0026-895X/92/040609-06$03.00/0

MOLECULAR PHARMACOLOGY, 41:609-614

A 94-kDa Protein, Identified as Neutral Endopeptidase-24.11, Can Inactivate Atrial Natriuretic Peptide in the Vascular Endothelium

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Received August 7, 1991; Accepted January 15, 1992

SUMMARY

Neutral endopeptidase 24.11 (EC 3.4.24.11) inactivates atrial natriuretic peptide by cleaving the hormone between Cys_7 and Phe_8, and inhibitors of the enzyme have consequent natriuretic and diuretic properties. The *in vivo* sites of degradation of this peptide by the zinc-metallopeptidase, however, remain to be established. Because an endopeptidase-24.11-like activity has recently been reported in the rat mesenteric artery, we have further investigated the degradation of atrial natriuretic peptide in vascular tissue. Endopeptidase-24.11 activity was detected in solubilized membrane preparations from rat and rabbit vascular tissue, using [^{3}H]D-Ala^2-leucine enkephalin as substrate, and both rabbit and rat aorta preparations were also found to cleave atrial natriuretic peptide between Cys_7 and Phe_8. In both cases, hydrolysis was inhibited by neutral endopeptidase inhibitors, with K_i values close to their K_i values for the pure enzyme. In preparations of rabbit aorta denuded of endothelium by saponin treatment, the hydrolysis of the Gly_3-Phe_4 bond of [^{3}H]D-Ala^2-leucine enkephalin and the Cys_7-Phe_8 bond of atrial natriuretic peptide was reduced by >90%. The high performance liquid chromatography method used to follow the degradation of atrial natriuretic peptide differed from previously published procedures, in that samples to be injected were first treated with excess dithiothreitol to reduce the Cys_7-Cys_{23} disulfide bridge. This facilitated the separation of the intact peptide and its metabolites. The presence of the 94-kDa neutral endopeptidase in rabbit aortic tissue was definitively established using a new potent ^{125}I-labeled inhibitor, [^{125}I]RB104 [2-[(3-[^{125}I]iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-hydroxyamino-3-oxo-1-phenylmethyl propyl]amino-4-oxobutanoic acid] (K_i, 30 pM), which selectively labeled the enzyme after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the membrane preparations. Therefore, despite its low concentrations in the vasculature, the presence of endopeptidase-24.11 almost exclusively in endothelial tissue suggests that the enzyme is ideally localized to inactivate circulating atrial natriuretic peptide.

ANP is a 28-residue peptide hormone that is involved in cardiovascular homeostasis and, in the rat, has the sequence SLRRSSCFGGRIDRIGAQSGLGCNSFRY. This sequence is highly conserved between species (reviewed in Refs. 1 and 2), and the integrity of the 17-residue ring formed by the disulfide bridge between Cys_7 and Cys_{23} is essential for biological activity. $rANP_{1-28}$ and $\alpha hANP_{1-28}$ differ only in the residue at position 12, which is isoleucine in the former peptide and methionine in the latter.

Numerous tissues have been found to synthesize ANP (3), but it is primarily released into the circulation from the atrial myocytes, and its plasma levels are elevated in congestive heart failure, kidney failure, and essential hypertension. Specific ANP receptors have been detected in diverse tissues such as the kidney, where ANP increases natriuresis and diuresis and inhibits renin release, the adrenals, where it inhibits aldosterone synthesis and release, and the vasculature, where it acts as a vasorelaxant. Other regions with high receptor densities include the choroid plexus and the subfornical organ. The peptide and its receptors have also been found in the central nervous system (1, 3).

There appear to be two distinct mechanisms responsible for the short half-life of the peptide *in vivo*, reported as being <1 min in rats (4) and up to 3 min in humans (5). The first involves the ANP_C, or "clearance" receptor, which is the most abundant ANP receptor in many tissues, including the kidney and the

This work was partly founded by a grant from Rhône Poulenc Rorer.

ABBREVIATIONS: ANP, atrial natriuretic peptide; RB104, 2-[(3-iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-hydroxyamino-3-oxo-1-phenylmethyl propyl] amino-4-oxobutanoic acid; NEP, neutral endopeptidase-24.11; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; rANP, rat atrial natriuretic peptide; hANP, human atrial natriuretic peptide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; R_t, retention time.

vasculature. Peptide bound to ANP_C is rapidly internalized and, after dissociation of the receptor-peptide complex, is degraded in the lysosomes; the receptor is recycled to the cell surface (6).

ANP is also metabolized extracellularly by an ectoenzyme, NEP (EC 3.4.24.11). *In vitro*, this zinc-metallopeptidase has been shown to cleave rANP and αhANP preferentially between Cys_7 and Phe_8, thereby opening the disulfide-linked ring and inactivating the peptide (reviewed in Ref. 7). The importance of NEP in ANP metabolism *in vivo* has been demonstrated by numerous studies showing that NEP inhibitors, in both rats and humans, potentiate the half-life of exogenous ANP and its natriuretic and diuretic actions (8, 9). In addition, in some animal models of hypertension and in a few limited studies in humans, NEP inhibitors alone have been shown to raise basal ANP levels and to have consequent natriuretic and diuretic properties. This has led to considerable interest in these molecules as potential antihypertensive agents (reviewed in Ref. 10). Although the role of NEP in ANP metabolism seems to be clearly established, its sites of action are less clear. The enzyme has a wide tissue distribution, and highest levels are found in the kidney (11), where a high concentration of ANP receptors are also located (1, 2). However, these receptors are mainly concentrated in the glomeruli, whereas NEP is predominantly found on the apical side of the brush border membranes of the proximal tubules. In addition, nephrectomy has been found to have little effect on the half-life of ANP (12, 13). Because the vascular endothelium is one of the major targets of ANP and an NEP-like activity has been reported in the rat mesenteric artery (14), we have further investigated the degradation of ANP and, the possible presence of NEP in vascular tissue, in particular, by using a newly developed radioiodinated NEP inhibitor, RB104, which has a very high affinity (K_i, 30 pM) and selectivity for the enzyme (15).

Materials and Methods

$\alpha hANP_{1-28}$ was purchased from Neosystem Laboratories (Strasbourg, France), and the NEP inhibitors thiorphan and (*R*)-retrothiorphan were synthesized in our laboratory as previously described (16, 17). $rANP_{1-28}$, $rANP_{1-7}$, and $rANP_{8-28}$ were obtained by solid-phase synthesis in our laboratory, following 9-fluorenylmethoxycarbonyl chemistry. The synthesis and iodination (2100 Ci/mmol) of the NEP inhibitor RB104 were as described (15). Other products were from Sigma (la Verpillere, France). Male Sprague Dawley rats (125 g) and New Zealand rabbits (2.5 kg) were supplied by Depré (Saint Doulchard, France) and the Centre d'Elevage Contigné (Bray-Lu, France), respectively. Healthy human ileac arterial tissue was removed at post-mortem.

Membrane preparations. Membrane preparations from rat aorta, vena cava, and mesenteric artery, rabbit aorta, and human ileac artery were prepared essentially as previously described (18). Briefly, tissue that had been washed and cleaned of connective tissue was homogenized in 50 mM Tris·HCl, pH 7.4, and the homogenate was layered over 41% (w/v) sucrose (3 ml of sucrose/2 ml of homogenate) and centrifuged for 1 hr at 90,000 × *g*, in a Beckman SW 60 rotor. Plasma membranes were collected at the Tris/sucrose interface and washed twice in a large volume of buffer. The final pellet was resuspended in Tris buffer, and protein was solubilized by addition of *n*-octylglucoside to a final concentration of 1% (w/v), for a protein concentration of 1 mg/ml. After a 1-hr incubation at 4°, with gentle agitation, the preparation was centrifuged at 100,000 × *g* for 40 min, and the supernatant was stored at −80° until required. No change in enzyme activity was noted after up to 3 months of storage under these conditions.

Endothelium denudation. Rabbit thoracic aorta was denuded of endothelium as previously described (19), with slight modifications. Aortic rings (5 mm) were incubated in 50 mM Tris·HCl, pH 7.4, containing 0.3 mg/ml saponin, for 30 min at 37° in a shaking water bath. The tissue was then washed three times in 50 ml of Tris buffer. Membranes were then prepared as described above. To monitor endothelium removal, some aortic rings were opened, stained with $AgNO_3$ as previously described (20), and examined by light microscopy.

Enzyme purification. Rabbit kidney NEP was purified by affinity chromatography using a monoclonal antibody, as previously described (21).

Enzyme assays. Enzyme activity was assayed using either 20 nM [^{3}H]D-Ala^2-leucine enkephalin or 50 μM ANP as substrate. With the radioactive ligand, incubations were carried out in 100 μl of 50 mM Tris·HCl, pH 7.4, and contained 1 μM levels of the angiotensin-converting enzyme inhibitor captopril and 10 μM levels of the aminopeptidase inhibitor bestatin. The reaction was stopped by addition of 10 μl of 0.5 M HCl, and the product [^{3}H]tyrosyl-alanyl-glycine was isolated as previously described (22). K_m values were measured using D-Ala^2-leucine enkephalin over a 64-fold concentration range, with 20 nM [^{3}H]D-Ala^2-leucine enkephalin included as tracer. With $rANP_{1-28}$ as substrate, incubations were carried out at 37° in 100 μl of 50 mM Tris·HCl, pH 7.4, and normally contained 70 ng of pure rabbit kidney enzyme or between 5 and 10 μg of membrane protein. The reactions were stopped by addition of 5 μl of 0.1 M dithiothreitol, followed by immediate incubation at 85° for 5 min. Intact peptide and products were separated by HPLC using a 300 Å C_8 7-μm Nucleosil cartridge column (30 × 4.6 mm). The buffers used were 0.05% trifluoroacetic acid (solvent A) and 70% acetonitrile in 0.045% trifluoroacetic acid (solvent B), and the peptides were eluted with a gradient of 0–40% solvent B in 20 min, at a flow rate of 1.2 ml/min. The effluent was monitored at 214 nm, and peak areas were calculated by using a Shimadsu C-R4A integrator. K_i values were calculated from the equation $K_i = IC_{50}/1 + [L]/K_m$.

Inhibitor gel electrophoresis. Nonreducing SDS-PAGE was carried out using 9% polyacrylamide gels (70 × 50 × 0.75 mm). After electrophoresis at constant voltage (180 V), the gels were washed twice for 30 min in 50 ml of 10 mM Tris·HCl containing 0.5% CHAPS and finally in Tris buffer containing 0.5% CHAPS, 0.15 M NaCl, and 0.3 M urea. The gels were then incubated with 100 pM [^{125}I]RB104, in the presence or absence of 0.1 mM retrothiorphan, for 1 hr at room temperature. After washing (3 × 50 ml of Tris, 10 min), the gels were placed in sealed plastic bags and placed against Amersham Hyperfilm-β_{max} for 3–4 days at 4°. The films were developed in Kodak LX-24 developer (1.5 min at 20°), fixed in Kodak L-4 fixative, and analyzed by densitometry, using a Biocom 200 image analyzer. Alternatively, the gels were cut into 2-mm slices and the radioactivity was determined directly.

Protein concentration. Protein concentration was measured by the method of Bradford (23).

Results

Hydrolysis of [^{3}H]D-Ala^2-leucine enkephalin by vascular membrane preparations. NEP activity, defined as the hydrolysis of [^{3}H]D-Ala^2-leucine enkephalin inhibited by 1 μM levels of the NEP-inhibitor retrothiorphan, was found in the homogenates of all vascular tissues tested. When plasma membranes were prepared, 40% of this activity was recovered with the plasma membrane fraction, with a purification factor of 4, and 95% of this activity was recovered in the supernatant after solubilization with octylglucoside, with a final purification factor of 20.

Similar activity was observed in solubilized membranes from rat and rabbit aorta and rat vena cava, with 2–4-fold less activity being found in the rat mesenteric and human ileac arterial preparations (Table 1). Membranes prepared from the

TABLE 1

NEP activity in solubilized vascular membrane extracts

NEP activity was measured, as described in Materials and Methods, using 20 nM [^{3}H] D-Ala^2-leucine enkephalin as substrate. Blank tubes contained 1 μM retrothiorphan.

Tissue	NEP activity
	pmol/mg/min
Rabbit aorta	
Thoracic	0.110 ± 0.020
Thoracic, minus endothelium	0.005 ± 0.001
Abdominal	0.235 ± 0.026
Rat aorta	0.126 ± 0.015
Rat mesenteric artery	0.039 ± 0.005
Rat vena cava	0.124 ± 0.013
Human ileac artery	0.066 ± 0.013

TABLE 2

K_i values of the NEP inhibitors thiorphan and retrothiorphan and the K_m of D-Ala^2-leucine enkephalin for rat and rabbit aorta and pure rabbit kidney NEP

NEP activity was measured as described in Materials and Methods, using 20 nM [^{3}H] D-Ala^2-leucine enkephalin as substrate or as tracer when D-Ala^2-leucine enkephalin was used as substrate. Blank tubes contained 1 μM retrothiorphan.

Enzyme source	K_i		K_m, D-Ala^2-leucine enkephalin
	Thiorphan	Retrothiorphan	
	nM		*μM*
Rabbit aorta	1.6 ± 0.3	1.6 ± 0.3	50.0 ± 5
Rat aorta	1.2 ± 0.5	2.0 ± 0.1	44.0 ± 4
Rabbit kidney NEP	2.0 ± 0.3	1.9 ± 0.1	45.0 ± 5

rabbit abdominal aorta had twice the NEP activity of those from the thoracic aorta. The K_i values of the NEP inhibitors thiorphan and retrothiorphan in the rat and rabbit aorta preparations were identical to their K_i values for pure rabbit kidney NEP (Table 2). In addition, the K_m of D-Ala^2-leucine enkephalin in the two aorta preparations was close to its K_m for the pure enzyme (Table 2). In preparations of rabbit thoracic aorta that had been treated with saponin, substrate hydrolysis was <5% of that found in control tissue (Table 1). Saponin has been previously shown to remove the endothelial cell layer of the aorta while guarding the integrity of the underlying vascular smooth muscle (19), and light microscopic examination of the treated tissue confirmed that this had occurred (data not shown). At the concentration used, saponin did not inhibit NEP activity and, using a preparation of rabbit kidney membranes, it was also verified that saponin did not release the normally membrane-bound enzyme into the supernatant.

Hydrolysis of ANP by rabbit aorta membranes. After incubation of rANP$_{1-28}$ with pure rabbit kidney NEP and subsequent reduction by dithiothreitol, as described in Materials and Methods, three major peaks were observed by HPLC (Fig. 1), with R_t values of 6.3, 17.8, and 18.4 min, corresponding to those of the synthetic peptides ANP$_{1-7}$, ANP$_{8-28}$, and reduced ANP$_{1-28}$, respectively. Similar results were found with αhANP (data not shown), confirming that NEP preferentially cleaves both peptides between Cys$_7$ and Phe$_8$. By measuring the formation of ANP$_{1-7}$, the K_m of ANP was found to be 10.6 ± 2.8 μM, and the NEP inhibitor retrothiorphan inhibited hydrolysis with a K_i of 3.1 ± 0.3 nM.

Rabbit aorta membrane preparations were also found to hydrolyze ANP, generating the same peaks on HPLC as found with the pure enzyme, corresponding to ANP$_{1-7}$, ANP$_{8-28}$, and reduced ANP$_{1-28}$ (Fig. 2). The appearance of ANP$_{1-7}$ with time is shown in Fig. 3. A broad peak, appearing concomitantly with

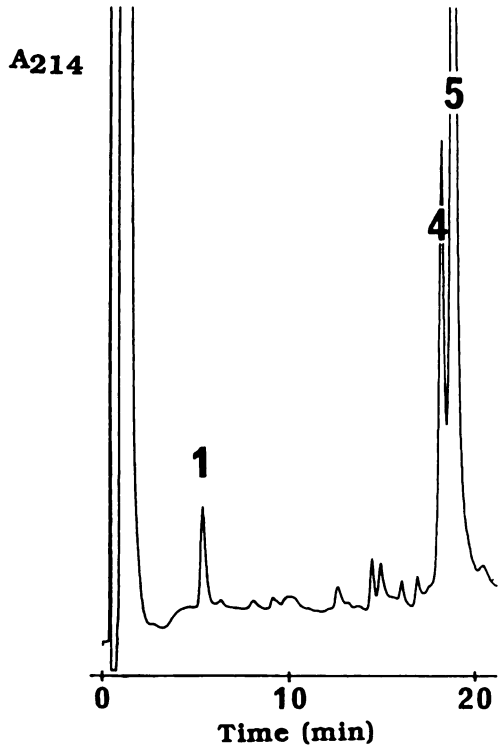

Fig. 1. HPLC profile obtained after incubating 50 μM rANP with pure rabbit kidney NEP, as described in Materials and Methods. Three major peaks were obtained, corresponding to rANP$_{1-7}$ (R_t, 6.1 min) (*peak 1*), rANP$_{8-28}$ (R_t, 18.5 min) (*peak 4*), and reduced rANP$_{1-28}$ (R_t, 19.2 min) (*peak 5*).

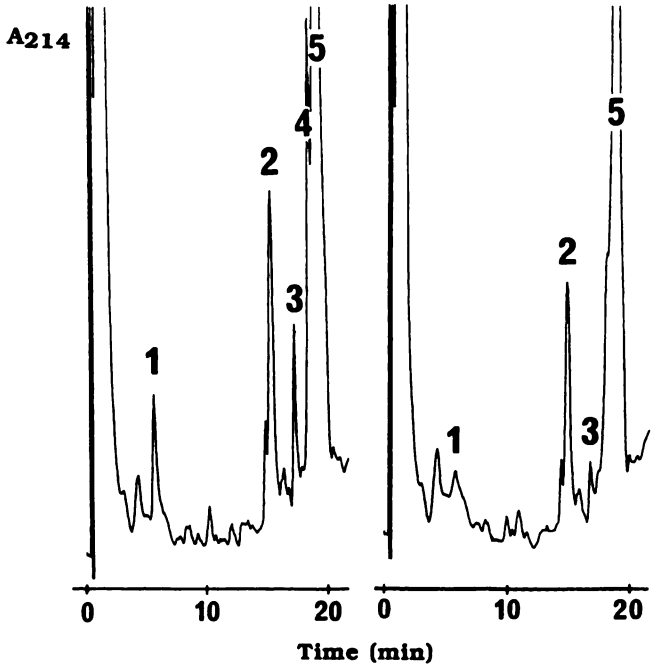

Fig. 2. HPLC profiles obtained after incubating 50 μM rANP with rabbit aorta membranes for 1 hr at 37°, as described in Materials and Methods. *Right*, five major peaks were obtained, which were identified, either by their R_t values or by sequence analysis, as rANP$_{1-7}$ (*peak 1*), rANP$_{1-11}$, rANP$_{17-28}$, and rANP$_{20-28}$ (*peak 2*), rANP$_{10-22}$ (*peak 3*), rANP$_{8-28}$ (*peak 4*), and reduced ANP$_{1-28}$ (*peak 5*). *Left*, the profile obtained when 1 μM retrothiorphan was included in the incubation.

ANP_{1-7} and having a R_t of 15 min, was also observed. An additional peak, with a R_t of 17 min, appeared with longer incubation times (>30 min), and after a 1-hr incubation a small peak with a R_t of 10 min was also observed. This latter peak had the same R_t as a tripeptide standard corresponding to the carboxyl terminus of ANP, Phe-Arg-Tyr. Retrothiorphan (1 μM) inhibited the formation of the peaks corresponding to ANP_{1-7} and ANP_{8-28} and that of the peak at 17 min and partially inhibited (35%) that of the peak at 15 min, whereas the peak at 10 min was unchanged. The K_i of retrothiorphan in inhibiting ANP_{1-7} formation, measured after a 20-min incubation, was 3.5 ± 0.4 nM, a figure close to the value reported above using [^{3}H]D-Ala^2-leucine-enkephalin as substrate.

In tissue that had been pretreated with saponin, the rate of appearance of ANP_{1-7} (Fig. 3), ANP_{8-28}, and ANP_{10-22} was <10% of control values and, again, their formation was inhibited by 1 μM retrothiorphan. The minor peak appearing at 10 min was unaffected by saponin treatment, whereas that at 15 min was reduced by about 45%. Neither of these latter peaks were affected by 1 μM retrothiorphan. ANP_{1-28} hydrolysis was not changed by either 10 μM bestatin or 1 μM captopril, inhibitors of aminopeptidases and angiotensin-converting enzyme, respectively.

Sequence and mass spectrometry analyses showed three major peptides in the broad peak eluting at 15 min. From their amino-terminal sequences, Ser-Leu-Arg-Arg-Ser, Ala-Gln-Ser-Gly-Leu-Gly, and Gly-X-X-X-Asn, and their masses of 1225.41, 1302.44, and 1016.6, respectively, it was determined that these peptides corresponded to ANP_{1-11}, ANP_{17-28}, and ANP_{20-28}. The peak at 17 min contained one peptide whose total sequence was determined and which corresponded to ANP_{10-22}.

Inhibitor gel electrophoresis. To further establish the presence of NEP in rabbit aorta membranes, the solubilized preparations were subjected to SDS-PAGE under nonreducing conditions. Attempts to detect NEP by Western blotting using two monoclonal antibodies, 2B12 (24) and 23B11 (25), which recognize the rabbit kidney enzyme under the same experimental conditions (data not shown), were unsuccessful, even when a ^{125}I-labeled second antibody against 2B12 was used. After electrophoresis the gels were, therefore, incubated with 100 pM [^{125}I]RB104, in the presence or absence of 0.1 mM retrothiorphan. RB104 is a recently designed inhibitor that has a K_i for NEP of 30 pM and is also highly specific; its K_i for another widely distributed zinc-metallopeptidase, angiotensin-converting enzyme, is 15 μM (15).

Gels incubated with [^{125}I]RB104 were subjected to autoradiography. Fig. 4 shows that this molecule also bound to a single protein in the preparation, with the same apparent molecular weight as the pure rabbit kidney enzyme, and this binding was completely inhibited by 0.1 mM retrothiorphan. Inhibitor binding to different quantities of the pure enzyme was found to be quantitative, and from the resulting calibration curve (Fig. 5) it was calculated that there was 0.3 μg of enzyme/mg of protein in the rabbit aorta membrane preparation. From gels that were sliced, with the radioactivity being determined directly, it was

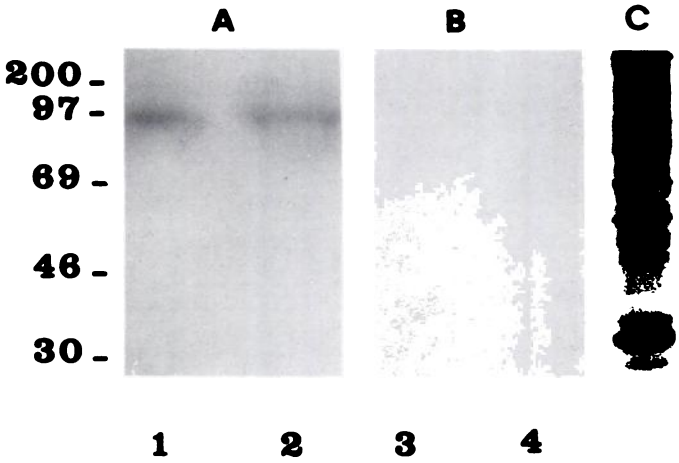

Fig. 4. Autoradiogram obtained after separation of solubilized membrane proteins from rabbit aorta by SDS-PAGE. After electrophoresis, the gel was divided into three parts. A and B were incubated with 100 pM [^{125}I] RB104, as described in Materials and Methods, with 0.1 mM retrothiorphan included in the incubation with B. C was stained with Coomasie Blue. *Lanes A1* and *B3*, 50 ng of pure rabbit kidney NEP; *lanes A2, B4*, and *C*, 100 μg of solubilized membrane protein. The running positions of molecular weight markers are indicated on the *left*.

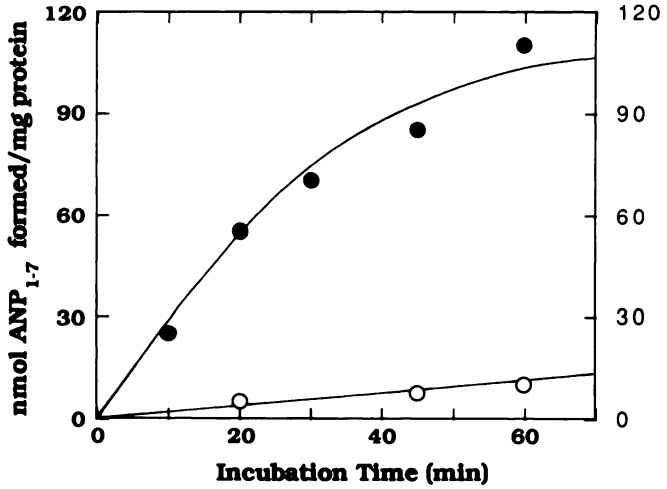

Fig. 3. Formation of ANP_{1-7} with time, during incubation of 50 μM rANP with solubilized rabbit aorta membranes, as described in Materials and Methods. The membranes were prepared either from total thoracic aorta (●) or from tissue denuded of endothelium by saponin treatment (○).

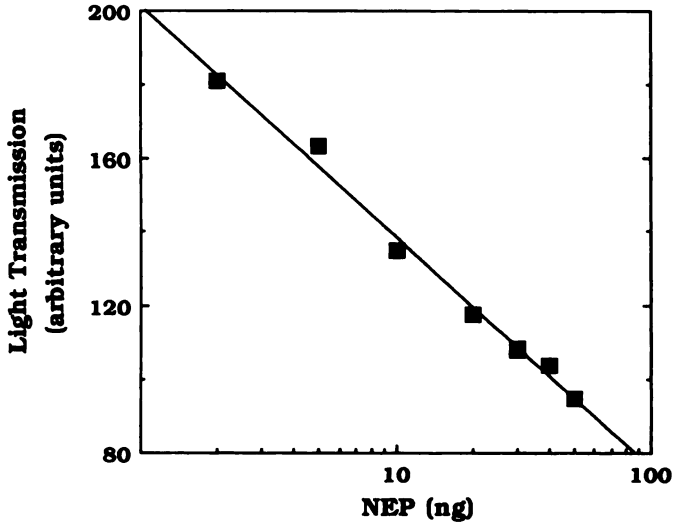

Fig. 5. Calibration curve of [^{125}I]RB104 binding to different quantities of pure rabbit kidney NEP after SDS-PAGE and autoradiography, as described in Materials and Methods. Light transmission (arbitrary units) is plotted against the logarithm of the enzyme concentration.

determined that, over the concentration range used, 0.25% of the enzyme was labeled by [^{125}I]RB104.

Discussion

Using the pentapeptide [^{3}H]D-Ala^2-leucine enkephalin as substrate, NEP-like activity was found in solubilized membrane extracts of rat aorta, mesenteric artery, and vena cava and rabbit aorta. Hydrolysis of this substrate in the aorta preparations was inhibited by the NEP inhibitors thiorphan and retrothiorphan, with K_i values similar to those found for the pure enzyme. The K_m of the substrate was also similar to that found with pure NEP. It is now well documented that NEP preferentially cleaves ANP_{1-28} between Cys_7 and Phe_8 (7, 10), as confirmed in this study. Both aorta preparations hydrolyzed $rANP_{1-28}$ at the same Cys_7-Phe_8 bond, and this cleavage was inhibited by retrothiorphan, again with a K_i similar to that obtained using pure enzyme. It should be noted that the method used here, of reducing the substrate and metabolites before HPLC, facilitates kinetic analysis, because ANP_{1-28} and its ring-opened metabolite have very similar retention times in a number of HPLC systems (14, 26).[1]

Prior removal of the endothelium of rabbit aorta, by saponin treatment, reduced the hydrolysis of both substrates by >90%. As previously discussed, saponin specifically removes the endothelial cell layer from aortic tissue (19). The results, therefore, suggest that most of the neutral endopeptidase activity detected in the total aorta extracts was present in the endothelial cell membranes.

The presence of NEP in the rabbit aorta was also directly demonstrated by its specific labeling with a radioactive inhibitor, after separation of rabbit aorta membrane proteins by SDS-PAGE. This method was adopted because, like others (14), we were unable to establish the presence of NEP in the membrane preparations by Western blotting, using antibodies that recognize the rabbit kidney enzyme. This may due to problems of sensitivity and/or differences in glycosylation between the enzyme from different tissues (27) affecting antibody recognition. The ability of NEP to bind the inhibitors after SDS-PAGE was not unexpected, because it has previously been shown that the enzyme retains some enzymatic activity under such conditions (28). Inhibitor binding, especially with the iodinated molecule, is, however, more sensitive and easier to quantify, and it was estimated that the solubilized rabbit aorta extract contained approximately 0.3 μg of enzyme/mg of protein.

Apart from being cleaved between Cys_7 and Phe_8, the HPLC profile showed additional hydrolysis of ANP by solubilized aorta membranes. The peptide eluting at 17 min, ANP_{10-22}, was not observed with short incubation times (<30 min) and, because its appearance was inhibited by both 1 μM retrothiorphan and saponin treatment, it was probably formed after the initial attack on ANP_{1-28} by NEP.

The broad peak eluting at 15 min contained three major peptides, ANP_{1-11}, ANP_{17-28}, and ANP_{20-28}, and its appearance was only partially inhibited by retrothiorphan and saponin treatment. ANP_{17-28} could well be formed by the action of NEP, because Gly_{16}-Ala_{17} has been shown to be a secondary NEP-susceptible bond (7). The enzymes responsible for the release of the other two peptides are unknown but, from the results with the denuded aorta, are probably not endothelium specific and seem to be mainly present in the smooth muscle layer. Other authors have noted an enzyme or enzymes, in both vascular smooth muscle and endothelium cells in culture, that releases the carboxyl-terminal tripeptide Phe-Arg-Tyr from ANP_{1-28} and is insensitive to NEP inhibitors (29, 30). Only low levels of this metabolite were observed in our study, but this could be due either to differences in tissue preparation or to the reported degradation of this peptide by amino- or carboxypeptidases (29, 30).

It can be concluded that NEP is present in vascular tissues and that, in the aorta at least, it is almost exclusively found in the endothelium, where a high concentration of ANP receptors are also located (31). It is, thus, ideally localized to regulate circulating ANP levels.

[1] J. M. Soleilhac et al., unpublished observations.

Acknowledgments

We would like to thank C. Dupuis for typing this manuscript and Drs. C. Durieux and J. Peyroux for their invaluable advice. We are grateful to Professor P. Crine for his gift of monoclonal antibodies. Sequencing and mass spectrometry were kindly carried out by Dr. P. Maes and the Institut Pasteur de Lille, France (Service du Professeur A. Tartar).

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