ELSEVIER

Peptides, Vol. 17, No. 4, pp. 709–720, 1996

0196-9781/96 $15.00 + .00

PII S0196-9781(96)00066-6

Proteases Involved in the Metabolism of Angiotensin II, Bradykinin, Calcitonin Gene-Related Peptide (CGRP), and Neuropeptide Y by Vascular Smooth Muscle Cells

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Received 6 December 1995

MENTLEIN, R. AND T. ROOS. *Proteases involved in the metabolism of angiotensin II, bradykinin, calcitonin gene-related peptide (CGRP), and neuropeptide Y by vascular smooth muscle cells.* PEPTIDES **17**(4) 709–720, 1996.—To understand the regulation of the vasoactive peptides bradykinin, angiotensin II, calcitonin gene-related peptide (CGRP), and neuropeptide Y (NPY), their proteolytic catabolism by cultured rat aortic vascular smooth muscle cells and A7r5 cells was investigated. Endopeptidase-24.11 (EC 3.4.24.11, CD 10) was responsible for the final inactivation of bradykinin, angiotensin II, and CGRP, but not of NPY, which was degraded by a different metallo-endopeptidase. Exopeptidases, namely the aminopeptidases A (EC 3.4.11.7), N (EC 3.4.11.2, CD 13), and P (EC 3.4.11.9) and the carboxypeptidases M (EC 3.4.17.12) and P (EC 3.4.17.16), were important for their differential, receptor subtype-specific activation or inactivation. Aminopeptidase A and N generated angiotensins III and IV from angiotensin II. Aminopeptidase P liberated the terminal amino acids from bradykinin and NPY, yielding the Y_2 receptor specific-agonist NPY(2–36). Carboxypeptidase P produced AT II(1–7) and carboxypeptidase M produced the BK_1 receptor agonist [des-Arg^9]bradykinin. Thus, peptidases at the surface of vascular smooth muscle cells exert a complex influence on the level of biologically active vasoactive peptides.

Angiotensin II Bradykinin Calcitonin gene-related peptide (CGRP) Neuropeptide Y Peptide degradation Aminopeptidase M Aminopeptidase P Carboxypeptidase M Carboxypeptidase P Endopeptidase-24.11 Vascular smooth muscle cells

VASODILATION and vasoconstriction are regulated by several endocrine, paracrine, and neurogenic factors. Perivascular nerves release, besides the classical transmitters acetylcholine or noradrenaline, the neuropeptides calcitonin gene-related peptide (CGRP), substance P, neuropeptide Y (NPY), and others as cotransmitters. Like the endocrine/paracrine peptides angiotensin II (AT II) and bradykinin (BK), these neuropeptides are strong vasoactive effectors. As other signal-transmitting substances, these peptides have to be inactivated after their interaction with receptors; otherwise, a constant stimulus would be produced, which permits no information transfer. Bioactive peptides are degraded and thereby mostly inactivated by cell surface-bound or soluble proteases (1,16,19). However, in some cases the extracellular proteolytic metabolism results in a modified or new receptor specificity (26). A number of proteases have been described to be implicated in the metabolism of regulatory peptides in various tissues (1,16), but the peptide-degrading proteases present at the smooth muscle cells rarely have been investigated. Moreover, despite various reports on proteases acting on small peptides like substance P with 11 residues (11,14,38), only a few proteases have yet been identified to be active on peptides with more than 30 amino acid residues, like NPY and CGRP with 36 and 37 residues. The inactivation mechanisms for NPY and CGRP in the vasculature are completely unknown.

Using smooth muscle cells in culture and membrane preparations, we therefore investigated 1) the proteolytic metabolism of the vasoactive peptides NPY, CGRP, BK, and AT II, and 2) identified the peptide-inactivating proteases by specific substrates, by analysis of peptide cleavage sites, and by effects of specific inhibitors on their fragmentation.

METHOD

Peptides and Inhibitors

Synthetic peptides (rat sequences) and fragments were purchased from Saxon Biochemicals (Hannover, Germany). BK(1–7), BK(5–9), and chromogenic peptidase substrates were from Bachem (Bubendorf, Switzerland). BK(2–9),

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BK(1–4), AT II(1–7), AT II(2–8), and AT(3–8) were from Calbiochem–Novabiochem (Bad Soden, Germany). AT II(1–4) and AT II(5–8) were also prepared by incubation of AT II with endopeptidase-24.18, highly purified from rat kidneys (17). Peptidase inhibitors were obtained from Calbiochem–Novabiochem. Captopril and enalaprilate were generous gifts from MSD Sharp & Dohme (Munich, Germany). Peptides were radioiodinated by the chloramine-T method and purified by reverse-phase HPLC (24). Neuropeptide Y was also radioiodinated by the iodogen method in Tyr^1 position, which was determined by cleavage with trypsin followed by HPLC analysis (34).

Cell Cultures

Vascular smooth muscle cells from rat aorta were prepared as described (5). The A7r5 cell line derived from the thoracic aorta of an embryonic DB1X rat was obtained from American Type Culture Collection (Rockville, MD). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Sigma, Munich, Germany). Subcultures were prepared by treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) for 4 min at 37°C, and cells were seeded at densities of 10^6 cells/25 cm^2 into culture flasks.

Preparation of Membranes

Aortic membranes. Dissected pig or rat aortae were carefully rinsed with PBS, cut longitudinally, and adventiva and intima were removed in strips. The media were cut into pieces and homogenized in PBS with a blender for 1 min at 4°C. Nuclei and debris were removed by centrifugation (7 min at 1000 × *g*), and the supernatant centrifuged at 48,000 × *g* for 2 h at 4°C to yield the membrane fraction as a pellet, which was resuspended in PBS.

Smooth muscle cell membranes. Confluent smooth muscle cell cultures were washed with PBS, the cells lysed with hypotonic 10 m*M* HEPES buffer, pH 7.4, scraped off with a rubber policeman, and the suspension homogenized by sonication. The membrane fraction was obtained by centrifugation as above.

Degradation Assays

Approximately 10^6 cells grown in 25-cm^2 culture flasks were carefully washed twice with 37°C thermostatted incubation medium that consisted of 145 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 20 m*M* glucose, and 20 m*M* HEPES adjusted with NaOH to pH 7.4 (14). After addition of 2 ml fresh incubation medium and 2–40 nmol of the unlabeled peptides (as 1 m*M* solutions in water) or 20 kBq of the radiolabeled peptides, cultures were incubated for 30–240 min at 37°C. Inhibitors were added as 100-fold concentrated stock solutions in water, and cultures were preincubated with inhibitors for 15 min at 37°C prior to addition of peptides. After different times of incubations, aliquots of the supernatants (500 μl) were withdrawn, acidified with 10% trifluoroacetic acid (50 μl), and degradation products separated by reversed-phase HPLC. For determination of protein, cells were lysed after incubation as described above.

Membrane fractions containing 5–20 μg protein were incubated with 2 nmol of peptides in 20 m*M* triethanolamine-HCl buffer, pH 7.4, in a total volume of 100 μl for 10–240 min at 37°C. Reactions were stopped by addition of 10 μl 10% trifluoroacetic acid; samples were cleared by centrifugation (5 min 14,000 × *g*) and analyzed by HPLC. Inhibitors were supplemented prior to addition of peptides and preincubated with membranes for 15 min at 37°C.

HPLC analyses were performed with Vydac C18 250 × 4.6-mm reversed-phase columns (Hesperia, CA) with linear gradients of 0–48% (BK, AT II) or 0–64% (NPY, CGRP) acetonitrile in 0.1% trifluoroacetic acid formed in 25 min at flow rates of 1 ml/min (25). Peptide fragments were detected and quantified in the eluates by their absorbance at 220 (peptide bonds) or 280 nm (aromatic amino acids), or in the case of radiolabeled peptides by collecting 0.5-ml fractions that were measured in a γ-counter.

Peptidase Assays

Aminopeptidase M was assayed with 200 μ*M* Leu-2-naphthylamide in 50 m*M* triethanolamine buffer, pH 7.4, at 37°C, and aminopeptidase A with 200 μ*M* α-Glu-2-naphthylamide in the presence of 0.1 m*M* bestatin in 50 m*M* Tris-HCl buffer, pH 7.4, containing 1 m*M* $CaCl_2$ at 37°C. Liberated naphthylamine was detected fluorometrically at excitation wavelength of 340 nm and emission wavelength of 450 nm (11,28). Aminopeptidase P was measured with 2 m*M* Arg-Pro-Pro in 40 m*M* Bicine buffer, pH 8.2, containing 1 m*M* $MnCl_2$ and 1 m*M* dithiothreitol at 37°C, and liberated Arg quantified by ninhydrin reaction (8). Dipeptidyl peptidase IV was monitored photometrically with 500 μ*M* Gly-Pro-4-nitroanilide in 100 m*M* Tris-HCl buffer, pH 8.5 (28). Carboxypeptidase P was assayed with 2 m*M* Z-Pro-Ala in 40 m*M* Bicine buffer, pH 7.8, supplemented with 1 m*M* $MnCl_2$ (27). Carboxypeptidase M was measured with 2 m*M* Bz-Gly-Lys in 40 m*M* Bicine buffer, pH 7.8, supplemented with 2 m*M* $CoCl_2$ (27). Endopeptidase-24.11 was assayed by HPLC with 100 μ*M* [D-Ala^2,Leu^5]enkephalin as substrate in 50 m*M* triethanolamine-HCl buffer, pH 7.4 (16,20). Blanks were performed with substrates and boiled membranes.

Solubilization and Partial Purification of Aminopeptidase P

Membranes of about 10^7 A7r5 cells in 500 μl 20 m*M* HEPES buffer, pH 7.4, were incubated for 2 h at 37°C with 5 U phosphatidylinosit-specific phospholipase C from *Bacillus cereus* (Boehringer, Mannheim, Germany). The mixture was then centrifuged (48,000 × *g*, 2 h) and the supernatant applied onto a 60 × 1.6-cm Superdex S-200 gel chromatography column (Pharmacia, Freiburg, Germany) equilibrated and eluted with 20 m*M* Bicine buffer, pH 7.8, containing 0.1 m*M* dithiothreitol, 1 m*M* $MnCl_2$, and 0.14 *M* NaCl. Fractions of 1 ml were collected and assayed for aminopeptidase P activity (8). For molecular mass determinations ferritin, catalase, IgG, bovine serum, and egg albumins served as standards.

Protein Quantification and Peptide Chemistry

Protein was quantified by a micromodification of the Coomassie brilliant blue binding assay for membrane-bound proteins (19). Peptide fragments collected after HPLC separation were analyzed after acid hydrolysis for amino acid composition (2).

RESULTS

General Characteristics of Peptide-Degrading Proteases of Vascular Smooth Muscle

Membranes from pig aortic smooth muscles, from rat vascular smooth muscle cells, and the rat embryonic vascular smooth muscle cell line A7r5 were used for a first characterization of peptidases involved in the degradation of vasoactive peptides. Because membranes from crude tissues like pig aorta could be contaminated with other cell types, no further attempt was made to purify plasma membranes. However, all proteases identified

TABLE 1

DEGRADATION RATES FOR VASOACTIVE PEPTIDES (20 μM) BY MEMBRANES FROM PIG AORTIC SMOOTH MUSCLE LAYER (pSM), MEMBRANES FROM CULTURED RAT VASCULAR SMOOTH MUSCLE CELLS (rVSMC), AND MEMBRANES FROM RAT A7r5 CELLS (n = 4, ± SD)

Peptide	Residues	Degradation Rate (nmol h^{-1} mg $Protein^{-1}$)		
		pSM	rVSMC	rA7r5
Angiotensin II	8	43 ± 3	8 ± 1	9 ± 1
Bradykinin	9	150 ± 28	18 ± 3	38 ± 6
Neuropeptide Y	36	20 ± 2	n.d.	4 ± 1
CGRP	37	24 ± 8	n.d.	4 ± 1

n.d., not done. Experiments were performed with nonradioactive peptides.

in experiments with intact cells proved to be surface bound. There were no significant differences in the fragmentation pattern between rat aortic smooth cells and the A7r5 cell line, only the degradation rates differed a bit (Table 1). Because A7r5 cells are easier to handle, we chose them for most further experiments.

Medium chain length peptides like BK or AT II were much faster hydrolyzed than long-chain vasoactive peptides like NPY and CGRP (Table 1). Degradation of all peptides could be completely blocked by incorporation of the metal-chelator 1,10-phenanthroline (1 mM), indicating that exclusively metallo-proteases were involved in peptide degradation by smooth muscle cells.

Exopeptidases present in the membrane preparations were identified by use of specific chromogenic substrates (Table 2). Leucyl-2-naphthylamide is a substrate for the plasma membrane-bound aminopeptidase N and the soluble or membrane-associated forms of the arylamidase. Both can be inhibited by 0.1 mM bestatin or amastatin, but only the arylamidase by 0.1 mM puromycin. The rapid hydrolysis of leucyl-2-naphthylamide by A7r5 and aortic membranes was completely blocked (less than 5% residual activities) by incorporation of bestatin or amastatin, but not by puromycin. Therefore, smooth muscle membranes should contain aminopeptidase N (or M, EC 3.4.11.2, CD 13), which is known to liberate neutral and basic N-terminal amino acids from peptides. Aminopeptidase A (EC 3.4. 11.7) releasing acidic N-terminal residues was detected by the specific substrate Glu-2-naphthylamide in comparable low activity. The tripeptide Arg-Pro-Pro is a specific substrate for aminopeptidase P (EC 3.4.11.9), a highly specialized exopeptidase liberating ultimate unblocked N-terminal amino acids only from peptides with penultimate proline. Considerable activity of this enzyme previously detected in kidney, lung, and brain was found in smooth muscle membranes. The proline-specific dipeptidyl peptidase IV (EC 3.4.14.5) was absent.

Besides aminopeptidases, two types of carboxypeptidases could be demonstrated in smooth muscle membranes. Bz-Gly-Lys can be hydrolyzed by four carboxypeptidases: the plasma membrane-bound carboxypeptidase M, the soluble carboxypeptidases B, N (both present in serum), and lysosomal, soluble, or membrane-bound carboxypeptidase H with acidic pH optimum. All four enzymes formerly identified in kidney or serum cleave C-terminal basic residues from peptides. The membrane localization and the activity at slightly alkaline pH identifies the Bz-Gly-Lys-cleaving activity of smooth muscle as carboxypeptidase M (EC 3.4.17.12). Z-Pro-Ala is a substrate for the lysosomal proline carboxypeptidase and the plasma membrane-bound carboxypeptidase P, both liberating ultimate C-terminal amino acids only from peptides with Pro (or Ala) in the penultimate position. Because the lysosomal enzyme is a soluble serine protease active at acidic pH, it can be excluded due to experimental conditions and inhibitory profile. Thus, smooth muscle membranes contain carboxypeptidase P (EC 3.4.17.16), which has been previously detected in kidney brush border membranes. In conclusion, at least five exopeptidases (aminopeptidases A, N, and P, carboxypeptidases N and P) are present in smooth muscle membranes.

Because specific chromogenic substrates are not available for most peptide-cleaving endopeptidases, we identified them in the next step by incubation of vasoactive peptides with intact A7r5 smooth muscle cells in culture and with membranes of these cells. In these experiments exo- and endopeptidases act together in the metabolism of peptides. Supernatants of whole cells (conditioned for up to 5 h in incubation medium) contained no proteolytic activity, indicating that no intracellular peptidase was released from intact or broken cells during the incubations.

Identification of Peptidases Involved in Degradation of Bradykinin and Angiotensin II by A7r5 Smooth Muscle Cells

Several exo- and endopeptidases that degrade BK and AT II have been isolated from tissues from different smooth muscle and thoroughly investigated (Fig. 1). Therefore, some peptidases present in A7r5 cells can simply be identified by i) analysis of cleavage sites and ii) incorporation of specific inhibitors. Both peptides were cleaved by cultivated vascular smooth muscles

TABLE 2

EXOPEPTIDASES ON A7r5 SMOOTH MUSCLE CELL MEMBRANES AS COMPARED TO MEMBRANES FROM PIG AORTA AND RAT KIDNEY (PEPTIDASE CONTROL)

Peptidase	Substrate	Activity With Membranes From (nmol h^{-1} mg $protein^{-1}$)		
		A7r5-Cells	Pig Aorta	Rat Kidney
Aminopeptidase A	0.2 mM α-Glu-NNap*	38	8,200	92,000
Aminopeptidase N	0.2 mM Leu-NNap	6,400	25,000	830,000
Aminopeptidase P	2 mM Arg-Pro-Pro	280	39	460
Dipeptidyl peptidase IV	0.5 mM Gly-Pro-$NPhNO_2$	<1	<1	36,000
Carboxypeptidase M/B	2 mM Bz-Gly-Lys	41	11	48
Carboxypeptidase P	2 mM Z-Pro-Ala	12	30	27

NNap, -2-naphthylamide; $NPhNO_2$, -4-nitroanilide; Z, benzoyloxycarbonyl, Bz, benzoyl.
* In the presence of 0.1 mM bestatin.

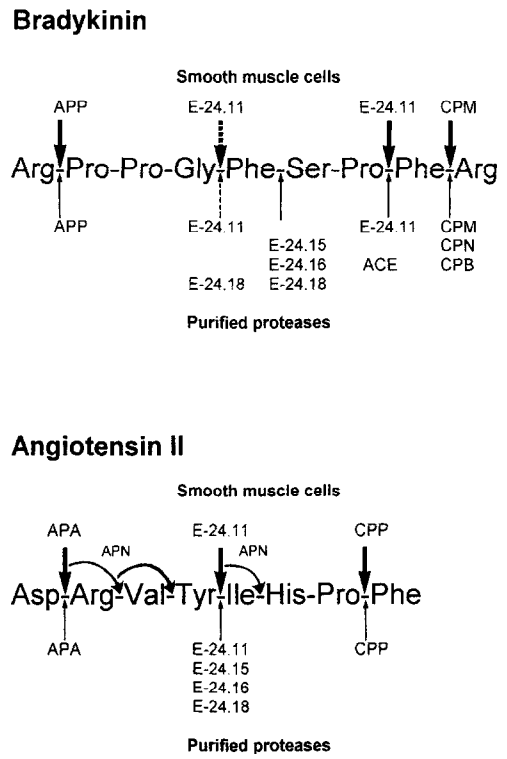

FIG. 1. Cleavage sites of bradykinin and angiotensin II by peptidases of cultivated A7r5 smooth muscle cells and by purified cell surface peptidases. AP, aminopeptidase indicated as P, A, or N. CP, carboxypeptidase indicated as M, N, B, or P. Endopeptidases specified by enzyme commission (EC) nomenclature numbers. Data for purified proteases compiled from (2,16,35,36).

cells and A7r5 cells to the same fragments that were identified by amino acid analysis and cochromatography with synthetic peptides (Table 3, Fig. 2). Intact A7r5 cells and their membrane preparations produced identical fragments, but in somewhat different yields (Table 3).

The major BK fragments, BK(1–7) and BK(8–9) (Fig. 1, Table 3), can result either from cleavage by endopeptidase-24.11 (EC 3.4.24.11, enkephalinase, atriopeptidase, neutral endopeptidase, neprilysin, CD 10), or angiotensin-converting enzyme (peptidyl dipeptidase A), or a yet unknown peptidase. Because for endopeptidase-24.11 or angiotensin-converting enzyme a number of specific inhibitors exist, the presence of the one or another peptidase should be easily detected by their influence on the generation of BK(1–7) and BK(8–9) fragments. The endopeptidase-24.11 inhibitors phosphoramidon and thiorphan, but not the angiotensin-converting enzyme inhibitors captopril or enalaprilate, nearly completely abolished their production (Table 4). Thus, endopeptidase-24.11 is the most important BK-degrading enzyme of A7r5 smooth muscle cells. Its presence is sustained by detection of BK(1–4) as a minor fragment (Figs. 1 and 2, Table 3), similar inhibitory profile for this cleavage (Table 4), and by hydrolysis of [D-Ala^2]enkephalin (see below) or *N*-[1-(*R,S*)-carboxy-3-phenyl-propyl-Ala-Ala-Phe-4-aminobenzoate (compare Table 4; inhibitor for endopeptidases-24.15/16, but substrate for endopeptidase-24.11). Two minor BK fragments, BK(1–8) and BK(2–9), must result from action of two exopeptidases that were already identified with chromogenic substrates. Production of BK(1–8) was greatly reduced by the carboxypeptidase inhibitor MERGEPTA (Table 4). Carboxypeptidase M should therefore be responsible for the liberation of *C*-terminal Arg from bradykinin. The only enzyme known to cleave the *N*-terminal Arg-Pro-bond in bradykinin is aminopeptidase P. No specific inhibitor is known, but very high concentrations of the angiotensin-converting enzyme inhibitor enalaprilate (but not captopril) are known to reduce its activity (13). Because such inhibitory profile was observed the BK(2–9) formation (Table 4), aminopeptidase P should be present on the surface of A7r5 cells. To verify this, aminopeptidase P was solubilized from A7r5 membranes by incubation with phosphatidylinosit-specific phospholipase C and partiallly purified. Gel chromatography yielded a molecular mass of 190 kDa. This enzyme preparation liberated Arg from BK.

The main fragment obtained after incubation of AT II with A7r5 cells was AT II(5–8) (Fig. 2, Table 3). The corresponding fragment AT II(1–4) was obtained in lower yield (probably due to higher degradation by aminopeptidases). The cleavage of the Tyr-Ile bond of AT II is known to be accomplished by several endopeptidases (Fig. 1), but only endopeptidase-24.11 inhibitors were effective suppressors for AT II(1–4) and AT II(5–8) production (Table 5). Beside endopeptidase-24.11, three exopeptidases are important for the metabolism of AT II by A7r5 cells. AT II(1–7) and Phe (detected in HPLC runs by the absorbance of this aromatic amino acid at 220 nm) as cleavage products of the *C*-terminal Pro-Phe bond were observed in considerable amounts. Because this prolyl bond is resistant to any metallocarboxypeptidase except carboxypeptidase P, this highly specific enzyme must be involved in AT II degradation by A7r5 cells (lysosomal prolyl carboxypeptidase can be excluded, because it is a serine-, not metallo-enzyme). In accordance with previous reports on the inhibitor spectrum of carboxypeptidase P (9), formation of AT II(1–7) and Phe by A7r5 cells was inhibited by phenanthroline, but not by MERGEPTA (Table 5); more specific inhibitors are unknown. The generation of other AT II fragments, AT II(2–8), AT II(3–8), AT II(4–8), and AT II(6–8), were inhibited by amastatin and bestatin (Tables 3 and 5). This shows that aminopeptidases A and N in succession significantly contribute to the degradation of AT II in A7r5 cells. In experiments with radioiodinated AT II (picomolar concentrations) two main radioactive degradation peaks were observed (not shown) whose generation could be inhibited by the endopeptidase-24.11 inhibitor phosphoramidon. Endopeptidase-24.11 could be directly measured in the cultures with [D-Ala^2]enkephalin as an established substrate (0.9 μmol h^{-1} mg protein^{-1}).

In conclusion, the vasoactive peptides BK and AT II are rapidly degraded in A7r5 smooth muscle cells by the endopeptidase-24.11 and to a lesser extend by the exopeptidases aminopeptidase P, carboxypeptidase P (which are both selective for prolyl-bonds), aminopeptidases A and N, as well as carboxypeptidase M.

Identification of Peptidases Involved in Degradation of NPY and CGRP

Due to the much lower degradation rates (and cleavage to many products) compared with AT II and BK, fragments of unlabeled NPY and CGRP could not be obtained after incubation with intact A7r5 cells in sufficient yield to allow sequencing. Only free Tyr as the major degradation product of unlabeled NPY was clearly detected by cochromatography with a standard (not shown). Therefore, the peptidases involved in degradation of NPY or CGRP were identified by the effects of inhibitors on the metabolism of the radioiodinated peptides.

In NPY five tyrosine residues can be radioiodinated (Fig. 3, bold letters). With polyiodinated NPY (not shown) or Tyr^1-

TABLE 3

FRAGMENTS PRODUCED BY DEGRADATION OF BRADYKININ (BK) AND ANGIOTENSIN II (AT II) BY A7r5 SMOOTH MUSCLE CELLS IN CULTURE AND MEMBRANES

Peptide/Fragment	Retention Time (min)	Yield* (%)		Inhibited by†
		Cells	Membranes	
Bradykinin (BK)				
BK (1–9) (complete)	21.5	66	66	—
BK (2–9)	23.1	2	1	enalaprilate
BK (1–8)	22.0	1	6	MERGEPTA
BK (1–7)	18.8	26	20	phosphoramidon, thiorphan
BK (5–7)	17.0	n.d.	1	phosphoramidon, thiorphan
BK (8–9)	13.4	3	5	phosphoramidon, thiorphan
BK (1–4)	11.8	2	1	phosphoramidon, thiorphan
Angiotensin II (AT II)				
AT II (1–8) (complete)	23.0	66	80	—
AT II (2–8) (AT III)‡	23.5	4	1	amastatin
AT II (4–8)	22.5	5	2	amastatin, bestatin
AT II (5–8)	21.1	12	7	phosphoramidon, thiorphan
AT II (1–7)	19.6	3	4	phenanthroline
AT II (6–8)	18.4	3	2	amastatin, bestatin
AT II (1–4)	16.7	2	3	phosphoramidon, thiorphan
AT II 8 = Phe	14.7	5	1	phenanthroline

All fragments were identified by correct amino acid composition and cochromatography with synthetic standards.

* Yield = peak areas (sum 100) after incubation of 10^6 cells with 10 μM of peptides for 30 min or 16 μg membrane protein with 20 μM of peptides for 2 h at 37°C.

† Compare Tables 4 and 5 and text.

‡ AT II (3–8) (AT IV) has identical retention time.

n.d., not detectable because of impurity released by cells.

monoradioiodinated NPY (Fig. 4) one main and several minor degradation products were detected after incubation with A7r5 cells. The main product was identified by cochromatography as [^{125}I]Tyr. Free Tyr may result from combined action of endo- and exopeptidases or by aminopeptidase liberation of *N*-terminal Tyr. Because proline is in penultimate *N*-terminal position, we evaluated whether aminopeptidase P as the only exopeptidase acting on such sequence might be involved in NPY degradation. Liberation of Tyr was completely blocked by incorporation of a metallo-chelator and partly by Arg-Pro-Pro [BK(1–3)] and Tyr-Pro, which act as competitive substrates (Table 6). The involvement of the metallo-aminopeptidase P could be verified by its partial purification from A7r5 membranes and incubation with NPY. Only Tyr was released from NPY, and after prolonged incubation NPY(2–36) could be analyzed (amino acid composition; NPY does not separate from the truncated fragement in the HPLC system used). The other, minor degradation peaks must result from cleavage by a metallo-endopeptidase (inhibition by phenanthroline, Table 6), which could not be further identified.

In CGRP only one histidine can be radioiodinated (Fig. 3, bold letters). Two main radioiodinated degradation peaks were detected when the peptide was incubated with A7r5 cells (Fig. 5). One was identified by cochromatography as [^{125}I]His. The generation of both peaks was abolished or significantly reduced by the endopeptidase-24.11 inhibitors phosphoramidon or thiorphan (Table 6). Therefore, endopeptidase-24.11 must be one of the peptidases responsible for the degradation of CGRP by A7r5 cells. However, the generation of free His cannot be explained by action of this peptidase alone, because endopeptidase-24.11 produces from CGRP as the smallest fragment only a His-containing tetrapeptide (Fig. 3). Incorporation of bestatin allows to test whether the combined cleavage of endopeptidase-24.11 followed by that of aminopeptidase N was responsible for the sequential release of free His. Indeed, bestatin blocked most of the formation of free His, but did not influence the other radiolabeled peak (Fig. 5).

In conclusion, NPY is primarily degraded by aminopeptidase P and CGRP by endopeptidase-24.11 in cultivated A7r5 smooth muscle cells. A further metallo-endopeptidase acts on NPY, and CGRP fragments are further catabolized by aminopeptidase N.

DISCUSSION

A variety of endocrine (AT II, vasopressin), paracrine (BK, endothelin), or neurogenic (NPY, CGRP, substance P, neurokinin A, vasoactive intestinal peptide) peptides participate in the modulation of peripheral vascular tone (10,31). We selected AT II, BK, NPY, and CGRP and the rat aortic embryonic cell line A7r5 as models for a search of peptide-metabolizing enzymes at the surface of smooth muscle cells. However, it should be kept in mind that the data obtained with aortic smooth muscle cells may not apply to smaller resistance vessels. Peptidases or their cleavage sites may be potential targets for the development of inhibitors as vasoactive drugs or for metabolically stable peptide agonists. Deficiency for peptidases could be related to augmented vasoconstrictor effect or hypertension.

With a minor exception peptide degradation could be completely traced by the action of previously known, but partly less characterized, membrane-bound surface peptidases. As found with other peptide target cells lysosomal/endosomal contribution was not relevant for their catabolism (19). Endopeptidase-24.11 was identified as the main degrading enzyme for BK, AT II, and CGRP. This enzyme, which has been localized on the surface of

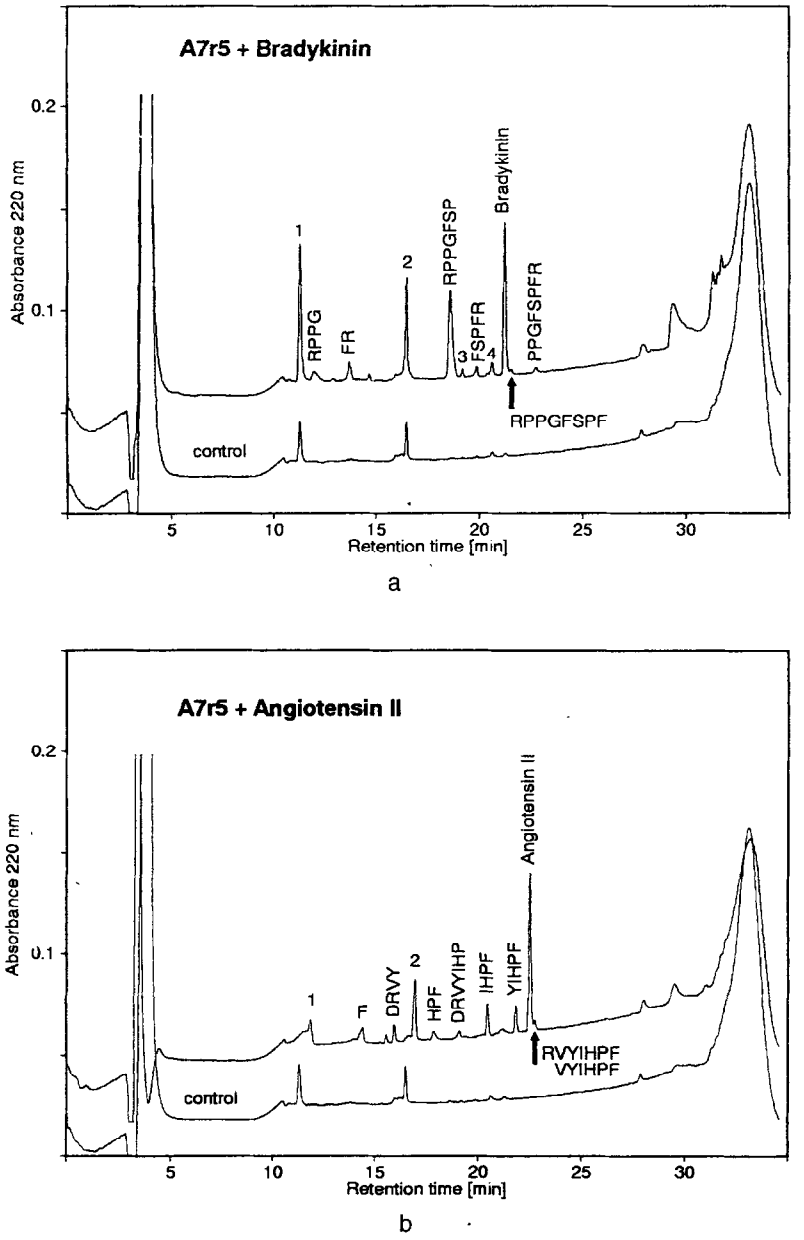

FIG. 2. Chromatographic separations of peptide fragments from (A) bradykinin or (B) angiotensin II obtained after incubation with A7r5 vascular smooth muscle cells. Cells (10^6) were incubated without (control) or with 10 μM of the peptides in synthetic medium, and after 30 min aliquots of the supernatants analyzed by reversed-phase HPLC as described under the Method section. Peptide fragments in the eluate detected by their absorbance at 220 nm were identified by amino acid composition and standards. 1 and 2 are impurities (compare controls), 3 and 4 unidentified fragments.

epithelial, endocrine, connective tissue, and Schwann cells, subpopulations of neurons and of B cells (16), as well as on smooth muscle cells (4,38), cleaves 5–37 amino acid residue peptides at bonds involving preferentially hydrophobic residues (e.g., natriuretic peptides, endothelins, substance P, BK, enkephalin) (16). The action on AT I, II, and III (35) as well as CGRP (18) has been reported, but not been shown to be relevant in cellular systems. On the level of vascular smooth muscle cells endopeptidase-24.11 degrades vasodilatory peptides like BK, CGRP (Fig. 6) and substance P (38) as well as the vasoconstrictory peptide AT II (Fig. 7). In any case, degradation results in a complete inactivation of these peptides, because all fragments are too small to interact with their receptors.

Apart from endopeptidase-24.11, several highly or less specialized exopeptidases are involved in the vascular smooth muscle cell metabolism of BK, AT II, CGRP, and especially NPY. Peptide bonds involving proline residues are often resistant to the attack of proteases due to its unique cyclic and imino structure (23). In particular, sequences with proline in the penultimate *N*- or *C*-terminal positions prevent the cleavage by nonspecialized

TABLE 4

INFLUENCE OF INHIBITORS ON THE DEGRADATION OF BRADYKININ (BK) BY CULTIVATED A7r5 SMOOTH MUSCLE CELLS

Inhibitor [I]	[I] (m*M*)	Residual Peak Area (Control = 100) of BK (1–7)	(8–9)	(1–4)	(1–8)	(2–9)	Inhibitor Spectrum
Bestatin	0.10	103	100	100	100	100	APN
Amastatin	0.05	n.d.	128	120	120	100	APN, APA
Captopril	0.10	92	97	105	90	120	ACE
Enalaprilate	0.10	108	100	100	105	85	ACE
	1.00	117	116	100	97	**35**	ACE, APP
MERGEPTA*	0.10	100	100	100	**20**	100	CPM, CPB
Phosphoramidon	0.05	**6**	**0**	**5**	76	n.d.	E-24.11
Thiorphan	0.05	**9**	**14**	**0**	130	68	E-24.11
Cpp-AAF-4-ab†	0.10	100	100	100	100	100	E-24.15/16

More than 60% inhibition is given in bold numbers. For abbreviations of proteases as targets of inhibitors see Fig. 1. Values are means of duplicate measurements from two individual cultures.

* MERGEPTA, mercaptomethyl-3-guanidino-ethylthiopropanoic acid.

† Cpp-AAF-4-ab, *N*-[1-(*R*, *S*)-carboxy-3-phenyl-propyl]Ala-Ala-Phe-4-aminobenzoate; this inhibitor is cleaved during incubation as a substrate for E-24.11; compare (39).

n.d., not detectable (interference with inhibitor).

exopeptidases. *N*-terminal Arg-Pro-Pro- in BK, Tyr-Pro-Ser- in NPY, or *C*-terminal -His-Pro-Phe in AT II are such protective structures. However, proline-containing sequences can be overcome by certain specialized proline-specific/-selective enzymes (23), namely aminopeptidase P (cleaving Xaa--Pro-), dipeptidyl peptidases II and IV (Xaa-Pro/Ala--Yaa-, Yaa not Pro), or carboxypeptidase P and lysosomal prolyl carboxypeptidase (-Pro/Ala--Xaa, Xaa Pro). Whereas dipeptidyl peptidase II and prolyl carboxypeptidase are soluble, lysosomal enzymes, the three others, are plasma membrane bound and therefore can act on intercellular signal molecules. Dipeptidyl peptidase IV (CD 26), which was absent in vascular smooth muscle cells, participates as a regulatory protease at the surface of endothelial and other cells in the degradation of bioactive peptides like substance P (11,38), NPY, peptide YY (26), or subsequent to action of aminopeptidase P on BK (30).

Purified aminopeptidase P liberates the *N*-terminal residues from BK and NPY (8,25,30), but this has not been shown to be relevant in cellular systems. The enzyme is anchored in plasma membranes by a covalently attached glycosyl-phosphatidyl inositol (GPI) moiety, but also soluble forms of the enzyme exist (8,33). Aminopeptidase P of vascular smooth muscle cells is of the GPI-anchored type, because it could only be solubilized by a GPI-specific phospholipase C. It purifies as a dimer [monomer reported to be 80–95 kDa depending on species and/or glycosylation (33)]. The enzyme has been suspected to participate in BK metabolism by vascular endothelium (30,32), and this physiological function can now be extended to smooth muscle cells. Liberation of *N*-terminal Arg from BK suffices to eliminate its biological activity to all receptor subtypes (31). However, aminopeptidase P appears less important than endopeptidase-24.11 in the inactivation of the vasodilatory BK.

TABLE 5

INFLUENCE OF INHIBITORS ON THE DEGRADATION OF ANGIOTENSIN II (AT II) BY CULTIVATED A7r5 SMOOTH MUSCLE CELLS

Inhibitor [I]	[I] (m*M*)	Residual Peak Area (Control = 100) of AT II (1–4)	(5–8)	(1–7)	Phe	(2–8)	Inhibitor Spectrum
Phenanthroline	1.00	**5**	**10**	**5**	**5**	**5**	Metallo-proteases
Bestatin	0.10	120	120	90	90	80	APN
Amastatin	0.10	105	120	100	80	**20**	APA, APN
Enalaprilate	0.10	100	n.d.	n.d.	110	115	ACE
MERGEPTA*	0.10	100	101	95	115	115	CPM, CPB
Phosphoramidon	0.05	**5**	n.d.	110	80	120	E-24.11
Thiorphan	0.05	**10**	**30**	130	80	120	E-24.11
Cpp-AAF-4-ab†	0.10	100	100	100	100	100	E-24.15/16

More than 70% inhibition is given in bold numbers. For abbreviations of proteases as targets of inhibitors see Fig. 1. Values are means of duplicate measurements from two individual cultures.

* MERGEPTA, mercaptomethyl-3-guanidino-ethylthiopropanoic acid.

† Cpp-AAF-4-ab, *N*-[1-(*R*, *S*)-carboxy-3-phenyl-propyl]Ala-Ala-Phe-4-aminobenzoate; this inhibitor is cleaved during incubation as a substrate for E-24.11; compare (39).

n.d., not determined (interference with inhibitor).

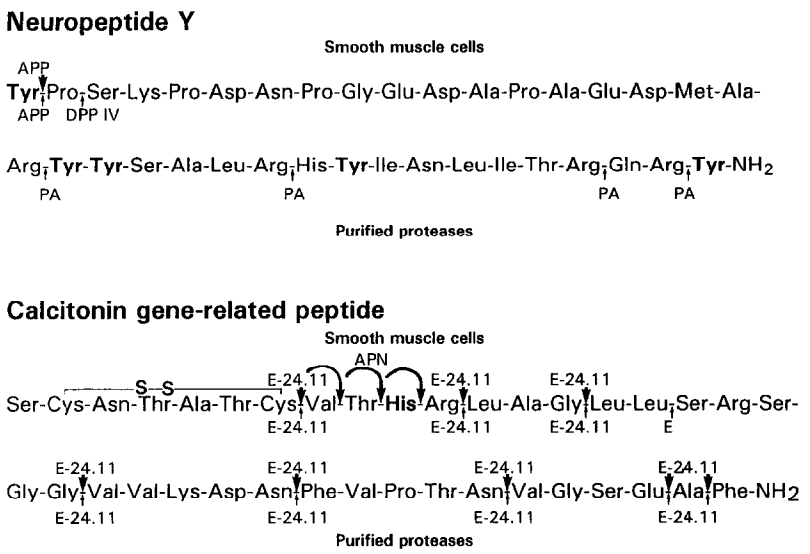

FIG. 3. Cleavage sites of neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) by peptidases of cultivated A7r5 smooth muscle cells and by purified peptidases. APP, aminopeptidase P; APN, aminopeptidase N; DPP IV, dipeptidyl peptidase IV. E-24.11, endopeptidase-24.11; PA, urokinase-type or tissue-type plasminogen activator (trypsin cleaves at same sites); E, undefined endopeptidase (substance P-degrading endopeptidase). Tyr residues radioiodinated in NPY and His residue radioiodinated in CGRP are indicated in bold. Data for purified proteases on NPY from (21,26) and CGRP from (3,18).

In contrast to this, NPY is inactivated by aminopeptidase P only with regard to one receptor subtype and turns into a natural agonist of other subtypes. NPY(2–36) is about 50-fold less potent to interact with the Y_1 receptor subtype, but nearly equally active on Y_2 receptors (and probably Y_3 receptors) than the complete peptide (7). The Y_1 receptor is the major vascular NPY receptor (6,37) by which NPY directly constricts, potentiates vasoconstriction evoked by noradrenaline, and exerts mitogenic effects on vascular smooth muscle cells (40). In particular, NPY(2–36) is nearly two magnitudes of orders less potent than NPY to increase arterial pressure in the anaesthetized rat or to displace the radioiodinated peptide from rat aortic smooth muscle cells (6). Thus, aminopeptidase P inactivates NPY concerning

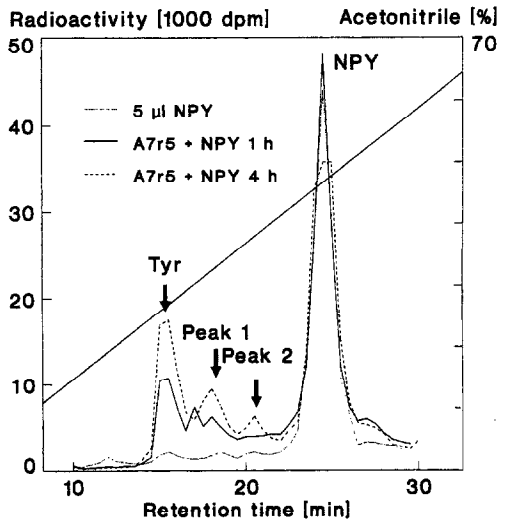

FIG. 4. Cleavage of [^{125}I-Tyr^1]neuropeptide Y (NPY) by cultivated rat A7r5 smooth muscle cells. After 1 h or 4 h of incubation cell supernatants were separated by reversed-phase HPLC, fractions of 0.5 ml collected, and radioactivity measured.

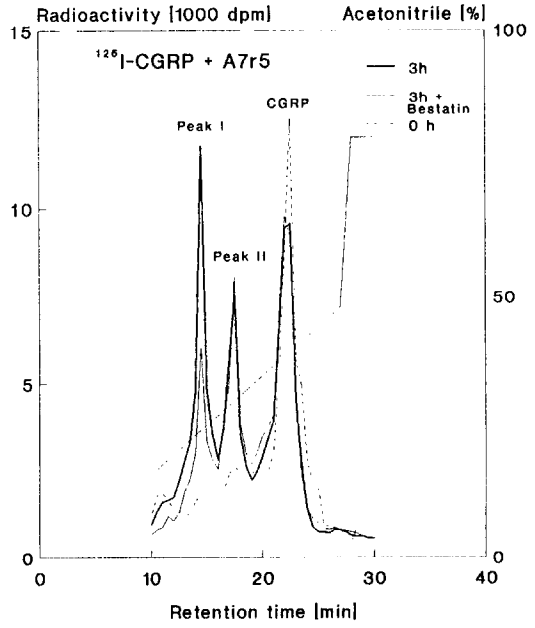

FIG. 5. Cleavage of radioiodinated calcitonin gene-related peptide (CGRP) by cultivated rat A7r5 smooth muscle cells. After 3 or 0 h of incubation in the absence or presence of the aminopeptidase inhibitor bestatin (3 h + BE) cell supernatants were separated by reversed-phase HPLC, fractions of 0.5 ml collected, and radioactivity measured. Peak I corresponds to radioiodinated histidine and peak II to an endopeptidase-24.11 fragment, probably CGRP(8–11) (compare Fig. 3).

TABLE 6

INFLUENCE OF INHIBITORS ON DEGRADATION OF [^{125}I-Tyr^1]NEUROPEPTIDE Y (NPY) AND [^{125}I]CALCITONIN GENE-RELATED PEPTIDE (CGRP) BY CULTIVATED A7r5 SMOOTH MUSCLE CELLS

Inhibitor [I]	[I] (m*M*)	Residual Peak Area (control = 100) NPY [^{125}I]Tyr	NPY Peak 1*	CGRP Peak I†	CGRP Peak II†	Inhibitor Spectrum
Phenanthroline	1	**0**	**0**	**0**	**0**	Metallo-proteases
Leupeptin	0.1	92	100	105	110	Ser-/Cys proteases
Bestatin	0.1	80	120	**29**	98	APN
Arg-Pro-Pro	1	**41**	56	n.d.	n.d.	APP
Tyr-Pro	1	**40**	90	n.d.	n.d.	APP
Phosphoramidon	0.05	90	90	**7**	**32**	E-24.11
Thiorphan	0.05	100	100	**12**	**28**	E-24.11

More than 60% inhibition is given in bold numbers.
* Compare Fig. 4; peak 2 was inhibited only by phenanthroline.
† Peak I = [^{125}I]His; compare Fig. 5.
n.d., not determined. For abbreviations of proteases as targets of inhibitors see Fig. 1.

its direct, postjunctional effects mediated by Y_1 receptors on vascular smooth muscle cells, but does not affect its prejunctional effects mediated by Y_2 receptors. The Y_2 receptors are located on the sympathetic nerves innervating the vasculature. Their activation results in suppression of stimulated noradrenaline and NPY release from the sympathetic nerves and thus exerts a negative feedback effect. In a simplified model aminopeptidase P would terminate the vasoconstriction after release of NPY at two stages (Fig. 8): NPY is inactivated in its direct, vasoconstrictive effects of smooth muscle cells, but the inactivation product NPY(2–36) still reduces the further release of NPY and noradrenaline. In conclusion, smooth muscle aminopeptidase P abolishes the vasoconstrictory action of NPY and potentiates its vasodilatory effect.

Although NPY functions largely in a paracrine fashion, it is released into the blood under certain clinical conditions (15,40). It has not yet been determined whether immunoreactive NPY in plasma consists of *N*-terminal truncated forms such as NPY(2–36) or NPY(3–36) (which should be considered in immunoassays). The fomer would result from the action of vascular smooth muscle aminopeptidase P, the later by endothelial or soluble plasma dipeptidyl peptidase IV (compare Fig. 8), which is far more active on NPY (26). Both proline-specific aminopeptidases should be important regulators of Y_1 reactive NPY levels and may be implicated in certain clinical conditions such as hypertension.

Carboxypeptidase P is a rarely investigated integral membrane metallo-enzyme detected previously in renal and in-

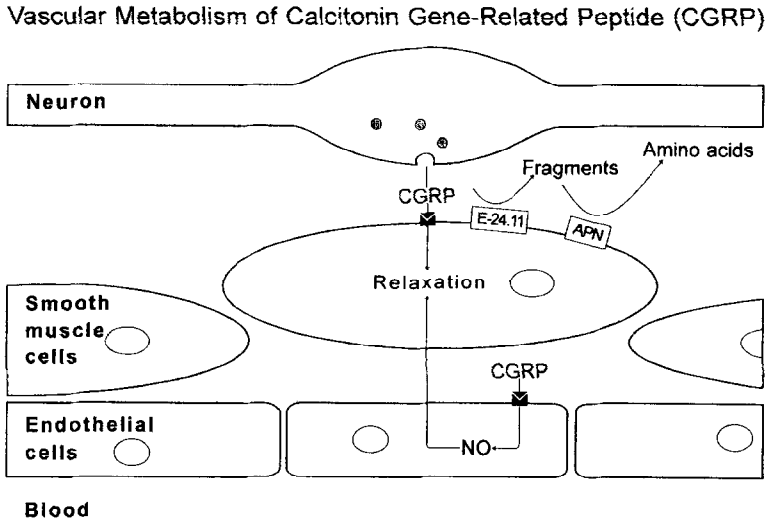

FIG. 6. Simplified scheme for the vascular metabolism of calcitonin gene-related peptide (CGRP). CGRP released from perivascular parasympathetic nerves at arteries and arterioles causes vasodilation by direct action on vascular smooth muscle cells and by NO synthase stimulation in endothelial cells. CGRP is inactivated by smooth muscle endopeptidase-24.11 (E-24.11) and fragments are further hydrolyzed by aminopeptidase N (APN).

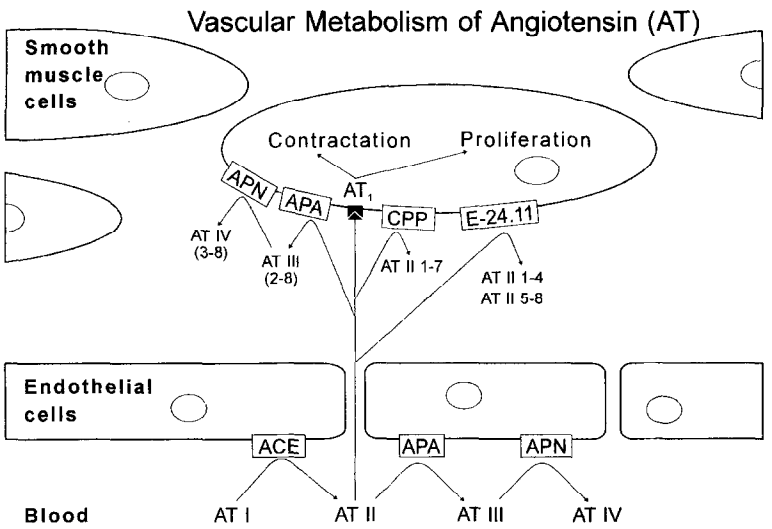

FIG. 7. Simplified scheme for the vascular metabolism of angiotensin (AT). AT I produced from angiotensinogen by renin is metabolized to active AT II by endothelial angiotensin-converting enzyme (ACE). AT III and AT IV are further biologically (on different receptors) active fragments produced by aminopeptidases A (APA) or N (APN) at endothelial or smooth muscle cells. Endopeptidase-24.11 (E-24.11) and carboxypeptidase P (CPP) at the surface of smooth muscle cells metabolize AT II to inactive [or in case of AT II(1–7) receptor specificity altered?] fragments.

testinal brush border membranes (22). Although the action of purified pig kidney carboxypeptidase P on AT II and III has been demonstrated (9), its significance has been obscure. Because *C*-terminal truncated AT II and III are ineffective at most, but not all, receptor subtypes (31), liberation of *C*-terminal Phe by smooth muscle cells should inactivate or modulate AT II. Carboxypeptidase P may also cleave BK after removal of *C*-terminal Arg by carboxypeptidase M.

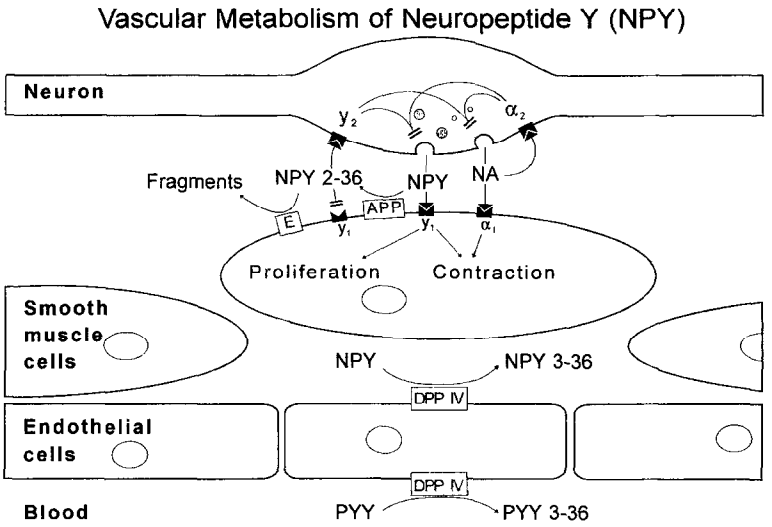

FIG. 8. Simplified scheme for the vascular metabolism of neuropeptide Y (NPY). NPY is released from perivascular sympathetic nerves and acts via Y_1 receptors on smooth muscles cells (contraction, proliferation) and Y_2 autoreceptors on the sympathetic neuron (inhibition of NPY and noradrenaline = NA release; NA inhibits in turn NPY and NA release by prejunctional α_2 receptors). Aminopeptidase P (APP) terminates the action of NPY on smooth muscle cells, but not neurons, which results in a prolonged reduction of vascular active NPY [by inactivation and by suppression of further release through the newly generated Y_2 agonist NPY(2–36)]. An unidentified metallo-endopeptidase metabolizes NPY or NPY(2–36). Endothelial cells cleave NPY or the structurally related peptide hormone peptide YY (PYY) by dipeptidyl peptidase IV [compare (26)].

Aminopeptidase N, A, and carboxypeptidase M are further surface peptidases of smooth muscle cells, which are implicated in initial or in subsequent degradation steps of vasoactive peptides. Aminopeptidase N liberates *N*-terminal amino acids (acidic ones and Pro with only low activities) from peptides up to about 30 residues provided that they are unblocked, have no Pro in the penultimate position, or a *N*-terminal cystine ring (12,22,29). Therefore, smooth muscle aminopeptidase N does not initially attack the vasoactive peptides investigated, but cleaves fragments generated by other peptidases (AT II truncated by aminopeptidase A, CGRP fragments produced by endopeptidase-24.11).

Carboxypeptidase M, previously detected in membranes from placenta, lung, and kidney, anchored probably by a GPI moiety (1), is structurally, immunologically, and catalytically distinct from carboxypeptidase N (kininase I), which is a soluble, serum, or cytosolic glycoprotein. However, both carboxypeptidases liberate *C*-terminal basic residues from peptides (16). Carboxypeptidase N has been shown to be responsible for the generation of [des-Arg^9]BK by human plasma. We now demonstrate that at smooth muscle cells carboxypeptidase M is present and takes the same role. [des-Arg^9]BK is a far better ligand for BK_1 receptors than intact BK, and the reverse is true for the BK_2 receptor subtype (31). Whereas BK_2 receptors are quite widely distributed in many tissues, BK_1 receptors are found primarily in the vasculature. Therefore, with respect to its direct vasoactive effects, the metabolism of BK by carboxypeptidase M is not an inactivation, but an activation. [des-Arg^9]BK may be further metabolized and thereby inactivated by endopeptidase-24..11, aminopeptidase P, or carboxypeptidase P at the surface of smooth muscle cells.

Conclusions

Vasoactive peptides are metabolized by more or less specialized endo- and exopeptidases at the surface of smooth muscle cells. Endopeptidases, especially endopeptidase-24.11, are involved in the final inactivation of these peptides, whereas exopeptidases differentially activate or inactivate them. By proceeding together, these proteases have a complex influence on the level of biologically active vasodilatory and vasoconstrictory peptides.

ACKNOWLEDGEMENTS

We thank Dagmar Freier and Martina Burmester for their expert technical assistance and Clemens Franke for drawing the figures. This work was supported by grant Me 758/2-4 from the Deutsche Forschungsgemeinschaft.

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