Biochemical Pharmacology, Vol. 42, No. 4, pp. 721–727, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

METABOLISM OF BRADYKININ AGONISTS AND ANTAGONISTS BY PLASMA AMINOPEPTIDASE P

PATRICK E. WARD,* ANA CHOW and GUY DRAPEAU
Department of Physiology, The Ohio State University, Columbus, OH 43210, U.S.A.

(*Received* 7 *November* 1990; *accepted* 1 *February* 1991)

Abstract—In addition to angiotensin I converting enzyme (ACE; EC 3.4.15.1) and carboxypeptidase N (CPN; EC 3.4.17.3), other peptidases contribute to bradykinin (BK) degradation in plasma. Rat plasma degraded BK by hydrolysis of the N-terminal Arg^1-Pro^2 bond, and the characteristics of hydrolysis are consistent with identification of aminopeptidase P (APP; EC 3.4.11.9) as the responsible enzyme. BK and BK[1–5] N-terminal hydrolysis was optimal at neutral pH, was inhibited by 2-mercaptoethanol, dithiothreitol, *o*-phenanthroline and EDTA, but was unaffected by the aminopeptidase inhibitors amastatin, puromycin and diprotin A, the endopeptidase-24.11 inhibitors phosphoramidon and ZINCOV, and the ACE and CPN inhibitors captopril and D,L-mercapto-methyl-3-guanidino-ethylthiopropanoic acid (MERGETPA), respectively. Although kallidin (Lys-BK) was not metabolized directly by APP, conversion to BK by plasma aminopeptidase M (EC 3.4.11.2) resulted in subsequent degradation by APP. BK analogs containing N-terminal Arg^1-Pro^2 bonds, including [Tyr^8-(OMe)]BK and [$Phe^8\psi(CH_2NH)Arg^9$]BK (B_2 agonists), des-Arg^9-BK and [D-Phe^8]des-Arg^9-BK (B_1 agonists), and [Leu^8]des-Arg^9-BK (B_1 antagonist), were degraded by APP with K_m and V_{max} values comparable to those found for BK (K_m = 19.7 ± 2.6 μM; V_{max} = 12.1 ± 1.2 nmol/min/mL). In contrast, B_2 antagonists containing D-Arg^0 N-termini, including D-Arg[Hyp^3,$Thi^{5,8}$,D-Phe^7]BK and D-Arg[Hyp^3,D-Phe^7,$Phe^8\psi(CH_2NH)Arg^9$]BK, were resistant to APP-mediated hydrolysis. These data support a role for plasma aminopeptidase P in the degradation of circulating kinins, and a variety of B_2 and B_1 kinin agonists and antagonists. However, APP does not participate in the degradation of D-Arg^0-containing antagonists.

Bradykinin (BK) and kallidin are potent vasoactive peptides [1–4] which are metabolized rapidly in the circulation by angiotensin converting enzyme (ACE; EC 3.4.15.1) and carboxypeptidase N (CPN; EC 3.4.17.3) [5–10]. Kinin metabolism by ACE results in inactivation, whereas hydrolysis by CPN forms des-Arg^9-BK and des-Arg^{10}-kallidin which, although inactive on the B_2 kinin receptor, are the natural agonists of the B_1 kinin receptor [11–13].

In addition to these naturally occurring kinins, Vavrek and Stewart [14] have developed a number of D-Phe^7-kinin analogs which are effective kinin antagonists, and Regoli *et al.* [15] have identified des-Arg^9-kinin analogs specific for the B_1 receptor. Although recent studies have demonstrated that these and other analogs demonstrate differential sensitivity to metabolism by ACE and CPN, and that such differential metabolism can be related directly to potency and receptor specificity considerations [10, 16–21], significant kinin and kinin analog degradation occurs which cannot be accounted for by ACE and CPN [22–24]. Thus, the present study was conducted to identify and characterize the enzymes other than ACE and CPN which contribute to kinin and kinin analog metabolism in plasma.

MATERIALS AND METHODS

Materials. Bradykinin (BK), BK[2–9], des-Arg^9-BK, BK[1–5], BK[1–7], BK[2–7], kallidin, dithiothreitol, *o*-phenanthroline, EDTA, amastatin, puromycin and phosphoramidon were obtained from the Sigma Chemical Co. (St. Louis, MO). D-Arg[Hyp^3,D-Phe^7]BK and D-Arg[Hyp^3,$Thi^{5,8}$,D-Phe^7]BK were gifts from Drs. Stewart and Vavrek (University of Colorado, Denver, CO). [Tyr^8(OMe)]BK, [$Phe^8\psi(CH_2NH)Arg^9$]BK, D-Arg[Hyp^3,D-Phe^7,Leu^8]BK, D-Arg[Hyp^3,D-Phe^7,$Phe^8\psi(Ch_2NH)Arg^9$]BK, [D-Phe^8]des-Arg^9-BK, [Leu^8]des-Arg^9-BK, and Phe-Arg were synthesized by the solid phase method as described previously [25]. The ACE inhibitor captopril was from Squibb (Princeton, NJ). The CPN inhibitor D,L-mercapto-methyl-3-guanidinoethylthiopropanoic acid (MERGETPA) [26], and the endopeptidase-24.11 (EC 3.4.24.11) inhibitor ZINCOV [27], were obtained from Calbiochem-Behring (San Diego, CA). The dipeptidylaminopeptidase IV (EC 3.4.14.5) inhibitor diprotin A [28] was obtained from Peninsula Laboratories (Belmont, CA). Amino acid standards, 2-mercaptoethanol, *o*-phthalaldehyde crystals and *o*-phthalaldehyde reagent solution were from the Pierce Chemical Co. (Rockford, IL).

Metabolism by rat plasma. The standard incubation (600 μL) consisted of plasma (1–4 μL), peptide substrate (25 μM), and 50 mM phosphate buffer (pH 7.5) containing captopril (10 μM) and MERGETPA (10 μM) to inhibit plasma ACE and CPN, respectively. In selected cases, diprotin A (10 μM) was also included to inhibit plasma dipeptidylaminopeptidase IV [28]. At sequential time intervals aliquots were immersed in a boiling water bath (5 min) to terminate the reaction, cooled on ice, and centrifuged in a

* Send reprint requests to: Dr. Patrick E. Ward, Department of Physiology, The Ohio State University, 4196 Graves Hall, 333 West 10th Ave., Columbus, OH 43210-1239.

table top centrifuge (5 min), and the supernate was collected for HPLC analysis [21, 29, 30]. Peptide hydrolysis was determined by the decrease in substrate (e.g. BK) and the increase in products (e.g. BK[2–9] and Arg). Rates of hydrolysis were calculated for the initial 30% of the reaction and were directly proportional to both time of incubation and amount of enzyme added. For inhibition studies, inhibitors were preincubated with enzyme and buffer for 10 min (37°) before addition of substrate. For K_m determinations, measurements of initial velocity were made over a range of substrate concentrations. Data were plotted as $1/V$ vs $1/[S]$ and fit to the best straight line.

HPLC analysis. 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 710B WISP™ Autosampler, a Lambda-Max model 481 LC Spectrophotometer (set at 210 nm) and a model 420 Fluorescence Detector were employed for HPLC analysis. Peptide substrates and metabolites (e.g. BK and BK[2–9]) were separated and quantitated as described previously [20, 21] on a reverse phase column (Vydac, 10 μm, C_{18}-μBondapak, 1.9 cm × 30 cm) at a constant flow rate of 2 mL/min using a linear gradient from 100% Solvent A to 35% Solvent A/65% Solvent B (21 min). After each separation, the column was re-equilibrated in Solvent A (3 min). Integration of sample peak areas and quantitation of peptide substrates and metabolites against the last-run standards (run every sixth injection) were calculated automatically by the data module. Solvent A was 0.05% trifluoroacetic acid (TFA) (v/v) in triple-distilled water and Solvent B was 0.04% TFA (v/v) in acetonitrile.

Amino acid metabolites (e.g. N-terminal Arg) were separated and quantitated as described previously [29, 30]. Briefly, samples (40 μL) were derivatized automatically with *o*-phthalaldehyde (20 μL) 3 min prior to chromatography (Pre-column Derivatization Program – Waters 710B WISP™ Autosampler) and separated subsequently on a reverse phase column (Waters, 10 μm, C_{18}-Radial-PAK™, 8 mm × 10 cm) at a constant flow rate of 5.0 mL/min utilizing a linear gradient from 100% Solvent A to 40% Solvent A/60% Solvent B (12 min). After each separation, the column was re-equilibrated in Solvent A (3 min). Solvent A was 10 mM sodium phosphate (pH 7.0) and Solvent B was a 50% (v/v) mixture of Solvent A and acetonitrile. As above, integration of sample peak areas and quantitation of amino acid metabolite against the last-run standard was calculated automatically by the data module.

RESULTS

In the presence of captopril (10 μM) and MERGETPA (10 μM), ACE- and CPN-mediated metabolism of BK (retention time = 13.4 min) was inhibited completely as demonstrated by the absence of the ACE metabolites Phe-Arg (RT = 8.7 min) and BK[1–7] (RT = 12.0 min), and the CPN metabolite des-Arg^9-BK (RT = 14.9 min). Rather, rat plasma hydrolyzed BK (Fig. 1A) to produce the metabolites BK[2–9] (RT = 13.8 min) (Fig. 1B) and Arg (determined by amino acid analysis, not shown). In addition, two small unidentified metabolite peaks with retention times of 11.5 and 12.3 min were observed (Fig. 1B). Since production of BK[2–9] and Arg indicated hydrolysis of the N-terminal Arg^1-Pro^2 bond of BK, the incubation was repeated in the presence of the aminopeptidase P (APP; EC 3.4.11.9) inhibitor 2-mercaptoethanol [31–33]. 2-Mercaptoethanol (4 mM) inhibited formation of both BK[2–9] (Fig. 1C) and Arg (not shown), but did not inhibit formation of the unidentified metabolites at 11.5 and 12.3 min (Fig. 1C). Consistent with hydrolysis of the N-terminal Arg^1-Pro^2 bond, incubation with des-Arg^9-BK produced comparable results (i.e. N-terminal Arg production inhibited by 2-mercaptoethanol). Since dipeptidylaminopeptidase IV has been shown to hydrolyze the APP metabolite BK[2–9] [31], subsequent incubations were conducted in the presence of the dipeptidyl-aminopeptidase IV inhibitor diprotin A (10 μM).

BK degradation was proportional to the time of incubation and the amount of plasma used. As shown in Fig. 2, the progressive decrease in BK over time was associated with nearly stoichiometric increases in both BK[2–9] and Arg. BK degradation, and production of the BK[2–9] and Arg metabolites, were inhibited nearly completely by 2-mercaptoethanol (Fig. 2). Since N-terminal hydrolysis of the Arg^1-Pro^2 bond of BK was the predominant degradative activity detected, subsequent studies were performed to characterize this APP-like activity (i.e. Arg^1-Pro^2 hydrolysis inhibited by 4 mM 2-mercaptoethanol).

N-Terminal hydrolysis of BK was optimal at pH 7.3. As shown in Table 1, hydrolysis of BK and BK[1–5] was inhibited by 2-mercaptoethanol, dithiothreitol, *o*-phenanthroline, and EDTA, but was unaffected by inhibitors of aminopeptidase A and M (amastatin) [34, 35], aminopeptidase B (puromycin) [36], dipeptidylaminopeptidase IV (diprotin A) [28], neutral endopeptidase-24.11 (phosphoramidon, ZINCOV) [27, 37], CPN (MERGETPA) [26] or ACE (captopril). Collectively, these data are consistent with identification of APP as the responsible enzyme.

Plasma APP hydrolyzed the Arg^1-Pro^2 bond of a variety of BK analogs at comparable rates (Table 2). In addition to BK[1–5] and BK[1–7], APP hydrolyzed the B_2 agonists [Tyr^8(OMe)]BK and [Phe$^8\psi$(CH$_2$NH)Arg^9]BK, the B_1 agonists des-Arg^9-BK and [D-Phe^8]des-Arg^9-BK, and the B_1 antagonist [Leu^8]des-Arg^9-BK [4, 14, 15]. However, consistent with a specificity for N-terminal X-Pro bonds [31–33], all of the D-Arg^0-containing B_2 antagonists examined were resistant to APP (Table 2). As shown in Table 3, the K_m and V_{max} values found for all of the APP-susceptible kinin agonists and antagonists were comparable to those for BK.

In view of the requirement for N-terminal X-Pro bonds, kallidin (Lys-BK) was expected to be resistant to plasma APP. However, incubations with kallidin (RT = 13.1 min) resulted in production of BK and, with continued incubation, BK[2–9] and Arg (not shown). Since plasma aminopeptidase M (EC

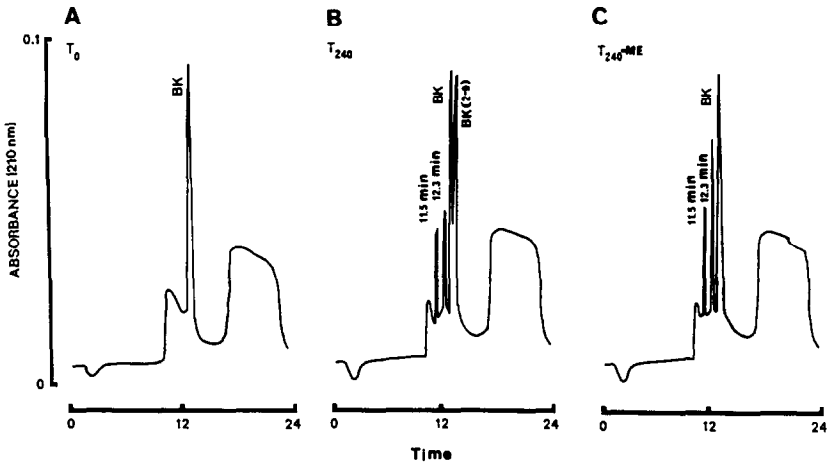

Fig. 1. Elution pattern of (panels A and B) bradykinin (BK; retention time = 13.4 min) degradation to des-Arg^1-BK (BK[2–9]; RT = 13.8 min) and unidentified metabolites by rat plasma containing captopril (10 μM) and MERGETPA (10 μM) as assessed by HPLC on a Vydac C_{18}-μBondapak column. During the 240-min incubation the decrease in BK was reflected by decreased peak area. In panel C, 2-mercaptoethanol (4 mM) was included in the incubation. Peaks due to incubation buffer have been deleted.

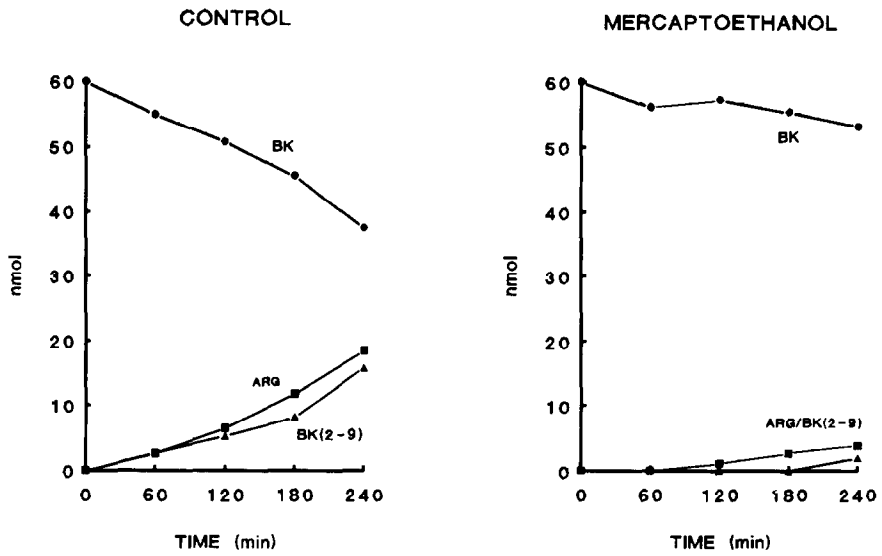

Fig. 2. Rat plasma degradation of bradykinin (BK) and production of des-Arg^1-BK (BK[2–9]) and Arg under control conditions and in the presence of 2-mercaptoethanol (4 mM). Separation and quantitation of substrates and products were assessed by HPLC on a Vydac C_{18}-μBondapak column and a Waters C_{18}-Radial-PAK column, as described in Materials and Methods.

3.4.11.2) has been shown to convert kallidin to BK [10, 35], these data indicate sequential conversion (kallidin to BK) and degradation (BK to BK[2–9]) by aminopeptidase M and APP, respectively. Consistent with this interpretation, the aminopeptidase M inhibitor amastatin (10 μM) completely inhibited conversion of kallidin to BK (and indirectly, subsequent APP-mediated degradation to BK[2–9]). 2-Mercaptoethanol, in turn, had no effect on conversion of kallidin to BK, but completely inhibited subsequent degradation by APP.

DISCUSSION

Despite the established importance of plasma ACE and CPN in kinin metabolism, recent studies have demonstrated that significant kinin degradation occurs which cannot be accounted for by either of these two kininases, or by neutral endopeptidase-24.11 or post-proline cleaving enzyme (EC 3.4.21.26) [22–24]. The results of the present study demonstrate that rat plasma degrades BK by hydrolysis of the N-terminal Arg^1-Pro^2 bond. Hydrolysis was optimal at

Table 1. Inhibition profile of rat plasma aminopeptidase P

Inhibitor	Concentration	Relative activity (%) Substrate: Bradykinin	Relative activity (%) Substrate: Bradykinin[1–5]
None		100	100
2-Mercaptoethanol	5.0 mM	10	3
	0.5 mM	21	28
Dithiothreitol	1.0 mM	21	14
o-Phenanthroline	1.0 mM	0	0
EDTA	1.0 mM	10	31
Amastatin	10 μM	100	118
Puromycin	100 μM	127	113
Diprotin A	100 μM		104
Phosphoramidon	10 μM	105	110
ZINCOV	10 μM	114	118
MERGETPA	10 μM		120
Captopril	10 μM		105

Bradykinin incubations were carried out in the presence of captopril (10 μM), MERGETPA (10 μM) and diprotin A (10 μM). Inhibitors were preincubated with enzyme and buffer for 10 min at 37° before addition of substrate. Values given are the averages of two to three determinations which did not differ by more than 15%. Control rates of hydrolysis of the Arg^1-Pro^2 bonds of bradykinin (50 μM) and bradykinin[1–5] (50 μM) were 13.1 and 15.4 nmol/min/mL, respectively.

Table 2. Metabolism of bradykinin analogs by rat plasma aminopeptidase P

	Rate (nmol/min/mL)	Relative rate
BK	6.6 ± 0.6 (N = 5)	100
N-Terminal fragments		
BK[1–5]	8.2 ± 2.0 (N = 3)	124
BK[1–7]	7.9 ± 1.8 (N = 3)	120
B_2 Agonists		
[Tyr^8(OMe)]BK	5.9 ± 0.2 (N = 3)	89
[$Phe^8\psi(CH_2NH)Arg^9$]BK	5.2 (5.3/5.0)	78
B_2 Antagonists		
D-Arg[Hyp^3,D-Phe^7]BK	0	0
D-Arg[Hyp^3,$Thi^{5,8}$,D-Phe^7]BK	0	0
D-Arg[Hyp^3,D-Phe^7,Leu^8]BK	0	0
D-Arg[Hyp^3,D-Phe^7,$Phe^8\psi(CH_2NH)Arg^9$]BK	0	0
B_1 Agonists		
des-Arg^9-BK	8.9 ± 0.6 (N = 4)	134
[D-Phe^8]des-Arg^9-BK	7.4 ± 1.6 (N = 3)	112
B_1 Antagonist		
[Leu^8]des-Arg^9-BK	7.8 ± 1.0 (N = 3)	118

Rat plasma N-terminal hydrolysis of bradykinin (BK) analogs was determined at a substrate concentration of 25 μM in the presence of captopril (10 μM), MERGETPA (10 μM) and diprotin A (10 μM). Values are means ± SEM or the average of two determinations.

neutral pH, was inhibited by 2-mercaptoethanol, dithiothreitol, *o*-phenanthroline and EDTA, but was unaffected by inhibitors of aminopeptidase A (EC 3.4.11.7) and aminopeptidase M (amastatin) [34, 35], aminopeptidase B (EC 3.4.11.6) (puromycin) [36], and dipeptidylaminopeptidase IV (diprotin A) [28]. In addition, only BK fragments and analogs containing Arg^1-Pro^2 bonds were subject to N-terminal

Table 3. Kinetics of bradykinin analog metabolism by rat plasma amino peptidase P

	K_m (μM)	V_{max} (nmol/min/mL)
BK	19.7 ± 2.6 (N = 5)	12.1 ± 1.2
N-Terminal fragments		
BK[1–5]	43.6 ± 5.2 (N = 3)	20.7 ± 3.2
BK[1–7]	38.1 ± 4.4 (N = 3)	18.9 ± 4.7
B_2 Agonists		
[Tyr^8(OMe)]BK	25.2 ± 1.5 (N = 3)	11.9 ± 0.3
[Phe$^8\psi$(CH$_2$NH)Arg^9]BK	21.1 (22.2/20.0)	11.0 (10.9/11.1)
B_1 Agonists		
des-Arg^9-BK	19.5 ± 1.7 (N = 4)	14.5 ± 1.4
[D-Phe^8]des-Arg^9-BK	31.6 ± 5.5 (N = 3)	16.4 ± 4.3
B_1 Antagonist		
[Leu^8]des-Arg^9-BK	32.7 ± 5.3 (N = 3)	17.4 ± 3.2

Assays were performed over a range of substrate concentrations (12.5 to 125 μM) and plotted as $1/V$ vs $1/[S]$. Values are means ± SEM or the average of two determinations.

degradation. Collectively, these data are consistent with identification of aminopeptidase P (APP; EC 3.4.11.9) [31–33] as the responsible enzyme.

APP is an aminoacylproline aminopeptidase specific for N-terminal X-Pro bonds [10, 33, 36]. In contrast, none of the other aminopeptidases noted above hydrolyze N-terminal bonds involving proline [10]. Thus, APP is the only characterized aminopeptidase [36] capable of degrading BK. Further, APP may be considered somewhat specific for BK in that few vasoactive peptides contain the necessary N-terminal X-Pro sequence. Although substance P has an X-Pro N-terminus, the majority of biologic activity of substance P is associated with the C-terminal sequence [38], and blood pressure responses to SP are not reduced significantly in the absence of N-terminal Arg [32].

The affinity of BK for APP found in the present study (K_m = 19.7 μM) is comparable to that reported for angiotensin I for ACE (K_m = 30 μM), and BK for CPN (K_m = 25–45 μM) [5–8]. Although the affinity for BK for purified ACE is higher (K_m = 1–2 μM) [5, 6], K_m values obtained in unfractionated plasma (i.e. comparable to the present studies) are similar (K_m = 17 μM) [20, 21].

In addition to plasma, Simmons and co-workers [31] have detected APP-mediated hydrolysis of Arg^1-Pro^2 bond of BK in pulmonary microsomes and in isolated perfused rat lungs. N-Terminal metabolism has also been found in a preparation enriched in cerebral microvasculature [29]. Consistent with an *in vivo* role for APP in BK metabolism, early studies of BK metabolism in the rat found that 2-mercaptoethanol potentiated the depressor action of BK [39] and, more recently, Ryan *et al.* [40] have reported potentiation of BK during co-infusion of alternate APP substrates (presumably via competitive inhibition).

Although the present data and much of the above-noted published data have been obtained in the rat, plasma and vascular APP is also present in other species. In a recent abstract, Chen *et al.* [41] reported hydrolysis of the APP-specific synthetic substrate Arg-Pro-Pro-[^{3}H]benzylamide by dog, rabbit, pig, cow, human and rat plasma, and by rabbit and cow pulmonary arterial endothelial cell monolayers. Nevertheless, the significance of APP to BK metabolism may vary considerably between species due to the range of plasma APP activity found in different species [41], and the relative contribution of ACE and CPN. For instance, despite the report that APP is higher in human plasma than in cat, dog, rabbit, pig and cow plasma [41], the majority of BK metabolism by human plasma/serum is due to C-terminal hydrolysis [9], and converting enzyme inhibitors potentitate the depressor action of BK *in vivo* [1]. Although these data suggest that APP may not be a primary factor in the metabolism of circulating BK under normal conditions, APP may be significant in patients receiving converting enzyme inhibitors, or in the metabolism of BK analogs resistant to ACE and CPN (see below).

Although we previously found that plasma and vascular aminopeptidase M converts kallidin to BK [10, 35, 42], the physiologic significance of this conversion was questionable since kallidin and BK display comparable potencies at B_2 receptors [10]. However, the present results suggest that despite its inability to degrade BK, aminopeptidase M can act indirectly as a kininase by converting the APP-resistant kallidin to the APP-susceptible BK. Thus, in conjunction with aminopeptidase M, APP can

degrade both of the naturally occurring B_2 kinins kallidin and BK. Similarly, both of the naturally occurring B_1 kinins, des-Arg^{10}-kallidin and des-Arg^9-BK, are susceptible to APP-mediated degradation. APP-mediated degradation may be particularly important for these B_1 agonists since they are resistant to degradation by ACE and CPN [10, 43].

In view of the rapid degradation of BK *in vivo*, the development of potent kinin analogs based on modifications of the parent molecule has included factors relevant not only to receptor interactions, but also stability from enzymatic degradation [44]. In recognition of the importance of ACE and CPN, kinin agonists and antagonists have been developed which are partially or completely resistant to degradation by ACE and/or CPN, and such differential sensitivity to metabolism can be related directly to their relative potencies and receptor specificities [10, 16–21]. ACE-resistant analogs such as D-Phe^7-containing B_2 antagonists are more potent and longer acting, whereas CPN-resistant analogs are more specific for B_2 receptors. Nevertheless, in the rat, D-Phe^7-containing kinin antagonists display relatively short half-lives despite their resistance to ACE [18]. Similarly, the B_2 kinin agonist [Phe$^8\psi$(CH$_2$NH)Arg^9]BK also has a relatively short duration of action despite its resistance to both ACE and CPN [17]. These data indicate that kinin-degrading peptidase(s) other than ACE and CPN may significantly affect the potency and duration of action of kinin agonists and antagonists.

Based on the results of the present study, existing kinin analogs can be grouped into two classes regarding their sensitivity to degradation by APP: (1) those completely resistant to APP, and (2) those degraded by APP with K_m and V_{max} values comparable to those for BK.

Consistent with the rationale of Stewart and Vavrek [44] for the use of D-amino acids at the N-terminus of BK antagonists, all of the D-Arg^0-containing B_2 antagonists examined were resistant to metabolism by APP. Thus, the enzyme(s) responsible for the degradation of these APP/ACE-resistant (e.g. D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK) and APP/ACE/CPN-resistant (e.g. D-Arg[Hyp^3,D-Phe^7,Phe$^8\psi$(CH$_2$NH)Arg^9]BK) analogs [10, 17–21] remains to be determined.

In contrast, APP may play a role in the degradation of analogs containing unmodified Arg^1-Pro^2 N-termini such as the B_1 agonist [D-Phe^8]des-Arg^9-BK, the B_1 antagonist [Leu^8]des-Arg^9-BK, and the B_2 agonists [Phe$^8\psi$(CH$_2$NH)Arg^9]BK and [Tyr^8(OMe)]BK. With the exception of the latter peptide, these analogs are resistant to degradation by both ACE and CPN [10, 15, 20, 43]. Although we are not aware of any attempts to potentiate B_1 analogs via APP inhibition, our preliminary studies (in progress) have found that the depressor action of the B_2 agonist [Phe$^8\psi$(CH$_2$NH)Arg^9]BK is potentiated significantly by 2-mercaptoethanol.

Acknowledgements—This work was supported by Grant-In-Aid 891032 from the American Heart Association, by Grants R01 DK 28184 and R01 HL 45791, and a Postdoctoral Fellowship to G.D. from the Fonds de la Recherche en Sante du Quebec (890242).

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