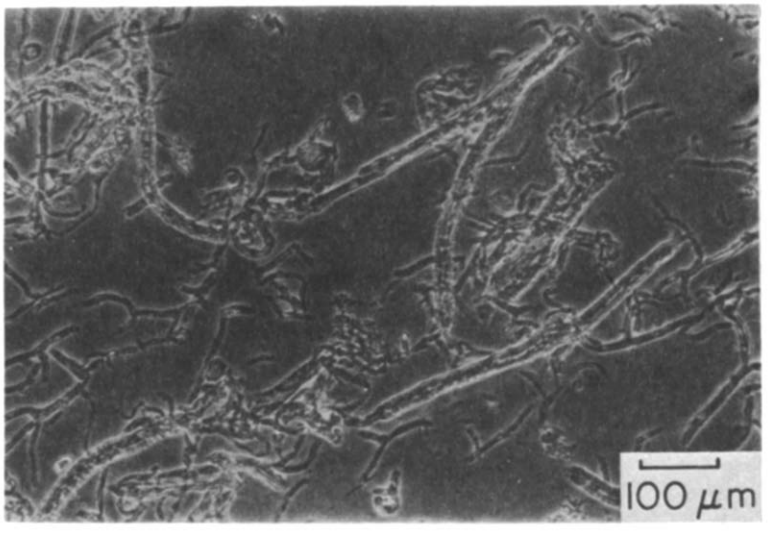

Fig. 1. Phase contrast micrograph of isolated cerebral microvessels.

omous branching and numerous bifurcations. At higher magnifications, endothelial cell nuclei could be visualized.

The extent of microvessel purification was determined by enrichment of γ-glutamyltransferase activity. As seen in Table 1, cerebral microvessel γ-glutamyltransferase activity (3.5 ± 1.1 nmol/min/mg protein) was enriched 25-fold over original homogenate (0.14 ± 0.02 units/mg). Microvessels were also enriched (12.6-fold) in AmA-like activity (α-glutamyl-2-naphthylamide hydrolysis). Homogenate and microvessel activities were 0.38 ± 0.08 and 4.8 ± 0.6 nmol/min/mg protein respectively. After centrifugation (20,000 *g*, 15 min) of the freeze-powdered microvessels, more than 70% of the AmA-like activity was detected in the pelleted membrane fraction. After solubilization with Triton X-100 (5%), this activity hydrolyzed α-glutamy-2-naphthylamide 4.9-fold more rapidly than α-aspartyl-2-naphthylamide. Further, the hydrolysis of α-glutamyl-2-naphthylamide was competitively inhibited by angiotensin II (200 μM).

Immunologic analysis. Kidney brush border (KBB) aminopeptidase A and solubilized cerebral microvessel (CMV) membrane were analyzed by fused-rocked immunoelectrophoresis. As shown in Fig. 2A, fused, rocket-shaped precipitates were visualized with no evidence of spurring. Shadowing of the precipitin lines resulted from overstaining in an effort to detect even minimal spurring. Thus, in addition to the previously identified aminopeptidase M [8], cerebral microvessel membrane contained an enzyme immunologically indistinguishable from renal aminopeptidase A.

To determine if immunoreactive-AmA was enriched in cerebral microvessels as suggested by the high levels of microvessel AmA-like activity (Table 1) rocket-immunoelectrophoretic studies were conducted. Cerebral microvessel protein (28 μg) produced a strong, rocket-shaped precipitate (Fig. 2B; right side), and sequential dilutions resulted in peak areas proportional to the amounts of microvessel protein used. Even a 1/16th dilution of microvessel protein (1.7 μg) produced a small peak (visible in the original gel). Conversely, 28 μg of brain homogenate protein (Fig. 2B; left side) produced only a faintly detectable precipitin peak which was even smaller than that seen for 1.7 μg of microvessel protein.

Angiotensin metabolism—TLC. Consistent with the known substrate specificity of AmA for N-terminal acidic amino acids, incubation of angiotensin II with cerebral microvessel membrane and subsequent separation of metabolites by TLC revealed that the peptide was rapidly metabolized, producing the N-terminal Asp within 15–30 min. A second metabolite (Arg) was also detected, indicating subsequent N-terminal hydrolysis. Similarly, in the presence of 10 μM captopril to inhibit ACE, N-terminal Asp was generated from angiotensin I. Although microvessels also hydrolyzed the N-terminal Asn^1-Arg^2 bond of (Asn^1,Val^5)angiotensin II, no hydrolysis of the angiotensin antagonist (Sar^1,Ala^8)angiotensin II was found.

Angiotensin metabolism—HPLC. Quantitative analysis of cerebral microvessel membrane hydrolysis of angiotensin I, angiotensin II and (Asn^1,Val^5)-angiotensin II established that the rates of production of N-terminal Asp or Asn were directly proportional to both time of incubation (0–90 min) and amount of microvessel membrane used. N-Terminal hydrolysis was optimal at pH 8.5 (Fig. 3) and was inhibited by 1 mM EDTA (90%), 1 mM *o*-phenanthroline (100%) and amastatin (I_{50} = 1–2 μM) (Fig. 4). Inhibitors of neutral endopeptidase (phosphoramidon), post-proline cleaving enzyme (Z-Pro-prolinal), carboxypeptidase N (MERGETPA) and ACE (captopril) had no effect (less than 7% inhibition at 10 μM final concentration).

As shown in Fig. 5 (individual experiment) and Table 2, maximal velocities of hydrolysis were higher for angiotensin II (16.1 ± 1.7 nmol/min/ml) than for angiotensin I (6.1 ± 0.4 units/ml), while the apparent K_m values of the two peptides were comparable (35.3 ± 4.3 and 40.1 ± 8.2 μM respectively). Although (Asn^1,Val^5)angiotensin II was rapidly metabolized (37.2 ± 6.4 nmol/min/ml), affinity was low (K_m = 156 ± 22 μM). As expected from the results of the preliminary TLC studies, no N-terminal hydrolysis of (Sar^1,Ala^8)angiotensin II could be detected.

DISCUSSION

Angiotensin II is a potent constrictor of numerous vascular beds including isolated cerebral vessels [6]. Renin or renin-like activity has been reported in

Table 1. Distribution of γ-Glutamyltransferase and aminopeptidase A-like activity in homogenates of whole brain and isolated cerebral microvessels

Enzyme	Enzyme specific activity in: Brain homogenate	Cerebral microvessels	Enrichment
γ-Glutamyltransferase	0.14 ± 0.02	3.5 ± 1.1	25.0
Aminopeptidase A-like activity	0.38 ± 0.08	4.8 ± 0.6	12.6

Specific activities are calculated as nmol substrate hydrolyzed per min/mg protein. Values given are the means ± SE from four experiments. Enrichment is calculated as (mean specific activity of microvessel fraction)/(mean specific activity of brain homogenate).

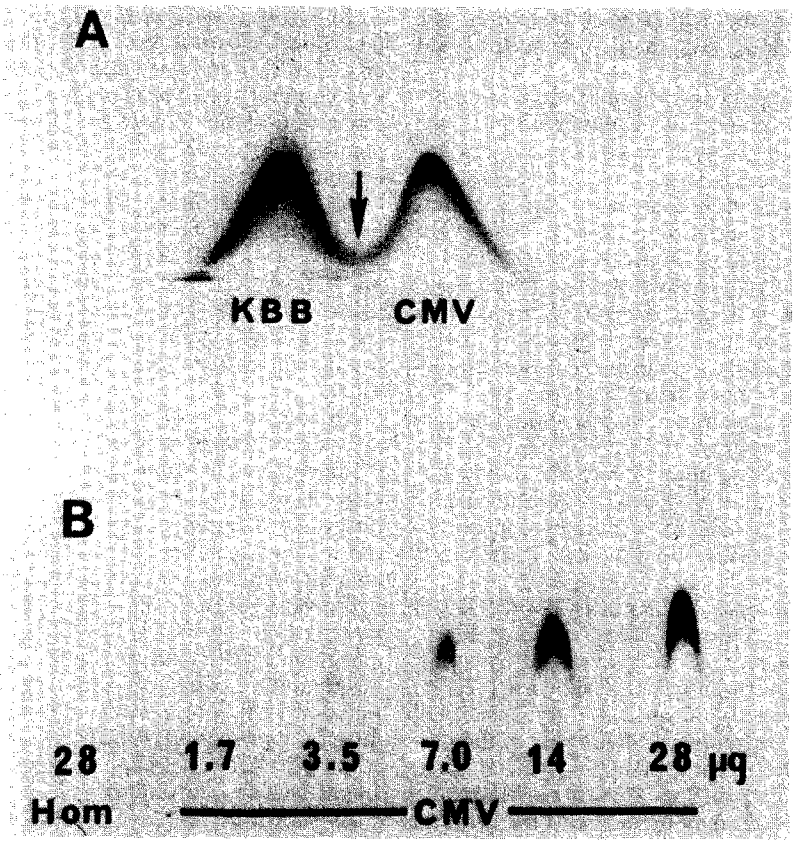

Fig. 2. (A) Fused-rocket immunoelectrophoresis of papain/detergent-solubilized porcine kidney brush border (KBB) AmA (left side) with cerebral microvessels (CMV) (right side). After 1 hr for diffusion, samples were vertically electrophoresed into gel containing anti-porcine AmA immunoglobulin at 2 V/cm for 18 hr. The vertical arrow indicates area of fusion. (B) Rocket immunoelectrophoresis of solubilized cerebral homogenate (Hom; 28 μg) and microvessel membrane (CMV; 1.7 to 28 μg). Samples were vertically electrophoresed into gel containing anti-porcine aminopeptidase A immunoglobulin at 20 V/cm for 2 hr. Preciptin lines were visualized by staining for enzyme activity as described in Materials and Methods.

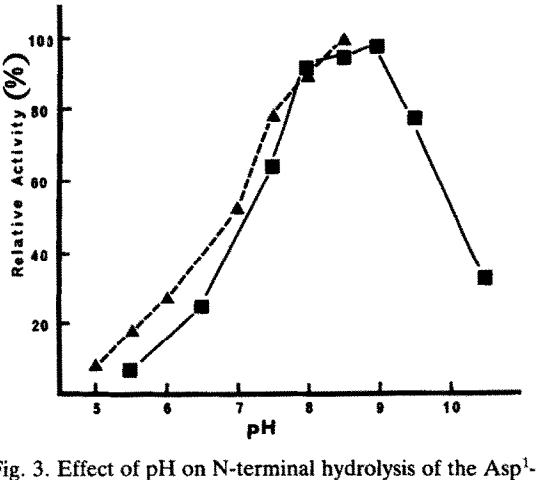

Fig. 3. Effect of pH on N-terminal hydrolysis of the Asp^1-Arg^2 bond of angiotensin II by cerebral microvessels. Activity is expressed as percent maximal activity in either sodium acetate/phosphate buffer (▲) or Britton and Robinson universal buffer (■). Values given are the averages of three determinations.

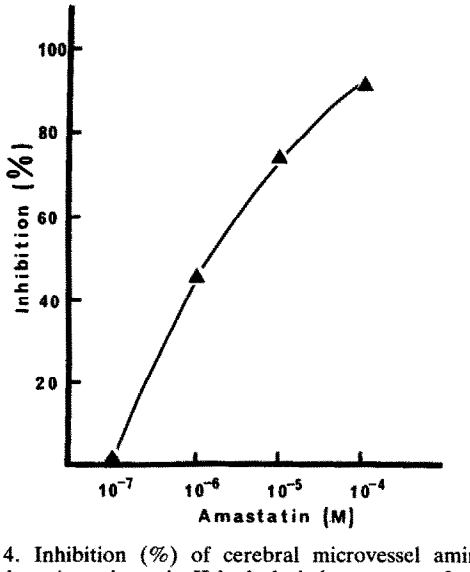

Fig. 4. Inhibition (%) of cerebral microvessel aminopeptidase A angiotensin II hydrolysis by a range of concentrations of amastatin. Values shown are averages of two determinations.

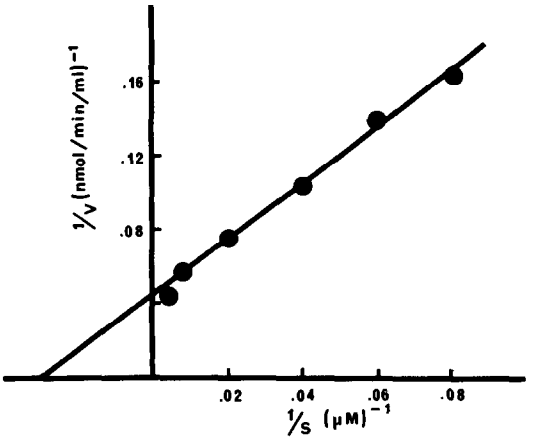

Fig. 5. Lineweaver–Burk plot of the rate of N-terminal hydrolysis of angiotensin II by cerebral microvessels.

large vessels, cultured endothelium and smooth muscle [2, 3] and isolated cerebral microvessels [4]. Cerebral microvessels also contain ACE [5], and inhibition of ACE blocks the constrictor effect of angiotensin I on cerebral arteries [6]. Little is known, however, regarding cerebral microvessel degradation of angiotensin II. Although aminopeptidase M (EC 3.4.11.2) has been found in cerebral microvessels [8], it cannot account for angiotensin II degradation since the acidic N-terminus of angiotensin II (Asp) makes it resistant to aminopeptidase M-mediated degradation [9].

The present data demonstrate that cerebral microvessels contain membrane-bound aminopeptidase A (EC 3.4.11.7) and that the principal route of angiotensin II degradation is N-terminal hydrolysis of the Asp^1-Arg^2 bond. Although participation of other microvascular aminopeptidases cannot be ruled out completely, such metabolism is consistent with the substrate specificity of AmA for N-terminal acidic amino acids from both synthetic substrates [18, 19] and angiotensin II [20]. Like purified renal AmA [18], the cerebral vascular enzyme hydrolyzed α-glutamyl-2-naphthylamide nearly 5-fold faster than α-aspartyl-2-naphthylamide. Other similarities to renal AmA include sensitivity to inhibition by both chelating agents and amastatin [18–20]. While such agents may also inhibit cytosolic aspartyl-aminopeptidase (EC 3.4.11.__) [21, 22], the present study used solubilized microvessel membrane. Collectively, these data support identification of aminopeptidase A as the enzyme responsible for microvascular angiotensin II hydrolysis.

Although aminopeptidase A (or -like) activity has been reported in plasma, endothelium [23], glomeruli [20] and adrenal vasculature [24], we are not aware of any study that has examined the kinetics of AmA metabolism of angiotensins. The K_m found with angiotensin II for AmA (35 μM) is comparable to that reported with angiotensin I for ACE (30–70 μM) [25, 26]. AmA also converted angiotensin I to des(Asp^1)angiotensin I (K_m = 40 μM). Since Ryan and co-workers [27] have demonstrated that the K_m of the reaction of des(Asp^1)angiotensin I with ACE is relatively low (11 μM), direct formation of angiotensin III may occur. Since angiotensin III may also affect cerebral vascular tone, studies are in progress to investigate its degradation by microvascular aminopeptidase M [8, 9].

The subcellular and cellular localization of AmA remains to be determined. Like renal and vascular ACE and AmM [10, 11, 21], it is probable that AmA is a plasma membrane enzyme. Such a localization would allow for metabolism of angiotensins in the microenvironment of vascular cell surface receptors. In addition to plasma AmA (angiotensinase A), endothelial AmA could modulate the effects of circulating angiotensins on local blood flow and, as a component of the blood–brain barrier, prevent peripheral angiotensins from entering the central nervous system [28] where they could interfere with the biologic actions of angiotensins generated within the CNS [29]. An endothelial localization would seem likely considering the epithelial localization of renal AmA [21] and the epithelial properties of cerebral capillary endothelium [30]. Further, AmA-like activity has been identified in adrenal capillary endothelium [24]. Nevertheless, like vascular smooth muscle AmM [31], AmA may also be present within the vascular wall where metabolism of locally released angiotensins and/or other perivascular peptides containing N-terminal acidic amino acids (e.g. CCK-8) [32] may occur.

Acknowledgements—This work was supported by Grants R01 DK 28184 and P01 HL 34300. We would like to thank the Karl Ehmer Co. for supplying fresh hog tissues.

Table 2. Kinetics of N-terminal hydrolysis of angiotensins by detergent-solubilized cerebral microvessel aminopeptidase A

Peptide	K_m (μM)	V_{max} (nmol/min/ml)
Angiotensin I	40.1 ± 8.2 (N = 3)	6.1 ± 0.4
Angiotensin II	35.3 ± 4.3 (N = 9)	16.1 ± 1.7
(Asn^1,Val^5)Angiotensin II	156 ± 22 (N = 3)	37.2 ± 6.4

Assays were performed over a range of substrate concentrations (12.5 to 250 μM) and plotted as in Fig. 5. Values are the means ± SE.

REFERENCES

1. R. L. Soffer, *A. Rev. Biochem.* **45**, 73 (1976).
2. V. J. Dzau, *J. cardiovasc. Pharmac.* **6**, S377 (1984).
3. V. J. Dzau, *Hypertension* **8**, 553 (1986).
4. H. Kowaloff, H. Gavras and P. Brecher, *Am. J. Physiol.* **238**, H384 (1980).
5. P. Brecher, A. Tercyak and A. V. Chobanian, *Hypertension* **3**, 198 (1983).
6. E. T. Whalley, M. Wahl and C. A. M. Sampaio, *Hypertension* **5**, V-34 (1983).
7. H. H. Bausback and P. E. Ward, *Biochim. biophys. Acta* **882**, 437 (1986).
8. L. Churchill, H. H. Bausback, M. E. Gerritsen and P. E. Ward, *Biochim. biophys. Acta* **923**, 35 (1987).

9. F. E. Palmieri, J. J. Petrelli and P. E. Ward, *Biochem. Pharmac.* **34**, 2309 (1985).
10. P. E. Ward and M. A. Sheridan, *Biochem. Pharmac.* **32**, 265 (1983).
11. P. E. Ward, *Biochem. Pharmac.* **33**, 3183 (1984).
12. K. Brendel, E. Meezan and E. C. Carlson, *Science* **185**, 953 (1974).
13. D. P. Selivonchick and B. I. Roots, *Lipids* **12**, 165 (1977).
14. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
15. F. E. Palmieri, H. H. Bausback, L. Churchill and P. E. Ward, *Biochem. Pharmac.* **35**, 2749 (1986).
16. R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, *Data for Biochemical Research*,, 2nd Edn. Oxford University Press, Oxford (1969).
17. M. Dixon and E. C. Webb, *Enzymes*, 3rd Edn. Academic Press, New York (1979).
18. E. M. Danielsen, O. Noren, H. Sjostrom, J. Ingram and A. J. Kenny, *Biochem. J.* **189**, 591 (1980).
19. H. Tobe, F. Kojima, T. Aoyagi and H. Umezawa, *Biochim. biophys. Acta* **613**, 459 (1980).
20. P. Kugler, *Histochemistry* **74**, 229 (1982).
21. A. J. Kenny, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), p. 393. Elsevier/North Holland Biomedical Press, Amsterdam (1977).
22. J. K. McDonald and C. Schwabe, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), p. 311. Elsevier/North Holland Biomedical Press, Amsterdam (1977).
23. A. R. Johnson, *J. clin. Invest.* **65**, 841 (1980).
24. P. J. Del Vecchio, J. W. Ryan, A. Chung and U. S. Ryan, *Biochem. J.* **186**, 605 (1980).
25. F. E. Dorer, J. R. Kahn, K. E. Lentz, M. Levine and L. T. Skeggs, *Circulation Res.* **34**, 824 (1974).
26. M. Das and R. L. Soffer, *J. biol. Chem.* **250**, 6762 (1975).
27. A. T. Chiu, J. W. Ryan, J. M. Stewart and F. E. Dorer, *Biochem. J.* **155**, 189 (1976).
28. G. Meisenberg and W. H. Simmons, *Life Sci.* **32**, 2611 (1983).
29. D. Ganten, R. E. Lang, E. Lehmann and T. Unger, *Biochem. Pharmac.* **33**, 3523 (1984).
30. A. L. Betz, *Fedn Proc.* **44**, 2614 (1985).
31. F. E. Palmieri, P. M. Luckett, S. A. Stalcup and P. E. Ward, *Fedn Proc.* **43**, 651 (1984).
32. J. McCulloch, *Nature, Lond.* **304**, 120 (1983).