Depressor Action of Bradykinin Agonists Relative to Metabolism by Angiotensin-Converting Enzyme, Carboxypeptidase N, and Aminopeptidase P (43402)

SALEEM AHMAD AND PATRICK E. WARD[1]
Department of Physiology, The Ohio State University, Columbus, Ohio 43210-1239

Abstract. **Bradykinin (BK) receptor agonists and antagonists contain modifications that confer resistance to specific peptidases. In control studies, rat plasma degraded BK (10.3 ± 0.3 nmol/min/ml) via angiotensin-converting enzyme (ACE; EC 3.4.15.1; 5.2 ± 0.3 nmol/min/ml), carboxypeptidase N (CPN; EC 3.4.17.3; 3.2 ± 0.4 nmol/min/ml), aminopeptidase P (APP; EC 3.4.11.9; 0.6 ± 0.2 nmol/min/ml), and other (unidentified) activity (2.1 ± 0.6 nmol/min/ml). In contrast, BK agonist analogs were hydrolyzed more slowly due to selective resistance to these plasma peptidases. In addition to Lys-Lys-BK (B1087), which is partially resistant to ACE, [Hyp^3,Phe^8-*r*-Arg^9]BK (B7642) was completely resistant to ACE, CPN, and the unidentified plasma activity (1.9 ± 0.3 nmol/min/ml), and D-Arg^0[Hyp^3,Phe^8-*r*-Arg^9]BK (B7644) was resistant to all plasma hydrolysis, including APP (<0.2 nmol/min/ml). *In vivo* ACE-resistant B1087 exhibited a depressor potency and duration of action greater than BK and equivalent to that of BK in the presence of the ACE inhibitor enalapril. Although the B7642 and B7644 agonists were also more potent and longer acting than BK, the increases were no more than that seen for B1087, despite their additional resistance to CPN (B7642) and CPN and APP (B7644). The duration of action of these analogs was, however, increased after renal ligation. These data demonstrate the importance of ACE to the metabolism of circulating BK and BK analogs. In contrast, resistance to CPN and APP are not associated with further potentiation. Beyond ACE resistance, it is likely that the development of more potent, longer-acting BK agonists and antagonists will relate to other factors, such as renal processing independent of CPN and APP.** [P.S.E.B.M. 1992, Vol 200]

Bradykinin (BK) is hydrolyzed by plasma angiotensin I-converting enzyme (ACE; EC 3.4.15.1) and carboxypeptidase N (CPN; EC 3.4.17.3) (1). However, recent studies have questioned the importance of CPN *in vivo* and have shown that degradation also occurs via other unidentified peptidases (2, 3). Neutral endopeptidase-24.11 (NEP-24.11; EC 3.4.24.11) and post-proline-cleaving enzyme (EC 3.4.21.26) can degrade BK, but inhibitors of these enzymes do not potentiate BK (3, 4). Furthermore, aminopeptidases such as aminopeptidase A (EC 3.4.11.7), aminopeptidase M (EC 3.4.11.2), aminopeptidase B (EC 3.4.11.6), leucine aminopeptidase (EC 3.4.11.1), and dipeptidyl amino peptidase IV (EC 3.4.14.5) can be ruled out based on their localization and/or inability to hydrolyze N-terminal Arg-Pro bonds (1, 5).

In contrast, aminopeptidase P (APP; EC 3.4.11.9) is an aminoacylproline aminopeptidase specific for N-terminal X-Pro bonds (5, 6). APP activity is present in pulmonary microsomes and isolated perfused lungs (7, 8), and plasma contains APP that degrades BK *in vitro* by hydrolysis of the Arg^1-Pro^2 bond (9–11). Although ACE-mediated metabolism may predominate under normal conditions, CPN- and APP-mediated metabolism could be significant in patients receiving ACE inhibitors, or in the metabolism of BK analogs resistant to ACE (12–14). Thus, we examined the relative contribution of ACE, CPN, and APP to BK and BK analog metabolism *in vitro*, and the relationship of such me-

[1] To whom requests for reprints should be addressed at Department of Physiology, 4196 Graves Hall, 333 West 10th Avenue, Columbus, OH 43210-1239.

Received September 9, 1991. [P.S.E.B.M. 1992, Vol 200]
Accepted January 2, 1992.

0037-9727/92/2001-0115$3.00/0

tabolism to depressor potency and duration of action *in vivo*.

Materials and Methods

Materials. BK, des-Arg^9-BK, and des-Arg^1-BK were obtained from Sigma Chemical Co. (St. Louis, MO). Lys-Lys-BK (B1087), [Hyp^3,Phe^8-*r*-Arg^9]BK (B7642), and D-Arg^0[Hyp^3,Phe^8-*r*-Arg^9]BK (B7644) were obtained from Drs. Stewart, Gera, and Vavrek (University of Colorado Medical School, Denver, CO) (12, 15). Phe-*r*-Arg represents the pseudopeptide in which C=O is reduced to —CH_2—. BK[1–7] and Phe-Arg were provided by Drs. Regoli and Drapeau (University of Sherbrooke, Sherbrooke, Quebec, Canada). The ACE inhibitor enalapril maleate was obtained from Merck (Rahway, NJ) and the CPN inhibitor D-L-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA) was from Calbiochem-Behring (San Diego, CA) (16).

Peptide Metabolism. Blood was obtained from anesthetized (sodium pentobarbital; 65 mg/kg, ip) male Sprague-Dawley rats via the carotid artery. Five milliliters of blood were mixed with 100 μl of heparin (100 units/ml) and centrifuged (10 min) in a bench-top clinical centrifuge, and the plasma was collected for analysis.

BK metabolism by ACE, CPN, and APP were determined as described previously (11, 14). The standard incubation (600 μl) consisted of rat plasma (2–15 μl), peptide substrate (10 μ*M*), and 50 m*M* phosphate buffer (pH 7.5) containing 30 m*M* NaCl and 100 μ*M* $CoCl_2$ incubated at 37°C. Metabolism by ACE, CPN, and APP were selectively inhibited by preincubation (10 min) with captopril (10 μ*M*), MERGETPA (10 μ*M*), and 2-mercaptoethanol (2-ME; 4 m*M*), respectively (11, 14). 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 table-top centrifuge (5 min), and the supernate was collected for high-performance liquid chromatography analysis. Peptide hydrolysis was determined by the decrease in substrate and the increase in products. Rates of hydrolysis were directly proportional to both time of incubation and amount of plasma used.

High-Performance Liquid Chromatography Analysis. Peptide substrates and metabolites (e.g., BK, des-Arg^9-BK, BK[1–7], and Phe-Arg) were separated and quantitated as described previously (11, 14) on a reverse-phase column (Vydac; 10 μm, C_{18}-μBondapak, 1.9 × 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 peak areas and quantitation of peptide substrates and metabolites (monitored at 210 nm) against the last-run standards (run every sixth injection) were calculated by the data module. Solvent A was 0.05% trifluoroacetic acid (v/v) in distilled water and solvent B was 0.04% trifluoroacetic acid (v/v) in acetonitrile. Amino acid metabolites (C- or N-terminal Arg) were separated and quantitated as described previously (17, 18).

***In Vivo* Studies.** *In vivo* studies using male Sprague-Dawley rats (270–300 g) were performed as described previously (19). Rats were anesthetized (sodium pentobarbital; 65 mg/kg, ip) and allowed to breathe spontaneously via a tracheostomy tube. Body temperature was maintained at 37°C using a heating lamp connected to a temperature regulator and thermistor probe. A jugular vein was cannulated with three catheters for supplemental anesthetic, infusion of 0.9% saline (30 μl/min), and phentolamine injections. Mean arterial pressure (MAP) was monitored from a femoral artery cannula using a Statham pressure transducer coupled to a Grass physiograph. A femoral vein was cannulated for peptide injections.

After completion of surgery and a 20-min stabilization period, α-adrenergic blockade was initiated with phentolamine mesylate (1.0 mg/kg, iv) and maintained by supplemental injections (0.5 mg/kg every 30 min) (20). Adrenergic blockade, which prevents secondary pressor responses to BK due to catecholamines (20), was confirmed by the loss of pressor response to phenylephrine. Peptide dose-response curves were obtained before and after ACE inhibition (enalapril maleate; 5 mg/kg) or attempted APP inhibition (2-ME; 60 mg/kg). They were also obtained before and 15 min after bilateral ligation of the renal artery and vein. For each dose, maximal change in MAP, duration of response, and area over the curve (AOC) were determined. AOC incorporates both the change in blood pressure and the duration of action (19). Area measurements were determined using Sigma Scan (Jandel Scientific, Corte Madera, CA). Data are expressed as mean ± SE and were evaluated using Student's paired *t* test. *P*-values less than 0.05 were considered statistically significant.

Results

Metabolism *In Vitro*. Incubation of BK (retention time [RT] = 12.4 min) with rat plasma produced the ACE metabolites Phe-Arg (RT = 8.4 min) and BK[1–7] (RT = 11.1 min) and the CPN metabolite des-Arg^9-BK (RT = 13.2 min). BK degradation was inhibited by captopril, and more so by captopril and MERGETPA (Fig. 1). The remaining metabolism of BK was not due to incomplete inhibition of ACE and CPN, since the production of BK[1–7], Phe-Arg, and des-Arg^9-BK were completely inhibited (not shown). Rather, this residual degradation was associated with the production of des-Arg^1-BK and N-terminal Arg, which could be completely inhibited by the APP inhibitor 2-ME. As shown in Figure 1, inhibition of APP further decreased BK

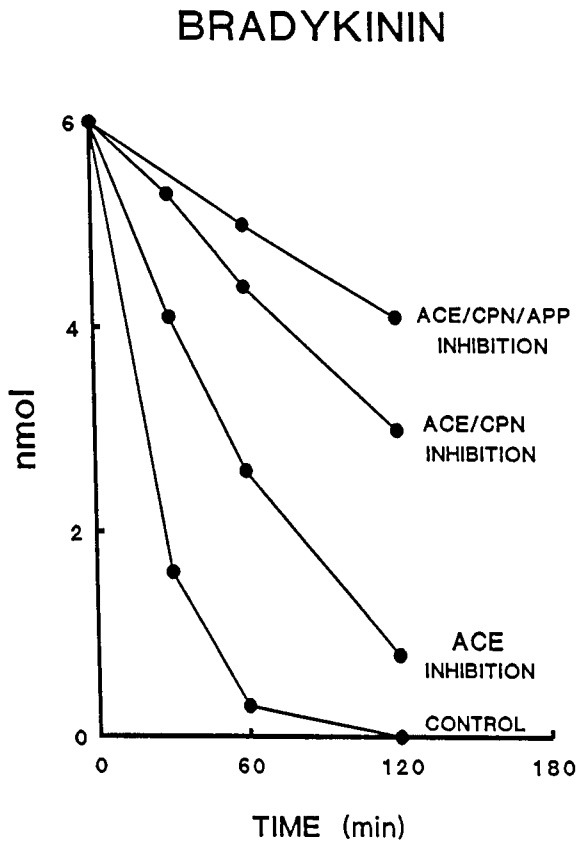

Figure 1. Rat plasma (15 μl) hydrolysis of bradykinin (10 μM) under control conditions, during ACE inhibition (captopril, 10 μM), ACE and CPN inhibition (captopril, 10 μM; MERGETPA, 10 μM), or ACE and CPN and APP inhibition (captopril, 10 μM; MERGETPA, 10 μM; 2-ME, 4 mM). Separation and quantitation of bradykinin and bradykinin metabolites were assessed by high-performance liquid chromatography as described in Materials and Methods.

degradation compared with that seen during ACE and CPN inhibition alone. Nevertheless, BK continued to by hydrolyzed despite complete inhibition of ACE, CPN, and APP. Thus, the combined actions of ACE and CPN accounted for the majority of BK metabolism, whereas the contribution of APP and other (unidentified) activity was significantly less (Table I).

As shown in Figure 2, B7642 (RT = 12.1 min), containing a modified Phe-*r*-Arg C terminus, was resistant to both ACE and CPN in that captopril and MERGETPA had little effect. However, consistent with its Arg-Pro N terminus, B7642 was degraded by APP (N-terminal Arg production; not shown) in that 2-ME inhibited degradation (Fig. 2). No hydrolysis by enzyme(s) other than APP was detected (i.e., APP accounted for essentially all degradation) (Table I).

In turn, B7644 (RT = 11.9 min), identical to B7642 except for its D-Arg^0 N terminus, was resistant not only to ACE and CPN, but also to APP (Fig. 2). Consistent with resistance to all three enzymes and the unknown activity, no significant degradation of B7644 was detected (Table I).

***In Vivo* Studies.** Previous studies found that MER-

Table I. Metabolism of Bradykinin and Agonist Analogs by Rat Plasma Peptidases[a]

	Bradykinin (nmol/min/ml)	B7642 (nmol/min/ml)	B7644 (nmol/min/ml)
Enzyme			
ACE	5.2 ± 0.3 (n = 3)	<0.1	<0.1
CPN	3.2 ± 0.4 (n = 3)	<0.1	<0.1
APP	0.6 ± 0.2 (n = 3)	1.7 ± 0.3 (n = 3)	<0.1
Other	2.1 ± 0.6 (n = 3)	<0.2	<0.2
Total	10.3 ± 0.3 (n = 4)	1.9 ± 0.3 (n = 3)	<0.2

[a] Metabolism of bradykinin and bradykinin agonists (10 μM) inhibited by captopril (10 μM; ACE), mergetpa (10 μM; CPN), 2-ME (4 mM; APP), or none of the above (Other). Total hydrolysis was determined in the absence of peptidase inhibitors. Values given are means ± SE.

GETPA cannot be used to specifically inhibit CPN *in vivo* (21). In an initial attempt to inhibit APP *in vivo*, rats were treated with 2-ME (60 mg/kg). 2-ME did not affect baseline mean arterial pressure nor the dose-response curves for acetylcholine (not shown). BK was potentiated regarding maximal change in MAP, duration of action, and AOC (Fig. 3). In order to determine whether potentiation was related to specific inhibition of APP, or nonspecific inhibition of ACE or other actions, studies were repeated subsequent to ACE inhibition with enalapril. As expected, ACE inhibition significantly potentiated BK. However, addition of 2-ME resulted in little further potentiation (Fig. 4). Thus, although confirming an early study of BK potentiation by 2-ME (20), these data demonstrated that such potentiation cannot be definitely related to APP inhibition *in vivo*.

In the absence of agents able to specifically inhibit CPN and APP *in vivo*, and in view of the differential sensitivity of BK, B7642, and B7644 to metabolism by ACE, CPN, and APP (Table I), we examined their relative potencies and durations of action *in vivo*. B1087, previously found to be partially resistant to ACE but metabolized as rapidly as BK by CPN (14, 22), was also examined.

Consistent with their partial or complete resistance *in vitro* to ACE, enalapril had only a minimal effect on B1087, and no effect on B7642 ([Hyp^3,Phe^8-*r*-Arg^9]BK) and B7644 (D-Arg^0[Hyp^3,Phe^8-*r*-Arg^9]BK) (not shown). As shown in Figure 5, the maximal change in MAP, duration of response, and AOC in response to B1087 was essentially equivalent to BK in the presence of enalapril. The dose-response curves of B7642 and B7644 were also similar, despite their additional resistance to CPN (B7642) and CPN and APP (B7644).

Since the kidney is reported to participate in the

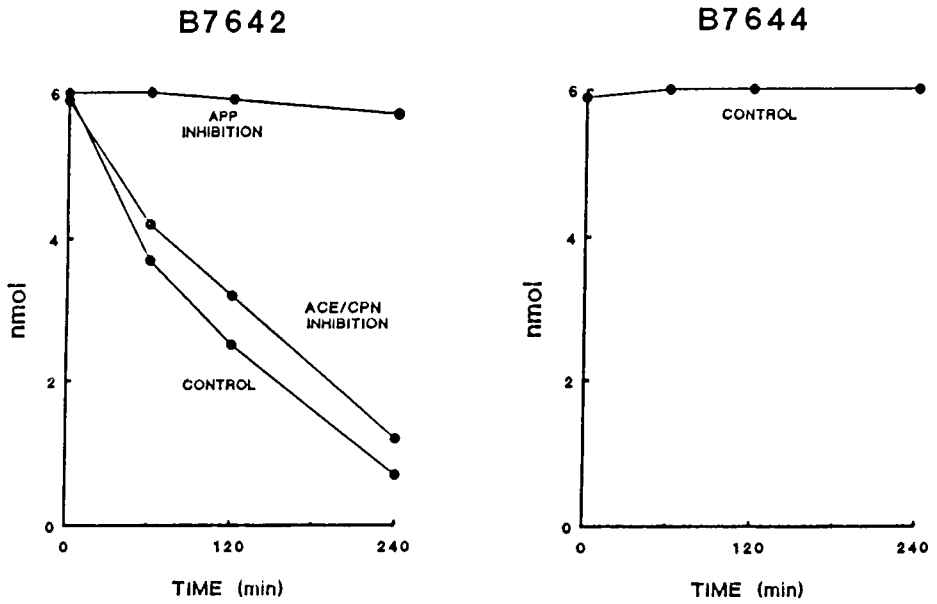

Figure 2. Rat plasma (15 μl) hydrolysis of 10 μ*M* B7642 and B7644 under control conditions, during ACE and CPN inhibition (captopril, 10 μ*M*; MERGETPA, 10 μ*M*) or APP inhibition (2-ME, 4 m*M*). Separation and quantitation of peptide hydrolysis were assessed by high-performance liquid chromatography as described in Materials and Methods.

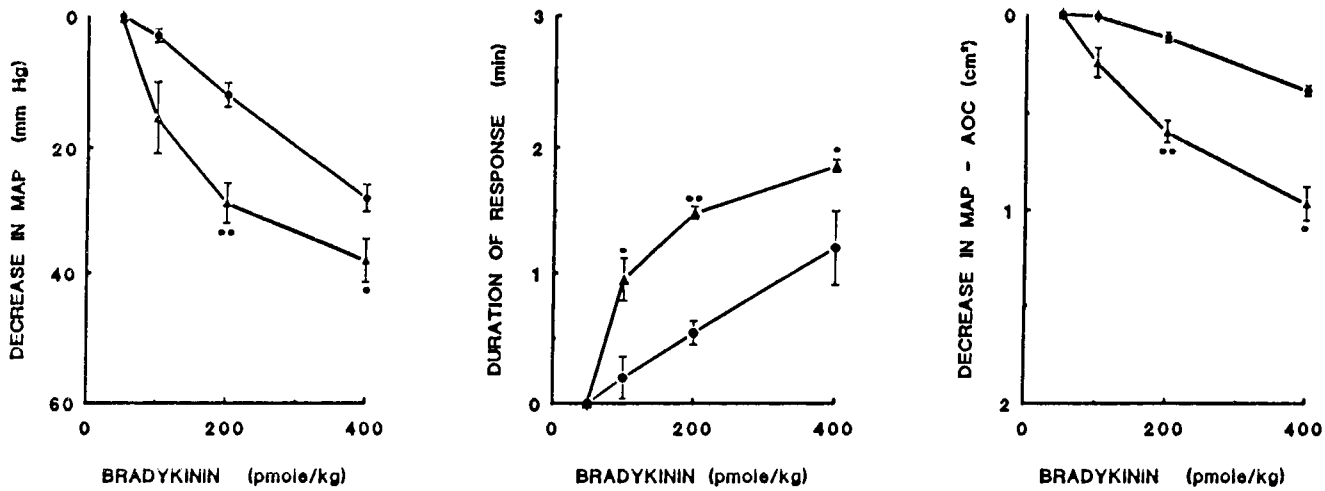

Figure 3. Response to intravenous bolus injections of bradykinin before (●) and during (▲) 2-mercaptoethanol (60 mg/kg) treatment in anesthetized rats. Values are means ± SE (n = 5). Significant differences from values before 2-mercaptoethanol: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ (Student's paired t test).

degradation of BK (2), studies were repeated before and after bilateral renal ligation. Ligation did not potentiate maximal changes in MAP (not shown), but significantly increased the duration of action of BK, B1087, and B7644 (Fig. 6). Dose-response curves to angiotensin II and acetylcholine were unaffected (not shown).

Discussion

The capacity of an enzyme to metabolize a peptide *in vitro* does not necessarily reflect physiologically significant metabolism *in vivo*. For instance, although both ACE and NEP-24.11 hydrolyze BK, the depressor action of BK is potentiated by ACE inhibitors but not by NEP-24.11 inhibitors (1, 2). Thus, if CPN and APP (or similar) activities play physiologically significant roles in the metabolism of circulating BK, inhibition of these activities and/or the use of analogs resistant to such metabolism should result in increased potency and duration of action.

In addition to plasma CPN, a serum carboxypeptidase B-like activity has been reported (23), vascular endothelium and microvasculature contain a CPN-like enzyme (17, 18), and a CPN-like enzyme known as carboxypeptidase M is widely distributed in tissues (24). Although interspecies variation has been reported in the patterns of BK hydrolysis by plasma ACE and CPN

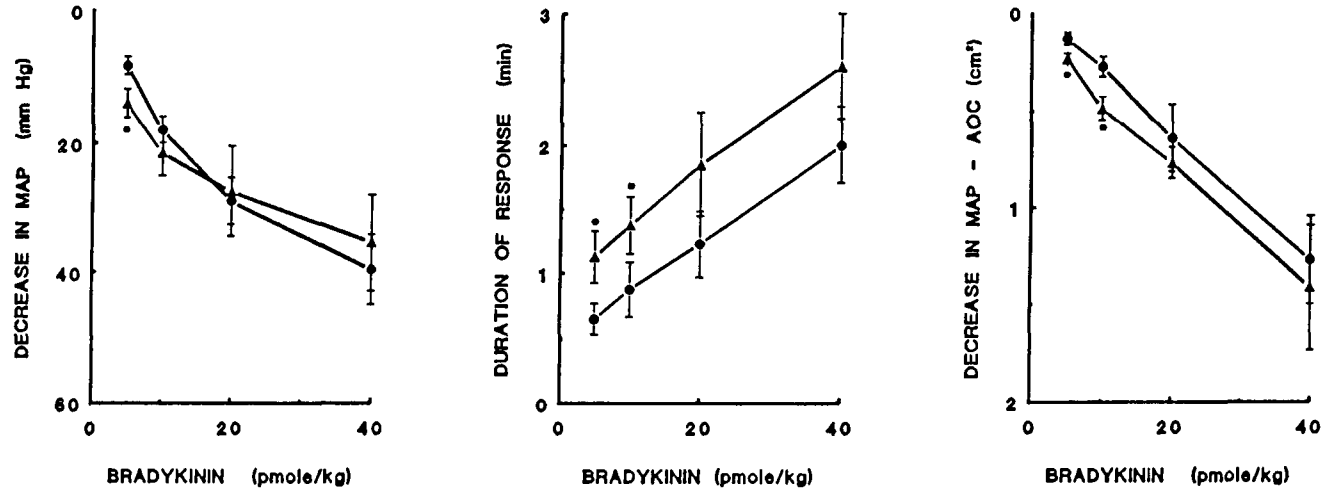

Figure 4. Response to intravenous bolus injections of bradykinin during enalapril maleate (5 mg/kg) (●) and subsequent to addition of 2-mercaptoethanol (60 mg/kg) (▲) in anesthetized rats. Values are means ± SE (n = 5). Significant differences from values before 2-mercaptoethanol: $^*P \leq 0.05$ (Student's paired t test).

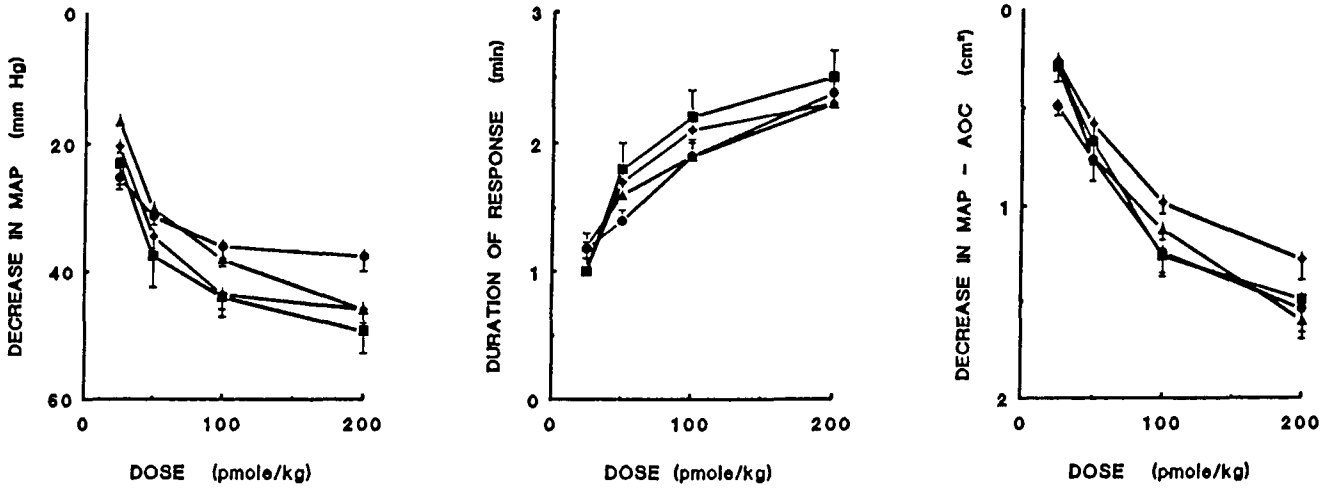

Figure 5. Response to intravenous bolus injections of bradykinin during enalapril maleate (5 mg/kg) (●) or B1087 (▲), B7642 (♦), and B7644 (■) under control conditions (no inhibitor) in anesthetized rats. Values are means ± SE (n = 5).

(14, 21, 23), all of these studies demonstrated MERGETPA-sensitive metabolism of BK to des-Arg^9-BK. MERGETPA specifically blocks BK stimulation of B_1 receptors via inhibition of des-Arg^9-BK production (25, 26), and Streeten *et al.* (27) found that patients with low plasma CPN exhibit elevated kinin levels and symptoms consistent with the biological actions of kinins.

A similar case has been made for APP-mediated BK metabolism *in vivo*. APP, an aminoacylproline aminopeptidase specific for N-terminal X-Pro bonds (5, 6), has been identified in pulmonary microsomes (7, 8), and hydrolysis of the Arg^1-Pro^2 bond of BK has been detected in plasma and during perfusion of isolated lungs (9–11). Chen *et al.* (28) have reported hydrolysis of an APP-specific synthetic substrate by plasma and pulmonary endothelium. Early studies found that 2-ME potentiated the depressor action of BK in the rat (20) and, more recently, Ryan and coworkers (9) have reported potentiation by alternate APP substrates.

These studies suggest that CPN and APP contribute to BK metabolism *in vivo* and that, even if ACE activity normally predominates, metabolism would still be significant in the presence of ACE inhibitors, or for ACE-resistant BK agonists and antagonists. However, unlike ACE, in which specific and effective inhibitors of *in vivo* metabolism are available, CPN- and APP-mediated metabolism of BK *in vivo* cannot be studied directly. Although the CPN inhibitor MERGETPA slightly potentiates BK *in vivo*, potentiation cannot be definitely related to CPN, since sodium nitroprusside, angiotensin I and II, and vasopressin are also potentiated (21). Although 2-ME is a selective inhibitor of APP *in vitro* (7, 11), and potentiates BK *in vivo* (20), we found that

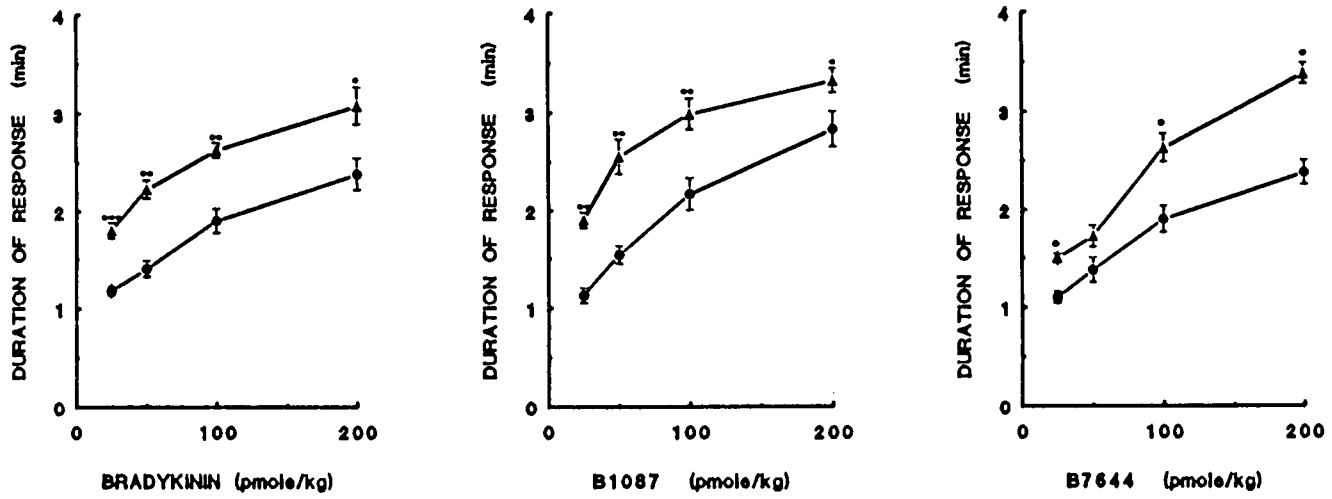

Figure 6. Duration of response to intravenous bolus injections of bradykinin during enalapril maleate (5 mg/kg) or B1087 and B7644 under control conditions (no inhibitor) before (●) and after (▲) bilateral renal ligation. Values are means ± SE (n = 4–6). Significant differences from values before ligation: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ (Student's paired t test).

potentiation is due to nonspecific inhibition of ACE and possible other effects. In view of these limitations, we comparatively examined several BK agonists to (i) establish selective resistance to ACE, CPN, and APP and (ii) to relate such resistance to potency and duration of action. We reasoned that analogs resistant to ACE, CPN, and APP would be more potent and longer acting than those resistant to ACE alone.

Consistent with previous data (11, 14), control studies found plasma BK metabolism due to ACE, CPN, APP, and other (unidentified) enzyme activity. ACE and CPN accounted for the majority of BK hydrolysis. We found previously that B1087 is resistant to ACE, but is metabolized as rapidly as BK by CPN (14). B7642, containing a modified C terminus, was resistant to both ACE and CPN and the unidentified plasma activity. Thus, plasma degradation of B7642 (1.9 nmol/min/ml), due entirely to APP hydrolysis of the N-terminal Arg-Pro bond, was one fifth that of BK (10.3 nmol/min/ml). In turn, B7644, containing an additional modification at its N terminus (D-Arg) which prevented hydrolysis by APP, was completely stable in plasma (<0.2 nmol/min/ml).

The potency and duration of action of these agonists emphasize the importance of ACE in the metabolism of circulating BK and ACE-susceptible BK analogs. The observed *in vitro* resistance to ACE was confirmed *in vivo* in that enalapril had little (B1087) or no (B7642 and B7644) potentiating action, and all ACE-resistant agonists were more potent and longer acting than BK. However, despite clear additional resistance to CPN (B7642 and B7644) and CPN and APP (B7644), these latter analogs were no more effective than BK during inhibition of ACE (i.e., accountable for by their resistance to ACE). Although we did not measure analogs and analog metabolites *in vivo*, others have shown that the fall in blood pressure in the rat correlates well with plasma kinin concentration (2). Although it could be argued that the CPN- and APP-resistant agonists were more slowly degraded *in vivo*, but that this potentiating action was countered by decreased receptor affinity/binding, several considerations suggest that this cannot account for the observed data. Regardless of the modification used to achieve resistance to ACE (N-terminal Lys-Lys extension/B1087, reduced C-terminal bond/B7642 and B7644), CPN (reduced C-terminal bond/B7642 and B7644), or APP (N-terminal D-Arg extension/B7644), the depressor potency, duration of action, and AOC of all analogs were equivalent with that of BK during ACE inhibition. Furthermore, amino acid N-terminal extensions have not been reported to significantly decrease BK analog receptor binding/affinity (12, 15).

Thus, in contrast to our original expectation, these data do not support a role for APP or CPN in the metabolism of circulating BK or BK analogs resistant to ACE. Although this conclusion may not apply to discrete sites of local synthesis and release where CPN or APP activities may predominate (1, 17), the finding that B7644 was completely resistant to plasma hydrolysis suggests that enzymatic or other factors not reflected in plasma metabolism play a significant role in terminating the action of circulating BK and BK analogs resistant to ACE. Whether these factors include metabolism by as yet unidentified vascular and tissue enzymes, removal by cellular processes, or other factors remains to be determined. Based on the present and other data, one such factor may involve renal processing. Ishida *et al.* (2) found that nephrectomy (but not ureteral ligation) potentiated BK levels *in vivo* despite

the presence of ACE, CPN, and NEP-24.11 inhibitors. Similarly, we found that the durations of action of BK analogs resistant to ACE, CPN, and APP were prolonged by renal ligation. Although other effects of renal ligation, such as altered renin and aldosterone, cannot be excluded, these data collectively indicate significant renal processing that cannot be accounted for by the actions of ACE, CPN, NEP-24.11, and APP.

This work was supported by National Institutes of Health Grant HL 45791 and American Heart Association Grant-in-Aid 891032.

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