Cellular and Molecular Neurobiology, Vol. 9, No. 3, 1989

Enzymatic Inactivation of Bradykinin by Rat Brain Neuronal Perikarya

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Received March 1, 1989; accepted March 5, 1989

KEY WORDS: isolated nuerons; bradykinin inactivation; thiol-endopeptidase; endopeptidase 24.11; angiotensin-converting enzyme; prolyl endopeptidase.

SUMMARY

1. Bradykinin (Bk; Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^8) inactivation by bulk isolated neurons from rat brain is described.

2. Bk is rapidly inactivated by neuronal perikarya (4.2 ± 0.6 fmol/min/cell body).

3. Sites of inactivating cleavages, determined by a kininase bioassay combined with a time-course Bk-product analysis, were the Phe^5–Ser^6, Pro^7–Phe^8, Gly^4–Phe^5, and Pro^3–Gly^4 peptide bonds. The cleavage of the Phe^5–Ser^6 bond inactivated Bk at least five fold faster than the other observed cleavages.

4. Inactivating peptidases were identified by the effect of inhibitors on Bk-product formation. The Phe^5–Ser^6 bond cleavage is attributed mainly to a calcium-activated thiol-endopeptidase, a predominantly soluble enzyme which did not behave as a metalloenzyme upon dialysis and was strongly inhibited by *N*-[1(*R,S*)-carboyx-2-phenylethyl]–Ala–Ala–Phe-*p*-aminobenzoate and endo-

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[4] Abbreviations used: ACE, angiotensin-I converting enzyme; AMC, 7-amino-4-methyl-coumarin; antiserum, rat brain endo-oligopeptidase A antiserum; Bk, bradykinin cF, *N*-[1(*R,S*)-carboxy-2-phenylethyl]; CNS, central nervous system; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; MCA, 4-methyl-coumarinyl-7-amide; MK 422, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-Ala-L-Pro; N-suc, *N*-succinyl; pAB, *p*-aminobenzoate; PCMB, *p*-mercuribenzoate; PE, prolyl endopeptidase; Z, *N*-benzyloxycarbonyl.

0272-4340/89/0900-0379$06.00/0

oligopeptidase A antiserum. Thus, neuronal perikarya thiol-endopeptidase seems to differ from endo-oligopeptidase A and endopeptidase 24.15.

5. Endopeptidase 24.11 cleaves Bk at the Gly^4–Phe^5 and, to a larger extent, at the Pro^7–Phe^8 bond. The latter bond is also cleaved by angiotensin-converting enzyme (ACE) and prolyl endopeptidase (PE). PE also hydrolyzes Bk at the Pro^3–Gly^4 bond.

6. Secondary processing of Bk inactivation products occurs by (1) a rapid cleavage of Ser^6–Pro^7–Phe^8–Arg^8 at the Pro^7–Phe^8 bond by endopeptidase 24.11, 3820ACE, and PE; (2) a bestatin-sensitive breakdown of Phe^8–Arg^9; and (3) conversion of Arg^1–Pro^7 to Arg^1–Phe^5, of Gly^4–Arg^9 to both Gly^4–Pro^7 and Ser^6–Arg^9, and of Phe^5–Arg^9 to Ser^6–Arg^9, Phe^8–Arg^9, and Ser^6–Pro^7, by unidentified peptidases.

7. A model for the enzymatic inactivation of bradykinin by rat brain neuronal perikarya is proposed.

INTRODUCTION

Bradykinin (Bk)[4] has been suggested to play a role as a neuromodulator and/or neurotransmitter in the CNS (Snyder, 1980; Kariya *et al.*, 1985) on the basis of several lines of evidence, such as biological activity, localization and distribution, and interaction with a specific receptor. Moreover, all the components of a kallikrein–kinin system, which include kallikrein (Chao *et al.*, 1983), kininogen (Shikimi *et al.*, 1973), kinin-converting (Camargo *et al.*, 1972), and Bk-inactivating (Carvalho and Camargo, 1981; cf. Orlowski, 1983) activities, and Bk (Corrêa *et al.*, 1979; Perry and Snyder, 1984; Kariya *et al.*, 1985) have been identified in the mammalian CNS.

Bk is rapidly inactivated after intracerebroventricular injection (half-life, 26.6 sec) (Kariya *et al.*, 1982), and its major inactivation mechanism appears to occur through peptide bond cleavage. Bk neuronal inactivation has been approached *in vitro* using homogenate preparations, purified peptidases, and cloned cell cultures, among other systems. Regarding the latter system, two cell lines from neural origin (neuro-2a neuroblastoma and C_8 glioma clones) were recently employed to study Bk inactivation (DelBel *et al.*, 1986). Although cell lines can provide useful models to study Bk enzymatic processing, it should be pointed out that they represent a transformed cell system. In this study, we have employed a preparation of bulk isolated neuronal perikarya (Sellinger *et al.*, 1971) from rat brain that is essentially devoid of nonneuronal cell types. This preparation was used to investigate the enzymatic mechanisms of Bk inactivation.

MATERIALS AND METHODS

Materials

Bk and its fragments were synthesized by Professors A. C. M. Paiva and L. Juliano, Escola Paulista de Medicina, São Paulo. *N*-Suc-Gly-Pro-MCA [7-(*N*-

succinyl-glycyl-L-proline)-4-methyl-coumarinamide] and AMC (7-amino-4-methyl-coumarin) were from Peptide Institute. Aminex A-5 and Durrum DC-6A resins were from Bio-Rad Laboratories and Durrum Chemical, respectively. All chemicals used for the amino acid analyzer buffer and ninhydrin solutions were from Pierce Chemical. Polyvinylpyrrolidone and bestatin were from General Aniline and Film and Sigma Chemical, respectively. Bovine serum albumin was fraction V from Pentex. MK 422 (*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-Ala-L-Pro) was a gift from Dr. L. J. Greene (Departamento de Farmacologica, Faculdade de Medicina de Ribeirão Prêto, Universidade de São Paulo). Rat brain endo-oligopeptidase A antiserum, rabbit IgG fraction (antiserum), was provided by Dr. A. C. M. Camargo, Departamento de Farmacologia, Instituto de Ciências Biomédicas, Universidade de São Paulo. cf-A-A-F-pAB (*N*-[1(*R,S*)-carboxy-2-phenylethyl]-L-Ala-L-Ala-L-Phe-*p*-aminobenzoate) and cF-F-pAB (*N*-[1(*R,S*)-carboxy-2-phenylethyl]-L-Phe-*p*-aminobenzoate), as well as Z-Pro-prolinal (*N*-benzyloxycarbonyl-L-prolyl-prolinal), were provided by Drs. M. Orlowski and S. Wilk (Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York).

Isolation of Neuronal Perikarya

Neuronal cell bodies were bulk isolated from brains of 18-day-old rats, according to a modification of the procedure of Sellinger *et al.* (1971). Brains from 13 Wistar rats were placed on an ice-cooled plate and cerebral cortices were dissected. The tissue (8.6 g wet weight) was minced using a razor blade, and 8.1 g was transferred into 120 ml ice-cold 7.5% (w/v) polyvinylpyrrolidone solution containing 1% (w/v) bovine serum albumin and 10 m*M* $CaCl_2$ (solution I). The mince was sieved through nylon bolting cloth (Tobler, Ernest and Traber, Elmsford, N.Y.) of 333-, 110- and 73-μm pore size. The volume of the last filtrate was brought to 130 ml with ice-cold solution I. Aliquots (20 ml) of the filtrate were layered on a two-step gradient (7 ml 1.0 *M* sucrose and 6 ml 1.75 *M* sucrose) and centrifuged at 41,000*g* for 30 min at 4°C, using a Beckman SW27 rotor. The pellet consisted of purified neuronal perikarya and its purity was assessed by phase-contrast microscopy. In fields comprising more than 50 structured particles, about 90% were neuronal perikarya. Contaminating structures consisted of bare nuclei and occasional capillary threads. Glial cells were not detected. Neuronal perikarya yield was $(4.4 \pm 0.45) \times 10^6$ cells/g wet brain cortex (mean ± SE; $N = 5$). Each neuronal pellet was either suspended in 2.0 ml/pellet of 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.32 *M* sucrose, and homogenized using a Potter–Elvehjem homogenizer for use on the same day or rapidly frozen to −70°C, stored at −20°C, and used within 1 week. Soluble and particulate fractions were prepared by centrifuging the homogenate at 105,000*g* for 1 hr at 4°C.

Prolyl Endopeptidase Assay

Prolyl endopeptidase (PE) activity was determined by a modification (Martins *et al.*, 1987) of the procedure of Kato *et al.* (1980). Initial rates (two or

three time points) of AMC release were measured by incubating 3.3 m*M* *N*-suc-Gly-Pro-MCA with the neuronal perikarya homogenate or homogenate-derived fraction in 100 μl 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl and 2 m*M* dithiothreitol (DTT), at 37°C for up to 30 min. The homogenate was preincubated with 2 m*M* DTT at 37°C for 5 min before the addition of substrate. The reaction was stopped by the addition of 2.0 ml 1.0 *M* sodium acetate buffer, pH 4.2, per 100 μl incubation medium. AMC was determined with an Aminco Model 125 spectrophotofluorometer at 380-nm excitation and 460-nm emission wavelengths on the supernatant (8300*g* for 10 min) of the acidified incubation medium. Blanks were prepared in the same manner as the incubates, except that 1.0 *M* sodium acetate buffer, pH 4.2, was added before the homogenate (zero time of reaction). Standard curves (0.3–3.0 nmol AMC/100 μl) presented a linear relationship between fluorescence intensity and AMC concentration. Homogenate (up to 50 μl) did not change the standard curve slope. In experiments designed to determine adequate assay conditions for measuring rat brain PE activity, it was shown that PE specific activity in a brain homogenate was maximal at a 2.5 m*M* substrate concentration. AMC release was linearly related to incubation time from 10 to 120 min, for 11–82 μg homogenate protein, and PE specific activity was independent of homogenate protein concentration in the above range. All measurements were carried out in duplicate. PE specific activity is expressed as nanomoles of AMC released per minute per milligram of protein.

Kininase Bioassay

The kininase bioassay (Camargo *et al.*, 1972) measures the rate of hydrolysis of the first peptide bond cleaved in Bk, because the hydrolysis of any peptide bond in the molecule leads to products essentially devoid of spasmogenic activity upon the isolated guinea pig ileum (Suzuki *et al.*, 1969). Initial Bk inactivation rates (two to four time points) were measured incubating 116 μ*M* Bk with a neuronal homogenate or homogenate-derived fraction in 250 or 1000 μl 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, at 37°C, for up to 60 min. The reaction was stopped by the addition of a 50-μl aliquot of the incubation medium to 0.95 ml 20 m*M* HCl. Residual Bk was determined with the isolated guinea pig ileum bathed in 10 ml Tyrode buffer containing 0.14 μ*M* atropine and 0.17 μ*M* diphenhydramine at 37°C. Controls were prepared in the same manner as the incubates, except that HCl was added before the homogenate (zero time of reaction). Control experiments showed that Bk was stable in the presence of acidified neuronal homogenate for up to 24 hr and that the incubation of homogenate in 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, without Bk, at 37°C for up to 2 hr did not lead to the release of material having spasmogenic activity upon the isolated guinea pig ileum. In experiments designed to determine adequate assay conditions for the measurement of rat brain kininase activity, it was shown that kininase activity was maximal at 100 μM Bk. Bk inactivation was linearly related to incubation time from 5 to 180 min, for 30–640 μg homogenate protein, and kininase specific activity was independent of

homogenate protein concentration over a 20-fold range. All measurements were carried out in duplicate. Kininase specific activity is expressed as nanomoles of Bk inactivated per minute per milligram of protein.

Bradykinin-Product Analysis

An amino acid analyzer was used to determine Arg^1–Pro^3, Arg^1–Gly^4, Arg^1–Phe^5, Arg^1–Pro^7, Gly^4–Arg^9, Phe^5–Arg^9, Ser^6–Arg^9, Phe^8–Arg^9, and Gly^4–Pro^7 (Oliveira *et al.*, 1976). Free amino acids were measured by the method of Spackman *et al.* (1958). The incubation conditions used for Bk-product analysis were similar to those used for the kininase bioassay, unless otherwise stated, and the reactions were stopped by the addition of 1.2 ml/ml incubation medium of a solution containing 69% (v/v) 0.2 *M* sodium citrate, pH 2.2, 1% (v/v) 6 *M* HCl and 30% (v/v) polyethylene glycol 400. Sample cleanup before amino acid and peptide analysis was carried out by filtration of the hydrolysate supernatant (8300*g* for 10 min) through a 0.45-μm filter (Millipore). The values reported for free amino acids were corected for the blank values obtained by incubating the homogenate or homogenate-derived fractions with or without peptidase inhibitors but without Bk. Control experiments showed that homogenate did not release material that eluted with the elution volume of the peptide standards.

Dialysis

Dialysis tubing (8/32 Nojax Visking Casing) was freed from contaminating substances according to the procedure of McPhie (1971). Water was purified using a four-cartridge Milli-Q system (Waters). Purified water (resistivity $\geq$10 MΩ-cm) showed the following metal concentration (g/liter), measured by atomic absorption spectrophotometry, $<4.5 \times 10^{-8}$, zinc, and 1.1×10^{-7}, calcium, and was used for the preparation of all solutions. The neuronal perikarya-soluble fraction was treated as described in the legend to Fig. 4.

DNA Determination

DNA was measured by the procedure of Croft and Luban (1965), using highly polimerized calf thymus DNA (California Corp. Biochemical Research) as the standard.

Protein Determination

Protein was measured by a modification (Bensadoun and Weinstein, 1976) of the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

RESULTS

Time Course of Bradykinin-Product Formation

The only peptide products detected at 10 min of Bk inactivation by the neuronal perikarya homogenate (Fig. 1A) were the complementary fragments Arg^1–Phe^5 | Ser^6–Arg^9, Arg^1–Pro^7 | Phe^8–Arg^9, and Arg^1–Gly^4 | Phe^5–Arg^9. At this time (about 10% bradykinin inactivation), the stoichiometry of the complementary peptide products Arg^1–Phe^5 | Ser^6–Arg^8 and Arg^1–Pro^7 | Phe^8–Arg^9 was essentially 1:1, whereas Arg^1–Gly^4 and Phe^5–Arg^9 were recovered in the molar proportion 1:0.2. The recovery of the hydrolysis products accounted for 117% of the Bk inactivated. These results indicate that isolated neurons hydrolyze Bk at the Phe^5–Ser^6, Pro^7–Phe^8, and Gly^4–Phe^5 peptide bonds. The initial rate of Phe^5–Ser^6 bond cleavage was about five fold higher than that of the Pro^7–Phe^8 and Gly^4–Phe^5 bonds. At >10% Bk inactivation, low amounts (<0.05 mol/mol Bk inactivated) of Gly^4–Arg^9, Gly^4–Pro^7, and Ser^6–Pro^7 were detected, whereas the low-color yield peptide Arg^1–Pro^3, which is complementary to Gly^4–Arg^9, was detected only at 40 and 60 min of incubation (<0.05 mol/mol Bk inactivated). These results suggest that isolated neurons also hyrolyze Bk at the Pro^3–Gly^4 bond.

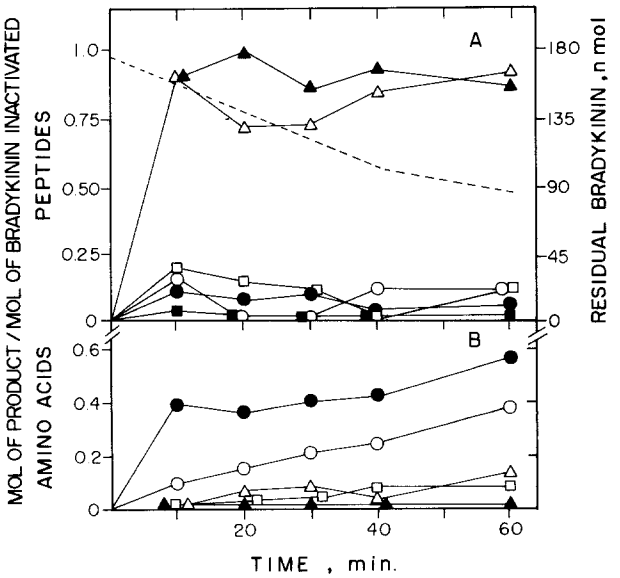

Fig. 1. Time course of Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^9 (bradykinin) hydrolysis by a neuronal perikarya homogenate. Bradykinin (176 μM) was incubated with 200 μl (21 μg DNA) homogenate in 1.0 ml 0.05 M Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl, at 37°C for up to 60 min. The reaction was stopped at the indicated times by acidification. Residual bradykinin was determined by bioassay. Bradykinin fragments and amino acids were measured using an amino acid analyzer. (A) Peptides: △——△, Arg^1–Phe^5; ▲——▲, Ser^6–Arg^9; ○——○, Arg^1–Pro^7; ●——●, Phe^8–Arg^9; □——□, Arg^1–Gly^4; ■——■, Phe^5–Arg^9; ----, bradykinin. (B) Amino acids: ●——●, Arg; ○——○, Phe; □——□, Ser; △——△, Pro; ▲——▲, Gly.

The rates of release of arginine and, to a lesser extent, phenylalanine during Bk inactivation (Fig. 1B) were much higher than those of glycine, serine, and proline. The profile of free amino acids released from Bk by isolated neurons is similar to that by neuro-2a neuroblastoma but not to that by C_8 glioma cells (DelBel *et al.*, 1986).

Processing of BK Hydrolysis Products

The increase in the relative amounts of free amino acids during Bk inactivation (Fig. 1B), concomitantly with a variation of the rate of release and a loss of the 1:1 stoichiometry of the complementary peptide products (Fig. 1A), was due to proteolytic processing of the primary hydrolysis products, possibly by endopeptidases and exopeptidases. Indeed, Table I shows that, in addition to the release of free amino acids, isolated neurons catalyzed the conversion of some primary inactivation products into shorter-chain primary and secondary peptide products. Thus, Arg^1–Pro^7 was extensively degraded to Arg^1–Phe^5 and, to a lesser extent, to Ser^6–Pro^7 and Gly^4–Pro^7. The major peptide fragments formed from Gly^4–Arg^9 were Ser^6–Arg^9 and Gly^4–Pro^7, and that from Phe^5–Arg^9 was Ser^6–Arg^9. Ser^6–Arg^9 and Phe^8–Arg^9 were almost completely degraded to free amino acids. At incubation times shorter than 30 min, in addition to free amino acids, Ser^6–Pro^7 and Phe^8–Arg^9 were released from Ser^6–Arg^9 by neuronal perikarya homogenate (data not shown), indicating that the homogenate hydrolyzes the Pro–Phe bond of Ser–Pro–Phe–Arg. In general, the N-terminal products of Bk inactivation were more resistant to degradation than the C-terminal fragments, possibly due to their Pro–Pro moiety.

Effect of Peptidase Inhibitors on Bk Processing

Table II shows that the metal chelator 1,10-phenanthroline exhibited a concentration-dependent inhibition of kininase activity, which was 93% inhibited by 1 m*M* chelator. In contrast, the other chelators used exerted either a slight (1 m*M* 8-hydroxyquinoline) or no (1 m*M* EDTA and 1 m*M* EGTA) inhibitory effect on kininase activity. PE activity was affected in the same manner as kininase activity by metal chelators. DTT (up to 2 m*M*) did not appreciably affect kininase activity, while it slightly activated PE activity. Both activities were strongly inhibited by 1 m*M* PCMB. The serine protease inhibitor diisopropyl-fluorophosphate (DFP; 0.2 m*M*) inhibited 7 and 99% kininase and PE activities, respectively. cF–Ala–Ala–Phe–pAB, an inhibitor of endopeptidase 24.15 (Chu and Orlowski, 1984), presented a concentration-dependent inhibition of kininase activity (data not shown), which was 90% inhibited by 1 m*M* inhibitor. Maximal inhibition (75%) of kininase activity by antiserum was attained at 16 μl antiserum/ml incubation medium. Z–Pro–prolinal (10 μM), a PE inhibitor (Wilk and Orlowski, 1983), inhibited 98% neuronal perikarya PE but did not appreciably affect kininase activity. Bestatin (50 μM), an aminopeptidase inhibitor, did not affect kininase activity.

Table III shows that 1,10-phenanthroline exhibited a concentration-dependent inhibitory effect on the release of the complementary peptide products

Table I. Degradation of Bradykinin Fragments by a Neuronal Perikarya Homogenate[a]

Substrate		mol of product/mol of substrate hydrolyzed									
		Peptides					Amino acids				
	Hydrolyzed (%/30 min)	$Arg^1 \rightarrow Phe^5$	$Gly^4 \rightarrow Pro^7$	$Ser^6 \rightarrow Arg^9$	Phe^8–Arg^9	Ser^6–Pro^7	Phe	Arg	Ser	Pro	Gly
$Arg^1 \rightarrow Pro^3$	44	0	0	0	0	0	0	0.32	0	0.95	0
$Arg^1 \rightarrow Gly^4$	24	0	0	0	0	0	0	0.52	0	0.05	0
$Arg^1 \rightarrow Phe^5$	28	0	0	0	0	0	0.25	0.75	0	0.15	0.05
$Arg^1 \rightarrow Pro^7$	100	0.37	0.11	0	0	0.15	0.27	0.45	0.26	0.76	0
$Gly^4 \rightarrow Arg^9$	57	0	0.45	1.1	0	0	0.55	0.19	0.36	0.43	0.58
$Phe^5 \rightarrow Arg^9$	84	0	0	0.44	0.03	0.13	0.60	0.10	0.10	0·12	0.01
$Ser^6 \rightarrow Arg^9$	72	0	0	—	0	0.2	0.70	0.80	0.50	0.60	0
Phe^8–Arg^9	98	0	0	0	—	0	0.80	0.70	0	0	0

[a] Peptides (46–66 μM) were incubated (30 min, 37°C) with the homogenate (20 μg DNA) in 0.5 ml 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl. The reaction was stopped by acidification. Hydrolysis products were measured using an amino acid analyzer.

Table II. Effect of Peptidase Inhibitors on Neuronal Perikarya Kininase and Prolyl Endopeptidase Activities[a]

Inhibitor	Kininase activity (%)	PE activity (%)
None	100	100
0.1 m*M* 1,10-phenanthroline	62	nd
1.0 m*M* 1,10-phenanthroline	7	20
0.1 m*M* 8-hydroxyquinoline	106	106
1.0 m*M* 8-hydroxyquinoline	80	80
1.0 m*M* EDTA	103	107
1.0 m*M* EGTA	103	106
2.0 m*M* dithiothreitol	110	125
1.0 m*M* PCMB	13	2
0.2 m*M* DFP	93	1
1.0 m*M* cF–Ala–Ala–Phe–pAB	10	nd
8 μl antiserum	25	nd
10 μM Z–Pro–prolinal	97	2
50 μM bestatin	107	nd

[a] The homogenate was preincubated (15 min, 37°C) with or without (control) inhibitor in 0.5 ml 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, and incubation (up to 30 min, 37°C) was started by the addition of either 116 μM bradykinin (kininase assay) or 3.3 m*M N*-suc–Gly–Pro–MCA (PE assay). The incubations were stopped by acidification. Kininase and PE activities were determined by bioassay and fluorimetry, respectively. The average values of four (kininase) and two (PE) determinations, which differed by 20% or less and 6% or less, respectively, are reported. Antiserum, endo-oligopeptidase A antiserum; nd, not determined.

Arg^1–Phe^5 | Ser^6–Arg^9 and Arg^1–Pro^7 | Phe^8–Arg^9 and of Ser^6–Pro^7 and free amino acids from BK. The formation of peptide products was essentially abolished by 1 m*M* 1,10-phenanthroline. EDTA (1.0 m*M*) and DFP (0.2 m*M*) did not inhibit the formation of Arg^1–Phe^5 | Ser^6–Arg^9 but partially blocked that of Arg^1–Pro^7 | Phe^8–Arg^9, Ser^6–Pro^7, and free amino acids. The formation of Arg^1–Phe^5 | Ser^6–Arg^9 was essentially abolished by 0.1 m*M* PCMB, while the release of Arg^1–Pro^7 was about 2.5-fold increased and that of Phe^8–Arg^9, Ser^8–Pro^7, and free amino acids decreased. The PCMB inhibition of Arg^1–Phe^5 | Ser^6–Arg^9 release was almost completely reversed by 0.5 m*M* DTT, and that of Phe^8–Arg^9, Ser^6–Pro^7, and free amino acids was partially reverted, but the formation of Arg^1–Pro^7 was strongly inhibited. cF–Ala–Ala–Phe–pAB, an inhibitor of the soluble (Chu and Orlowski, 1984) and membrane-bound (Acker *et al.*, 1987) forms of endopeptidase 24.15 blocked the release of Arg^1–Phe^5 | Ser^6–Arg^9 in a concentration-dependent manner (inhibition >90% by 1 m*M* inhibitor), whereas the concentration of Arg^1–Pro^7 increased up to 5.2-fold, and that of Phe^8–Arg^9, Ser^6–Pro^7, and free amino acids decreased as the inhibitor concentration increased. Rat brain endo-oligopeptidase A antiserum, which exhibits anticatalytic activity against endo-oligopeptidase A (Camargo *et al.*, 1987; Toffoletto *et al.*, 1988), strongly blocked the release of Arg^1–Phe^5 | Ser^6–Arg^9, increased twofold the concentration of Arg^1–Pro^7, and decreased that of Phe^8–Arg^9, Ser^6–Pro^7, and free amino acids. A twofold

Table III. Effect of Peptidase Inhibitors on the Formation of Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^9(Bradykinin) Hydrolysis Products by a Neuronal Perikarya Homogenate[a]

Inhibitor	Products							
	Peptides					Amino acids		
	$Arg^1 \rightarrow Phe^5$	$Ser^6 \rightarrow Arg^9$	$Arg^1 \rightarrow Pro^7$	Phe^8–Arg^9	Ser^6–Pro^7	Phe	Arg	Ser
None	100 (29.2)	100 (22.1)	100 (2.4)	100 (2.3)	100 (1.7)	100 (4.1)	100 (7.9)	100 (1.2)
0.1 m*M* 1,10-phenanthroline	62	63	25	96	48	24	46	0
1.0 m*M* 1,10-phenanthroline	0	+	0	0	0	9	38	+
1.0 m*M* EDTA	100	99	57	46	33	19	57	62
0.2 m*M* DFP	100	95	55	58	77	68	58	0
0.1 m*M* PCMB	0	+	244	10	0	+	21	+
0.1 m*M* PCMB + 0.5 m*M* DTT	94	87	+	31	40	31	30	+
0.1 m*M* cF–Ala–Ala–Phe–pAB	40	31	407	66	34	56	67	59
1.0 m*M* cf–Ala–Ala–Phe–pAB	7	8	520	29	0	40	48	+
16 μl antiserum	27	25	214	60	0	62	86	81
0.1 m*M* cF–Phe–pAB	97	121	+	20	25	37	40	57
0.1 m*M* MK 422	86	98	44	76	67	57	41	33
10 μM Z–Pro–prolinal	97	84	60	64	83	49	18	70

[a] The homogenate was preincubated (15 min, 37°C) with or without (control) inibitor in 1.0 ml 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, and incubation (30 min, 37°C) was started by the addition of 116 μM bradykinin and was stopped by acidification. For PCMB + DTT, the homogenate was preincubated (15 min, 37°C) with 0.1 m*M* PCMB and thereafter with 0.5 m*M* DTT (15 min, 37°C), before the addition of substrate. Unhydrolyzed bradykinin was determined by bioassay. Hydrolysis products were determined using an amino acid analyzer. The product concentration is reported as the percentage of that formed in the absence of inhibitor. Values in parentheses indicate product concentration expressed as nmol/ml incubation medium. The levels of free Pro and Gly released in the presence of inhibitors were too low for accurate determination, and therefore their values are not shown. +, the amount detected was too small for quantitation.

increase in antiserum concentration did not lead to a further decrease in Arg^1–Phe^5 | Ser^6–Arg^9 formation. Endopeptidase 24.11 inhibitor cF–Phe–pAB (0.1 m*M*) (Almenoff and Orlowski, 1983) strongly inhibited the formation of Arg^1–Pro^7, Phe^8–Arg^9, Ser^6–Pro^7, and free amino acids but increased 21% the Ser^6–Arg^9 concentration without affecting that of Arg^1–Phe^5. MK 422 and Z–Pro–prolinal, inhibitors of ACE (Patchett *et al.*, 1980) and PE (Wilk and Orlowski, 1983), respectively, exhibited inhibitory effects on the release of Bk fragments which were qualitatively similar to those of cF–Phe–pAB, but less intense, except that the Ser^6–Arg^9 concentration was not affected or decreased slightly. MK 422 (0.1 m*M*) and Z–Pro–prolinal (10 μM) blocked 56 and 40% respectively, the formation of Arg^1–Pro^7.

Effect of Peptidase Inhibitors on Ser–Pro–Phe–Arg Degradation

Ser^6–Pro^7–Phe^8–Arg^9, which is one of the primary products of Bk inactivation, contains the Pro–Phe bond that was primarily cleaved in Bk (cf. Fig. 1A) and was extensively degraded by the neuronal perikarya homogenate (cf. Table 1). Therefore, we have studied the effect of peptidase inhibitors on the proteolytic processing of this tetrapeptide by the neuronal homogenate. Figure 2, left, shows that 50 μM bestatin led to a sixfold increase in Phe–Arg release, while the phenylalanine and arginine concentrations decreased 94 and 78%, respectively. These results indicate that the hydrolysis of Phe–Arg was catalyzed to a large extent by a bestatin-sensitive aminopeptidase. Since arginine release was 4.4-fold higher than that of phenylalanine in the presence of bestatin, the involvement of a carboxypeptidase in arginine release from Ser^6–Arg^9 cannot be excluded. The effect of peptidase inhibitors on Pro–Phe bond hydrolysis was studied by measuring the release of Phe–Arg from Ser^6–Pro^7–Phe^8–Arg^9 in the presence of 50 μM bestatin. Figure 2, right, shows that with 50 μM bestatin, the formation of Phe–Arg from Ser^6–Arg^9 was 52, 27, and 22% inhibited by 0.1 m*M* cF–Phe–pAB, 0.1 m*M* MK 422, and 0.1 m*M* Z–Pro–prolinal, respectively. A mixture of these four inhibitors, at the above concentrations, abolished the release of Phe–Arg.

Characterization of the Major Bradykinin-Inactivating Endopeptidase Activity in Neuronal Perikarya

Occurrence in the Neuronal Soluble Fraction

Table IV documents the levels of kininase and PE activites in isolated neurons. The values of kininase and PE specific activities in the neuronal perikarya homogenate corresponded to 4.2 ± 0.56 and 0.4 ± 0.07 (aveage ±SE; $N = 9$), respectively, when expressed as femtomoles per minute per cell body. The yields of kininase and PE activities in the neuronal perikarya homogenate (relative to brain cortex, taken as 100%), were $1.9 \pm 0.2\%$ and $0.4 \pm 0.05\%$ ($N = 9$), respectively. Kininase activity recovery was 1.7-fold higher in the soluble (105,000*g* for 1 hr) than in the particulate fraction, whereas the distribution of PE activity was about the same in these fractions. These results suggest that

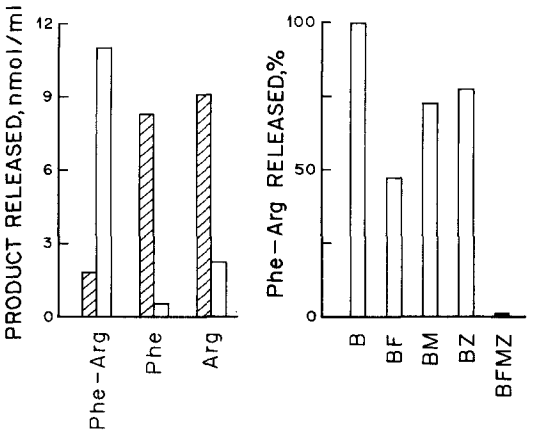

Fig. 2. Effect of peptidase inhibitors on Ser–Pro–Phe–Arg degradation by a neuronal perikarya homogenate. The homogenate was preincubated (15 min, 37°C) with or without inhibitor, in 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, and incubation (30 min, 37°C) was started by the addition of 54 μM Ser–Pro–Phe–Arg. The reaction was stopped by acidification. Degradation products were determined using an amino acid analyzer. Left: effect of bestatin on the release of Phe–Arg, Phe, and Arg from Ser–Pro–Phe–Arg. Hatched bars, no inhibitor; open bars, 50 μM bestatin. Right: effect of inhibitor combinations containing bestatin on Phe–Arg release from Ser–Pro–Phe–Arg. The amount of Phe–Arg released is reported as the percentage of that formed in the presence of 50 μM bestatin alone (B), taken as 100%. Inhibitor combinations used were 50 μM bestatin and 0.1 m*M* cF–Phe–pAB (F), 0.1 m*M* MK 422 (M), 0.1 m*M* Z–Pro–prolinal (Z), and a mixture of all these inhibitors at the above concentrations (BFMZ). The amounts of free amino acids released in the presence of inhibitor combinations were too low for accurate determination, and therefore their corresponding values are not shown.

Table IV. Specific Activity and Recovery of Kininase and Prolyl Endopeptidase Activities in Neuronal Perikarya Homogenate and Homogenate-Derived Fractions[a]

	Kininase activity		Prolyl endopeptidase activity	
Fraction	Sp act (nmol/min/mg protein)	Recovery (%)	Sp act (nmol/min/mg protein)	Recovery (%)
Homogenate	5.7 ± 0.3	|100|	0.48 ± 0.04	|100|
Soluble	5.7 ± 0.5	63 ± 9.1	0.42 ± 0.04	52 ± 4.5
particulare	4.5 ± 1	37 ± 9	0.60 ± 0.9	48 ± 4.9

[a] Kininase and prolyl endopeptidase activities were measured by bioassay and fluorimetry, respectively. Recoveries are reported relative to the total recovered activity |100%| in the soluble and particulate fractions. The yields relative to homogenate for kininase and prolyl endopeptidase activities were 121 and 119%, respectively. The average values ±SE of five independent determinations are reported.

neuronal perikarya kininase activity is mainly cytosoluble, in agreement with subcellular distribution studies carried out with whole brain (Camargo and Graeff, 1969). However, kininase activity is predominantly particulate in exponentially growing neuro-2a neuroblastoma cells (DelBel *et al.*, 1986). Neuronal PE activity is not predominantly cytosoluble, in contrast to the enzyme distribution reported using whole brain (Dresdner *et al.*, 1982) but not to that described in neuro-2a neuroblastoma cells (DelBel *et al.*, 1986).

A product-analysis of Bk degradation by soluble and particulate fractions of isolated neurons (data not shown) showed that the peptide- and amino acid-product composition of their hydrolysates was similar to that of the parent homogenate. The Phe^5–Ser^6 bond cleavage accounted for more than 95% of Bk inactivation by the soluble fraction, on the basis of the relative amounts of the complementary peptide products formed. Taken together, the above results and the data in Table IV suggest that the major neuronal Bk inactivating cleavage is catalyzed by a cytosoluble endopeptidase activity which hydrolyzes the Phe^5–Ser^6 peptide bond.

Effect of Peptidase Inhibitors

The susceptibility of the Phe^5–Ser^6 bond hydrolysis by the soluble neuronal fraction to peptidase inhibitors was studied next. Figure 3 shows that 0.5 m*M* DTT did not affect the release of the complementary peptide products Arg^1–Phe^5 | Ser^6–Arg^9 from Bk, whereas 0.1 m*M* PCMB inhibited 97% the formation of these products. The inhibition of the Phe^5–Ser^6 bond cleavage by 0.1 m*M* PCMB was surmountable by 0.5 m*M* DTT. Kininase activity in a soluble fraction dialyzed against 1.0 m*M* Tris–HCl buffer, pH 7.0, for 48 hr at 4°C, was activated by DTT in a concentration-dependent manner (data not shown). Activation was maximal (about twofold) at 0.5 m*M* DTT and decreased with higher DTT concentrations. At 2.0 m*M* DTT there was essentially neither activation nor inhibition of enzyme activity. These results indicate a requirement of a thiol group for enzyme activity. DFP (0.2 m*M*) did not affect the endopeptidase activity. Chelating agents differentially affected the Phe^5–Ser^6 bond cleavage. Thus, 1,10-phenanthroline exhibited a concentration-dependent inhibitory effect (>90% inhibition by 1.0 m*M*), whereas EDTA (1.0 m*M*) did not affect the peptidase activity. Both cF–Ala–Ala–Phe–pAB and antiserum inhibited the Phe^5–Ser^6 bond hydrolysis in a concentration-dependent manner, and the activity was about 90% blocked by 1.0 m*M* inhibitor and 20 μl antiserum/ml.

Effect of Metal Ions

Since metal chelators differentially effected the Phe^5–Ser^6 bond cleavage (Table III and Fig. 3), it was not clear whether or not this enzyme activity is metal dependent. In order to address this issue, the effect of dialysis of the neuronal soluble fraction against 1,10-phenanthroline and EDTA and against chlorides of divalent cations on kininase activity was studied. PE activity was also studied because the brain enzyme has been shown not to be a metalloprotease (cf. Orlowski, 1983); it occurs in the neuronal soluble fraction (cf. Table IV) and

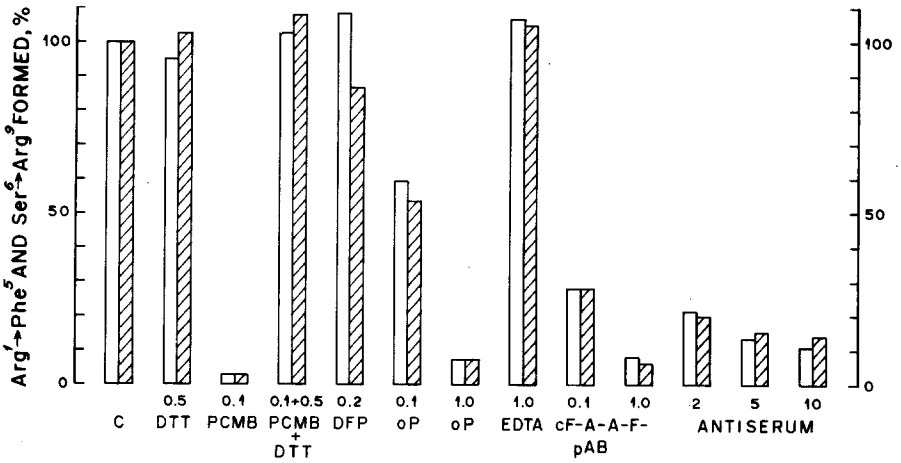

Fig. 3. Effect of peptidase inhibitors on the formation of Arg^1–Phe^5 (□) and Ser^6–Arg^9 (▨) from bradykinin by a neuronal perikarya-soluble fraction. The soluble fraction was preincubated (15 min, 37°C) with or without (C, control) inhibitor, in 250 μl (antiserum tubes) or 1.0 ml 0.05 *M* Tris–HCl buffer, pH 7.5, containing 0.1 *M* NaCl, and incubation (30 min, 37°C) was started by the addition of 116 μ*M* bradykinin. The reaction was stopped by acidification. The concentrations of Arg^1–Phe^5 and Ser^6–Arg^9 released are reported as the percentage of those formed without inhibitor (39 and 31 nmol/ml, respectively), taken as 100%. Inhibitor concentrations are reported as millimolar, except for those of antiserum, which are reported as microliters of antiserum per 250 μl of incubation medium. In a control experiment, it was shown that the concentrations of PCMB and DTT used here were the lowest concentrations that, upon preincubation (15 min, 37°C), essentially fully inhibit (0.1 m*M* PCMB) and completely restore (0.5 m*M* DTT) the PCMB-inhibited kininase activity in a neuronal soluble fraction. oP, 1,10-phenanthroline; antiserum, rat brain endo-oligopeptidase A antiserum.

is affected by metal chelators (cf. Table II) similarly to the soluble endopeptidase activity which hydrolyzes the Phe^5–Ser^6 bond of Bk (cf. Fig. 3). Figure 4A shows that the complete inhibition of the kininase activity in the soluble fraction by dialysis against 1.0 m*M* 1,10-phenanthroline was more than 95% reverted upon removal of the chelator by dialysis against 1 m*M* Tris–HCl buffer, pH 7.0; these dialysis procedures led to about a 20% decrease in PE activity. In contrast (cf. Fig. 4B), kininase activity was not affected by dialysis against 1 m*M* EDTA, whereas PE activity was about 40% activated. Figure 4A also shows that dialysis of the 1,10-phenanthroline-treated soluble fraction against 0.1 m*M* $CaCl_2$ led to 33 and 48% activation of kininase and PE activities, respectively, whereas dialysis against 0.1 m*M* $CoCl_2$ did not essentially affect or only slightly activated both kininase and PE activities. $ZnCl_2$ (0.1 m*M*) led to about 55% inhibition of both enzyme activities. The removal of excess and loosely bound metal ions by dialysis against 1.0 m*M* Tris–HCl buffer, pH 7.0, essentially reverted the activating and inhibitory effects of metal ions on kininase activity, whereas PE activation by $CaCl_2$, but not inhibition by $ZnCl_2$, was reverted.

Similar experiments, except that 1.0 m*M* EDTA replaced 1.0 m*M* 1,10-phenanthroline, led to comparable results with respect to the metal ion effect (data not shown). Alternatively, the neuronal soluble fraction was dialyzed

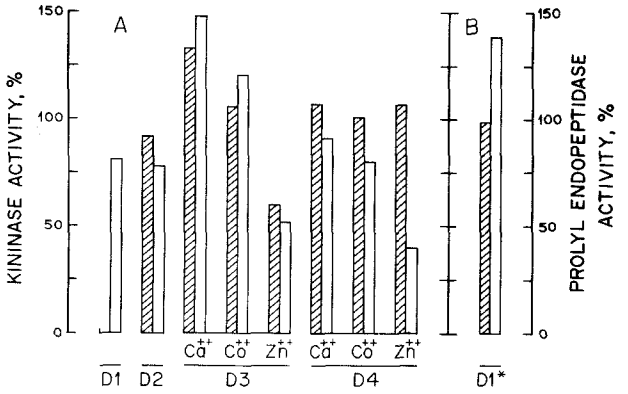

Fig. 4. Effect of dialysis against chelating agents and metal ions on kininase (▨) and prolyl endopeptidase (□) activities in a neuronal perikarya-soluble fraction. Kininase and PE activities in each dialysis-treated soluble fraction are reported relative to their specific activities in a soluble fraction dialyzed for the same time against 1.0 m*M* Tris–HCl buffer, pH 7.0 (controls), which were taken as 100%. Kininase and PE activities were determined (in the presence of 0.5 and 2.0 m*M* dTT, respectively) by bioassay and fluorimetry, respectively. (A) Soluble fraction (5.0 ml) was dialyzed at 4°C against four changes of 500 ml 1.0 m*M* 1,10-phenanthroline in 1.0 m*M* tris–HCl buffer, pH 7.0, for 24 hr (D1), and then against four changes of 500 ml 1.0 m*M* Tris–HCl buffer, pH 7.0, for 24 hr (D2). The resulting retentate was divided in three equal parts. Each part was dialyzed against two changes of 250 ml of 0.1 m*M* $CaCl_2$, or 0.1 m*M* $CoCl_2$, or 0.1 m*M* $ZnCl_2$ in 1.0 m*M* Tris–HCl buffer, pH 7.0, for 24 hr (D3), and then against six changes of 250 ml of 1.0 m*M* Tris–HCl buffer, pH 7.0, for 48 hr (D4). (B) Same procedure as for D1 in A, except that 1.0 m*M* EDTA was substituted for 1.0 m*M* 1,10-phenanthroline (D1*).

against 1.0 m*M* EDTA, pH 7.0, and then against 1.0 m*M* Tris–HCl buffer, pH 7.0. The preincubation (15 min, 37°C) of this EDTA-treated fraction with 1.0 m*M* $CaCl_2$ or with 25 μM $ZnCl_2$ led to 60% activation or 40% inhibition of kininase activity, respectively.

DISCUSSION

The present study shows that rat brain neuronal perikarya contain peptidases able to inactivate Bk through hydrolysis of the Phe^5–Ser^6, Pro^7–Phe^8, Gly^4–Phe^5, and Pro^3–Gly^4 peptide bonds (cf. Fig. 1 and Results). There is strong evidence that each of the four inactivating cleavages of Bk by the neuronal perikarya homogenate was catalyzed by a different peptidase, except for the Pro^7–Phe^8 bond cleavage, which appears to be catalyzed by at least three enzymes.

Neurnal Perikarya Inactivate Bradykinin Mainly Through Cleavage at the Phe^5–Ser^6 Bond by a Thiol-Endopeptidase

The hydrolysis of Bk at Phe^5–Ser^6 bond by the neuronal homogenate appears to be catalyzed mainly by an endopeptidase different from the multicatalytic protease complex (Wilk and Orlowski, 1980), endopeptidase 24.15 (Orlowski *et al.*, 1983), and endo-oligopeptidase A (Camargo *et al.*, 1973; Carvalho and Camargo, 1981), which hydrolyze the Phe^5–Ser^6 bond. Evidence to support this is as follows. The multicatalytic protease complex is strongly inhibited by 1 m*M* NaCl (Wilk and Orlowski, 1980) and in our enzyme assays 100 m*M* NaCl was employed. The active site-directed inhibitor of endopeptidase 25.15, cF–Ala–Ala–Phe–pAB (1 m*M*) (Chu and Orlowski, 1984), which does not inhibit endo-oligopeptidase A (Toffoletto *et al.*, 1988), almost completely inhibits the Phe^5–Ser^6 bond cleavage by the neuronal homogenate. Rat brain endo-oligopeptidase A antiserum, which exhibits anticatalytic and immunoprecipitating activity against endo-oligopeptidase A, but does not similarly affect endopeptidase 24.15 (Toffoletto *et al.*, 1988), strongly inhibits the Phe^5–Ser^6 bond hydrolysis by the neuronal homogenate. Endo-oligopeptidase A (Camargo *et al.*, 1987) and endopeptidase 24.15 (Orlowski *et al.*, 1983) are strongly inhibited by both 1 m*M* 1,10-phenanthroline and 1 m*M* EDTA, whereas the release of Arg^1–Phe^5 | Ser^6–Arg^9 from Bk by the neuronal homogenate is blocked by 1 m*M* 1,10-phenanthroline but not by 1 m*M* EDTA (cf. Table III). The hydrolysis of the Phe^5–Ser^6 bond seems to be catalyzed by a thiol-endopeptidase (cf. Table III). Endo-oligopeptidase A (Oliveira *et al.*, 1976; Camargo *et al.*, 1987), but not endopeptidase 24.15 (Orlowski *et al.*, 1983; Acker *et al.*, 1987), has been reported as a thiol-endopeptidase.

Arg^1–Phe^5 is also a degradation product of Arg^1–Pro^7 by neuronal perikarya homogenate (cf. Table I). The inhibition of Bk Phe^5–Ser^6 bond cleavage by cF–Ala–Ala–Phe–pAB, antiserum, and PCMB leads to a concomitant several-fold increase in Arg^1–Pro^7 formation (cf. Table III), suggesting that the same enzyme activity catalyzes the Phe^5–Ser^6 bond cleavage both of Bk and of its Arg^1–Pro^7 moiety. Endo-oligopeptidase A does not hydrolyze Arg^1–Pro^7 to an appreciable extent (Oliveira *et al.*, 1976).

About two-thirds of the neuronal kininase activity is soluble, and more than 95% of Bk inactivation by the soluble fraction is accounted for by cleavage of the Phe^5–Ser^6 bond (cf. Table IV and Results). Our studies on the effect of peptidase inhibitors upon the release of Arg^1–Phe^5 | Ser^6–Arg^9 from Bk by the neuronal soluble fraction (cf. Fig. 3) provide strong evidence that a thiol-endopeptidase inactivates Bk through cleavage of the Phe^5–Ser^6 bond and that this enzyme differs from previously described soluble peptidases which hydrolyze Bk at the same site. Thus, the nearly complete inhibition of the Phe^5–Ser^6 bond cleavage by 0.1 m*M* PCMB was fully reversed by 0.5 m*M* DTT, a DTT concentration that maximally activates the peptidase in a dialyzed soluble fraction. Both antiserum and cf–Ala–Ala–Phe–pAB almost completely inhibit thiol-endopeptidase activity in the neuronal perikarya soluble fraction.

The differential inhibitory effect of metal chelators on the Phe^5–Ser^6 bond

cleavage exhibited by the neuronal perikarya homogenate (cf. Tables II and III) is also displayed by the soluble fraction (cf. Fig. 3): the Phe^5–Ser^6 bond cleavage is blocked by 1 m*M* 1,10-phenanthroline, but not by 1 m*M* EDTA, as it occurs with carboxypeptidase B (Folk *et al.*, 1960), a metalloenzyme sensitive to 1,10-pnenanthroline but not to EDTA. However, peptidases reported as non-metalloenzymes, such as endo-oligopeptidase A (Camargo *et al.*, 1987) and PE (Andrews *et al.*, 1980), can be inhibited by metal chelators. By using a dialysis-based technique (Vallee *et al.*, 1960), it was shown (cf. Fig. 4 and Results) that the inhibition of soluble neuronal kininase and PE activities by 1,10-phenanthroline is fully reversible upon removal of the chelator. In contrast to endopeptidase 24.15 (initially called soluble metalloendopeptidase), whose activity is abolished by dialysis against 1 m*M* EDTA at pH 7 and restored by the addition of Zn^{2+} (Orlowski *et al.*, 1983), the EDTA-treated neuronal soluble activity is inhibited by Zn^{2+}. Thus, it seems clear that the thiol-endopeptidase activity described here cannot be classified as a metalloendopeptidase. However, soluble thiol-endopeptidase activity is activated by high Ca^{2+} concentrations, suggesting a possible role of calcium in the regulation of this activity. The evidence presented here suggests that neuronal perikarya thiol-endopeptidase activity differs from that of both endo-oligopeptidase A and endopeptidase 24.15, which were reported to be two different enzymes (Camargo *et al.*, 1987; Toffoletto *et al.*, 1988). Our data do not permit excluding a minor contribution of endo-oligopeptidase A and/or endopeptidase 24.15 to the cleavage of the Bk Phe^5–Ser^6 bond by neuronal perikarya.

Involvement of Endopeptidase 24.11, Angiotensin-Converting Enzyme, and Prolyl Endopeptidase in BK Neuronal Inactivation

Bk inactivation by the neuronal perakarya homogenate through Pro^7–Phe^8 bond cleavage appears to be catalyzed by at least three enzymes. Evidence to support this is as follows. The formation of the complementary peptide products Arg^1–Pro^7 | Phe^8–Arg^9 is partially inhibited by 1 m*M* EDTA and completely blocked by 1 m*M* 1,10-phenanthroline (cf. Table III), suggesting the involvement of metallopeptidase(s) in Pro^7–Phe^8 bond cleavage. The partial inhibition of this cleavage by endopeptidase 24.11 inhibitor cF–Phe–pAB (Almenoff and Orlowski, 1983; Matsas *et al.*, 1984) and by ACE inhibitor MK 422 (Patchett *et al.*, 1980; Matsas *et al.*, 1984) (cf. Table III) indicates the participation of both zinc-peptidases, endopeptidase 24.11 and ACE, in Pro^7–Phe^8 bond hydrolysis. PE is a third enzyme seemingly to cleave the same Pro^7–Phe^8 bond, since the PE inhibitor Z–Pro–prolinal (Wilk and Orlowski, 1983) partially inhibits Bk Pro^7–Phe^8 bond cleavage (determined by Bk-product analysis; Table III) and completely blocks PE activity (determined by fluorimetry; Table II). The serine-enzyme inhibitor DFP partially inhibits this cleavage and completely inhibits PE activity. PCMB strongly blocks Pro^7–Phe^8 bond cleavage and fully inhibits PE activity. PCMB inhibition of Pro^7–Phe^8 bond cleavage is partially reversed by DTT, which activates PE activity (Table II) and (Greene *et al.*, 1982) but inhibits ACE (Soffer, 1981) and endopeptidase 24.11 (Almenoff and

Orlowski, 1983). EDTA partially inhibits Pro^7–Phe^8 bond hydrolysis and does not affect PE activity, whereas 1,10-phenanthroline strongly blocks both activities. The effects of chelators on neuronal Pro^7–Phe^8 bond cleavage and PE activity are in agreement with those described for brain PE, which is not a metalloprotease (cf. reviews by Wilk, 1983; Orlowski, 1983). Neuronal soluble PE activity does not behave as a metalloprotease and is activated by Ca^{2+} (cf. Fig. 4). The activation of PE by dialysis against EDTA (cf. Fig. 4) is possibly due to the removal of inhibitory heavy metal. Indeed, some laboratories routinely include EDTA in the medium used to assay PE activity (Kato *et al.*, 1980; Hersch, 1981). PE is a DTT-activated (Oliveira *et al.*, 1976; Greene *et al.*, 1982) serine-endopeptidase (Andrews *et al.*, 1980) specifically inhibited by Z–Pro–prolinal (Wilk and Orlowski, 1983). The properties of PE in neuronal perikarya homogenate described here are similar to those reported for purified brain PE (cf. Wilk, 1983), except for calcium activation.

Bk inactivation by the neuronal perikarya homogenate through cleavage of Pro^3–Gly^4 and Gly^4–Phe^5 bonds seems to be catalyzed by PE and endopeptidase 24.11, respectively, as suggested: first by the evidence that both enzymes occur in the neuronal homogenate and hydrolyze the Bk Pro^7–Phe^8 bond (this report); and second, by the specificity of purified PE and endopeptidase 24.11, which hydrolyze the Bk Pro^3–Gly^4 (Orlowski *et al.*, 1979) and Gly^4–Phe^5 (Almenoff and Orlowski, 1983) bonds, respectively, in addition to the Pro^7–Phe^8 bond cleavage.

The use of a time course of Bk-product formation to identify the sites of peptide bond cleavage, combined with a peptidase inhibitor paradigm, in which the effects of selective inhibitors can be attributed to the blockade of a given bond cleavage, leads to the model for the mechanism of Bk inactivation by rat brain neuronal perikarya proposed in Fig. 5. Primary cleavages of Bk molecule should be distinguished from secondary cleavages of inactivation products. Primary cleavage at the Phe^5–Ser^6 bond is due almost entirely to the action of a calcium-activated thiol-endopeptidase. Cleavage at the Bk Pro^7–Phe^8 bond results mainly from the action of endopeptidase 24.11, ACE, and PE. The primary cleavages at Pro^3–Gly^4 and Gly^4–Phe^5 bonds appear to result from the action of PE and endopeptidase 24.11, respectively. Secondary cleavages of Bk inactivation products are clearly demonstrated for Ser^6–Arg^9, which is converted to Ser^6–Pro^7 and Phe^8–Arg^9 by endopeptidase 24.11, ACE, and PE, and for Phe^8–Arg^9, which is hydrolyzed by a bestatin-sensitive aminopeptidase.

Enzymatic degradation has been proposed as a major mechanism for the inactivation of neuropeptides. For a peptidase to exert such a role it should fulfill some requirements, which include the ability to hydrolyze a peptide bond(s) of its potential substrate(s), leading to inactive products, and appropriate localization, among others (Lynch and Snyder, 1986; Turner *et al.*, 1985; White *et al.*, 1985). The ability of neuronal perikarya ACE and endopeptidase 24.11 to inactivate Bk (this report), taken together with the neurochemical properties of these membrane peptidase (Turner *et al.*, 1985), suggests that they could play a role in Bk neuronal processing. Several possible roles have been proposed for PE such as neuropeptide processing (Martins *et al.*, 1980; Oliveira *et al.*, 1976, cf. Wilk, 1983), participation in intermediate steps of intracellular protein degradation

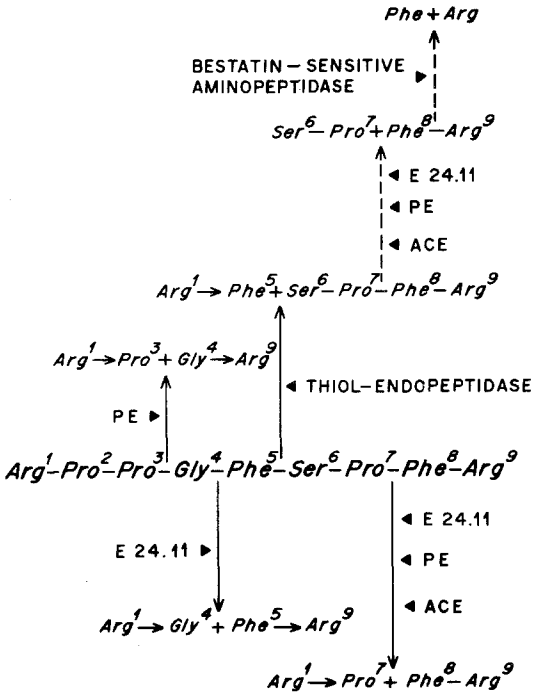

Fig. 5. Model for the mechanism of bradykinin inactivation by rat brain neuronal perikarya. Primary cleavages of bradykinin molecule are indicated by solid arrows, and secondary cleavages of inactivation products by dashed arrows. E 24.11, endopeptidase 24.11.

(Oliveira *et al.*, 1976; Camargo *et al.*, 1979), and participation in neural developmental processes (Kato *et al.*, 1980; Martins and De Mello, 1985; DelBel *et al.*, 1986; Martins *et al.*, 1987). Peptidases that cleave the Bk Phe^5–Ser^6 bond have been reported in adult brain (Oliveira *et al.*, 1976; Orlowski *et al.*, 1983; Camargo *et al.*, 1987; MacDermott *et al.*, 1987) and in neural systems used for developmental studies such as the chick retina (Martins and De Mello, 1985) and neuro-2a neuroblastoma cells (DelBel *et al.*, 1986), but their similarity to the thiol-endopeptidase shown here to occur in neuronal pericarya isolated from the developing rat brain is unclear. In addition to suggesting a possible participation, if any, of PE and thiol-endopeptidase in neuronal bradykinin inactivation, this report shows that bulk isolated neurons from rat brain could be used as a model to study neuronal peptide processing.

ACKNOWLEDGMENTS

We thank Dr. M. Orlowski for the generous gift of cF–Ala–Ala–Phe–pAB and cF–Phe–pAB, Drs. S. Wilk and A. C. M. Camargo for the generous gifts of Z–Pro–prolinal and endo-oligopeptidase A antiserum, respectively. We are

grateful to Drs. M. U. Sampaio and M. C. O. Salgado, Escola Paulista de Medicina and Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, respectively, for helpful review of the manuscript. We thank Dr. H. S. Pretel for helpful technical assistance and Ms. Issajuara Freire for typing the manuscript. E.A.D.B. is the recipient of FAPESP Predoctoral Fellowship 84/1968. This research was supported by Grants CNPq 30.0550-79 and 40.8428-85, FAPESP 84/1539-6, and FINEP 86/0849 (to A.R.M.).

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