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DEGRADATION OF ATRIAL NATRIURETIC PEPTIDES BY AN ENZYME IN RAT KIDNEY RESEMBLING NEUTRAL ENDOPEPTIDASE 24.11

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Abstract—The inactivation of rat atrial natriuretic factor (ANF) was studied using a bioassay, ANF-stimulated guanylate cyclase activity. Rat kidney membranes degraded ANF into biologically inactive forms. The primary cleavage site appears to be the Cys_{105}–Phe_{106} bond. The degradation, measured by HPLC, followed classical Michaelis–Menten kinetics. The sensitivity of the enzyme to inhibitors suggested it to be a metalloendopeptidase, resembling neutral endopeptidase 24.11. When this enzyme, characterised by its enkephalin-degrading activity, was compared to the enzyme responsible for ANF inactivation, striking differences were found in tissue distribution, pH-dependence and sensitivity to protein-modifying reagents. It is concluded that an enzyme similar to endopeptidase 24.11 may be responsible for inactivation of atrial peptides in the rat.

Atrial natriuretic peptides are a recently discovered family of hormones secreted from cardiac tissue which are thought to play an important role in the control of fluid balance and of blood pressure [1–3]. The main circulating form is a twenty-eight amino acid peptide ANF_{99-126} (henceforward referred to as ANF)* which is released from atrial cells in response to volume loading and atrial distension. Its effects are thought to be mediated by stimulation of a membrane-bound guanylate cyclase [4, 5].

Studies following the fate of injected atrial peptides suggest that elimination by the kidney may be an important factor in the removal of these peptides from the plasma [6–8] and that the peptides sequestered by the kidney may be metabolized rather than excreted [9], and a role for renal kallikrein has been suggested [10]. Recently, Stephenson and Kenny [11] have reported that ANF is a rather good substrate for neutral metalloendopeptidase 24.11 ("enkephalinase"; EP 24.11), an enzyme implicated in the degradation of many peptides.

The aim of the present study was to investigate further the inactivation of atrial peptides by their target organs in the rat. The approach used was to incubate the peptides [rat ANF_{99-126} (ANF) or rat $ANF_{103-126}$ (AP III)] with membrane preparations and measure the loss of bioactivity by bioassay, in this case, stimulation of guanylate cyclase activity. This approach was adopted in order to pinpoint the enzyme(s) responsible for terminating the biological activity of these peptides and to avoid possible confusion due to enzymatic interconversion between different biologically active forms. Due to limitations on the quantifiability of the bioassay, a parallel study was undertaken using HPLC in order to define the kinetic parameters of the degrading activity.

*Abbreviations: ANF, atrial natriuretic factor; AP III, atriopeptin III; [^{3}H]-DALE, [^{3}H]-(D-Ala^{2}Leu^5)enkephalin; TFA, trifluoroacetic acid; EP 24.11, neutral endopeptidase 24.11 (EC 3.4.24.11); HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Materials. Atrial peptides (rat) and angiotensin II (human) were obtained from Peninsula laboratories. [^{3}H]-(D-Ala^2, Leu^5)enkephalin ([^{3}H]-DALE, 51 Ci/mmol) was purchased from CEA. Radioimmunoassay kits for the measurement of cGMP and ANF were purchased from NEN and Amersham, respectively. Acetonitrile was HPLC grade from Carlo Erba. Trifluoroacetic acid and *N*-ethylmorpholine were obtained from Aldrich Chimie. All other reagents were obtained from Sigma Chemical Co. (Mo, U.S.A.) Thiorphan and kelatorphan were generous gifts of Pr. B. P. Roques, Université de Paris VII.

Preparation of renal membranes. Kidneys were dissected from male Sprague–Dawley rats (250–270 g). A crude membrane preparation was obtained by the method of Malfroy and Schwartz [12].

Measure of ANF-degrading activity. Membrane incubation: Renal membrane preparations (0.5 mg prot./ml) were incubated for 5 min at 37° with rat ANF or rat atriopeptin III (AP III) both at 5×10^{-6} M (final volume 50 μl). For the experiments using bioassay, aliquots (10 μl) were removed and placed in a boiling water bath for 3 min. For the experiments using radioimmunoassay or HPLC, the reaction was terminated by acidification with HCl (5 μl, 0.15 M). Furthermore, angiotensin II (100 ng) was added as an internal standard to the samples destined for HPLC analysis.

Assay of atrial peptides. (1) Bioassay: Rat adrenal glands (1:5, w/v) were homogenized in 50 mM Tris–HCl buffer pH 8 containing EDTA (1 mM), DTT

(1 mM) and sucrose (250 mM) using a Polytron (18,000 rpm). The homogenate was centrifuged (1500 *g* for 15 min) to eliminate cell debris and the supernatant recovered. This was then centrifuged at high speed (105,000 *g* for 60 min) and the pellet resuspended in Tris–HCl/EDTA/DTT/sucrose at a protein concentration of 5 mg/ml. The guanylate cyclase activity of these membranes (1 mg/ml) was measured using the method of Kimura *et al.* [13]. The cGMP so formed was measured by radioimmunoassay.

(2) Measure of ANF by radioimmunoassay: In certain samples, the atrial peptide content was estimated directly using [^{125}I]-ANF and a rabbit antiserum raised against rat ANF; this antiserum recognised the central portion of the peptide. Bound and free tracer were separated by charcoal adsorption. The samples containing atrial peptides were diluted five thousand-fold into the immunoassay buffer beforehand.

(3) HPLC: Peptides were separated by reversed phase chromatography using an LKB 2150 pump and a C_{18} μBondapak column (3.9 mm × 30 cm, Waters) protected by a C_{18} Guard-PAK precolumn (Waters). Angiotensin II was used as an internal standard. The samples were injected in a volume of 20 μl using a WISP 710B sample injector. Five minutes after injection, a gradient of H_2O:MeCN containing trifluoroacetic acid (TFA) was applied. The following gradients were used:

ANF: 17.5% to 27.5% MeCN over 25 min; TFA at 0.05%.

AP III: 20.0% to 28.0% MeCN over 16 min; TFA at 0.02%.

The eluted peptides were detected by their optical absorbance at 220 nm. The retention times for ANF and AP III were 23.9 and 16.9 min respectively. Angiotensin II eluted with a retention time of 21.5 min on the ANF gradient, and of 14.6 on the AP III gradient.

Measure of enkephalin-degrading activity. Enkephalin-degrading activity was measured by radioenzymatic assay according to the method of Llorens *et al.* [14] using [^{3}H]-(D-Ala^2,Leu^5)enkephalin as substrate ([^{3}H]-DALE: 20 nM; incubation time: 45 min).

Treatment with protein-modifying reagents. The assay was carried out using a modification of a previously described method [15]. Kidney membranes (1 mg/ml) were treated for various times with either diethylpyrocarbonate (2 mM) in Tris–HCl buffer (50 mM, pH 7.4) at 25°, butanedione (10 mM) in borate buffer (50 mM, pH 8.0) or phenylglyoxal (20 mM) in *N*-ethylmorpholine–acetate buffer (100 mM, pH 8.0), both at 37°. The reaction was stopped by the addition of an excess of histidine (diethylpyrocarbonate) or of L-arginine (phenylglyoxal and butanedione). Membranes were centrifuged at 15,000 *g* (20 min); the pellet was washed and dispersed in Tris–HCl buffer (50 mM, pH 7.6) at a protein concentration of 0.25 mg/ml.

Peptide sequencing. The peptide sequence of the principal metabolite was obtained using an Applied Biosystems 470A gas protein microsequencer. Edman degradation of the peptide yielded phenylthiohydantoin derivatives of its constituent amino acids, which were identified by reversed phase HPLC (Applied Biosystems 120A analyser).

Protein assay. Protein concentrations were estimated by the method of Lowry *et al.* [16], using bovine serum albumin as a standard.

RESULTS

Validation of the ANF bioassay

ANF markedly stimulated guanylate cyclase activity of adrenal membranes with an EC_{50} of 55 nM (13–78 nM, 95% confidence limits) and this stimulation reached a maximum at a concentration of 500 nM (data not shown); AP III was some two-fold more potent.

Degradation of atrial peptides by kidney membranes

Incubation of ANF with rat renal membrane preparations led to a rapid and total loss of bioactivity (Fig. 1). Similar results were obtained with AP III, but this peptide appeared to be degraded somewhat more slowly: ANF and AP III lost respectively 90 and 54% of their activity after 5 min.

These results were obtained using a crude kidney membrane fraction. In bioassay experiments, no enzymatic activity was detected in the soluble fraction.

No ANF-degrading activity was detectable in membranes from aorta, adrenal gland, liver, lung, striatum or testicle. These last two tissues could degrade [^{3}H]-DALE to a significant extent (kidney: 73 ± 8, striatum: 66 ± 7 and testicle: 12 ± 1 fmol/mg prot./min).

The loss of AP III was measured by HPLC. For each protein concentration tested, this followed a linear time-course whose slope corresponded to the initial velocity. Using a non-saturable concentration of substrate, the renal preparation transformed 26.7 ± 1.6 nmol AP III/mg prot./min (Fig. 2a). Using the same method, it was possible to determine

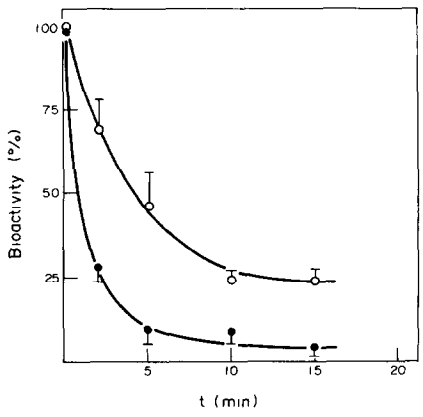

Fig. 1. Inactivation of atrial peptides by rat kidney membranes. Kidney membranes were incubated with 5 μM ANF (●) or AP III (○) for the indicated times. The loss of atrial peptides was measured by bioassay as described in the Methods. The results are expressed as the percentage of the control activity (i.e. in the absence of renal membranes). The data represent the mean ± SEM of three independent determinations, each performed in triplicate.

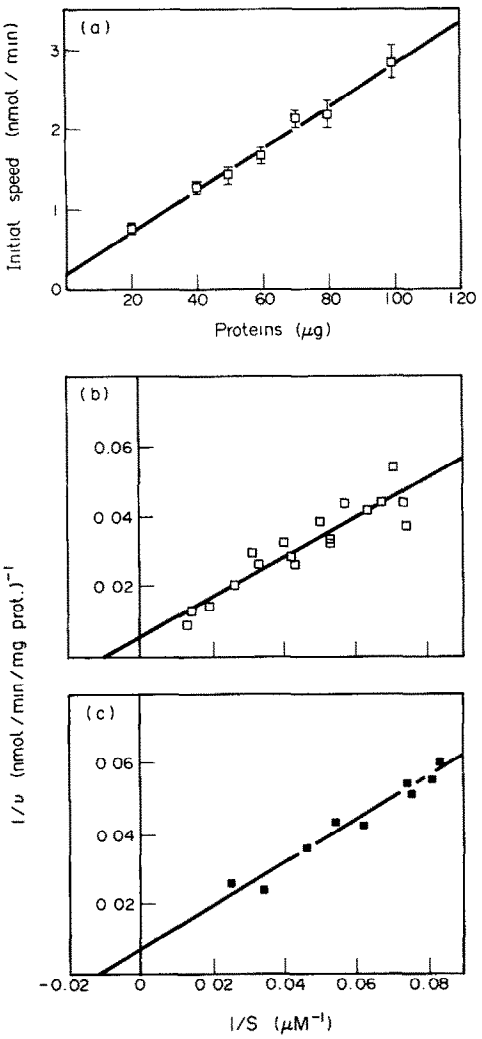

Fig. 2. Kinetics of the renal degradation of AP III. (a) The influence of protein concentration on the ANF-degrading activity of kidney membranes was examined. The initial reaction velocity was measured at different protein concentrations with 15 μM AP III as substrate. The data points represent the slopes (± SE) obtained by linear regression analysis of the time-course of degradation measured at five time points, each determined in duplicate by HPLC. The data show a linear relationship between protein concentration and degradation rate ($r = 0.98$). (b) Saturation analysis of AP III degradation by rat kidney membranes. For K_m and V_{max} determination, membranes (0.05 mg/ml) were incubated with different concentrations of AP III (8–50 μM). For each concentration, the initial reaction rate was calculated as above. The data were pooled from two independent experiments and are represented as a Lineweaver–Burk transformation. K_m and V_{max} values were thence obtained by linear regression analysis ($r = 0.95$). (c) Saturation analysis of ANF degradation by rat kidney membranes. Experimental conditions were as described for Fig. 2(b). Data are from a single experiment. K_m and V_{max} values were obtained by linear regression analysis ($r = 0.98$).

the kinetic parameters (K_m and V_{max}) by Lineweaver–Burk analysis (Fig. 2b). Apparently, the degradation of AP III follows classical enzyme kinetics for a single substrate with K_m and V_{max} values of 99 μM and 180 nmol/mg prot./mm respectively. Similar experiments with ANF (Fig. 2c) yielded values of 85 μM (K_m) and 140 nmol/mg prot./min (V_{max}).

pH-Dependence of renal peptidase activity

ANF-degrading activity was measured over a pH range from 6.0 to 9.5 using phosphate, Tris and borate buffers (Fig. 3). Certain differences were noted according to the buffer used, with greatest activity in borate and least in phosphate. At no pH was any loss of ANF observed in the absence of membranes. Data obtained with all three buffers were pooled to obtain a pH-activity curve, which showed a broad pH optimum, with no loss in activity at basic pH. However, little degradation occurred below pH 7.0. The pH-dependence of the enkephalin-degrading activity of the same membranes was also examined. This showed a pH optimum of 7.5 with decreased activity observed at both acid and basic pH. Appreciable degradation of this substrate was, however, still apparent at pH 5.0–6.0.

Effect of peptidase inhibitors

The effects of various peptidase inhibitors on ANF degradation were studied. Among the chelating agents tested, 1,10-phenanthroline (10^{-3} M) inhibited ANF degradation completely, whilst the same concentration of EDTA (30% inhibition), EGTA (7%) and dithiothreitol (14%) were much less efficacious. The most potent compounds were three inhibitors of EP 24.11, thiorphan ($IC_{50} = 2 \times 10^{-8}$ M), kelatorphan ($IC_{50} = 1 \times 10^{-7}$ M) and phosphoramidon ($IC_{50} = 5 \times 10^{-8}$ M). On the other hand, the following compounds (10^{-4} M unless otherwise indicated) had no effect on the degradation of ANF: captopril, pepstatin A, benzylsuccinic acid, bestatin, puromycin, PMSF (5×10^{-6}), bacitracin (1 U/ml).

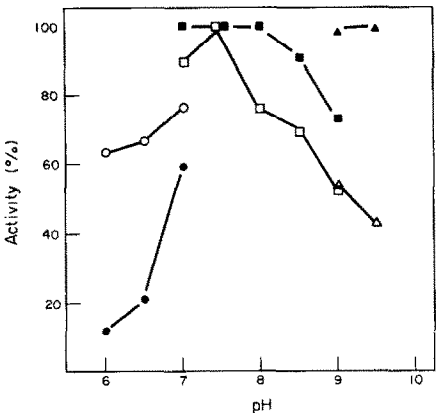

Fig. 3. pH dependence of renal enkephalin- and ANF-degrading activities. Peptidase activity of renal membranes was measured at different pHs using either 20 nM [^{3}H]-DALE (open symbols) or 5 μM ANF (closed symbols) as substrates. The following buffers were used: 50 mM sodium phosphate (pH 6–7, ○,●), 50 mM Tris–HCl (pH 7–9, □,■) and 50 mM sodium borate (pH 9–9.5, △,▲). ANF-degrading activity was measured by bioassay. The data are expressed as a percentage of the activity obtained at pH 7.5, and are the mean of three independent determinations.

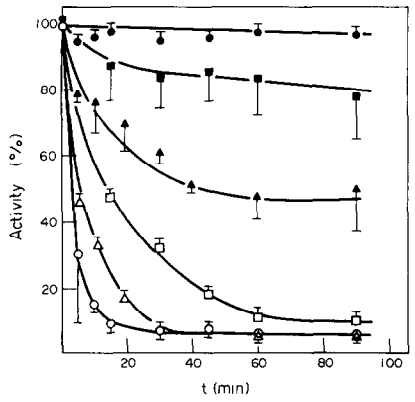

Fig. 4. Inactivation of renal peptidases by protein-modifying reagents. Renal membranes were incubated with diethylpyrocarbonate (2 mM, ○,●), butanedione (10 mM, □,■) or phenylglyoxal (20 mM, △,▲) for the indicated times. The treated membranes were assayed for enkephalin- (open symbols) and ANF- (closed symbols) degrading activities, the latter by radioimmunoassay. Data are expressed as the percentage of the activity found in non-treated membranes, and represent the mean ± SEM of three independent determinations, each performed in triplicate.

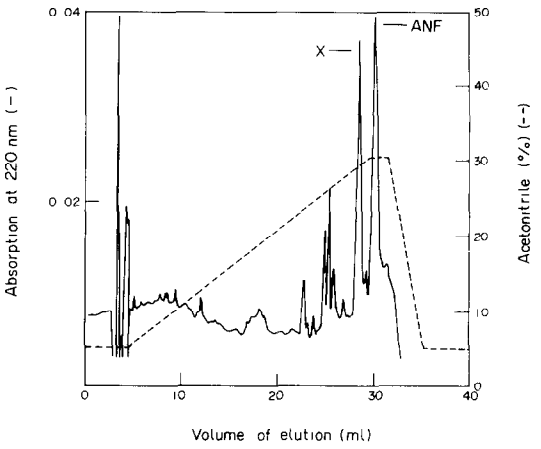

Fig. 5. Separation of ANF metabolites by HPLC. ANF (20 μM) was incubated with rat kidney membranes (0.05 mg prot./ml, 400 μl final volume) for 6 min, and the reaction terminated by acidification (40 μl) as described in the Methods. An aliquot (100 μl) of the mixture was injected onto the HPLC column, and subsequently eluted with a H_2O: MeCN gradient containing 0.05% TFA. The gradient is indicated by the dotted line. The positions of the unchanged peptide (ANF; retention time = 29.9 min) and the principal metabolite (X; retention time = 28.3 min) are noted by the arrows. The chromatogram is representative of four similar observations.

Effect of protein-modifying reagents

The degradation of ANF was not modified by incubation with sulphydryl reagents such as dithiothreitol and *N*-ethylmaleimide. Pretreatment of kidney membranes with the histidine reagent, diethylpyrocarbonate, did not affect their ANF-degrading activity, while their enkephalin-degrading activity was abolished rapidly (Fig. 4). Pretreatment with the arginine reagents, butanedione and phenylglyoxal, led to a partial decrease in ANF-degrading activity, but this effect was less marked and slower in onset than their effect on the enkephalin-degrading activity.

Identification of the principal metabolite

The degradation products obtained upon incubation of ANF with rat kidney membranes were separated by HPLC (Fig. 5). One major and several minor degradation products were detected. The principal metabolite was sequenced and identified as the parent peptide cleaved within the disulphide loop between Cys_{105} and Phe_{106}, the resulting two strands remaining joined by the disulphide bridge.

DISCUSSION

These results demonstrate that the rat kidney contains a membrane-bound enzyme that avidly degrades atrial peptides to biologically inactive forms. Both ANF and atriopeptin III were degraded by the kidney membrane fraction according to classical Michaelis–Menten kinetics, consistent with the activity of a single enzyme. This enzyme appears to hydrolyse ANF by firstly cleaving the Cys_{105}–Phe_{106} bond; such an opening of the disulphide ring would be sufficient to explain the loss of bioactivity of the peptide [3].

Somewhat surprisingly, no evidence was found for ANF-degrading activity in any other rat tissue, in spite of the presence of large numbers of ANF receptors in many of them, e.g. the adrenal gland [17]. It is possible that in these other tissues, the interaction of ANF with its receptors is terminated either by the removal of ANF into the circulation for subsequent degradation in the kidney, or by rapid internalisation of the ANF–receptor complex, as has been demonstrated for aortic myocytes studied in culture [18].

Since Stephenson and Kenny [11] have shown human ANF to be a substrate for purified pig EP 24.11, and the kidney is an exceptionally rich source of this enzyme, it seemed logical to enquire whether the membrane-bound activity described in fact corresponded to EP 24.11. The inhibitors used did not distinguish the enzyme responsible for ANF inactivation from EP 24.11, whether these were simple metal chelators or more specific substrate analogues such as thiorphan [19]. However, it should be pointed out that the IC_{50}s obtained were an order of magnitude higher than those generally observed with EP 24.11.

Other types of experiment, however, show striking differences between the ANF-degrading activity and EP 24.11. Whilst the latter enzyme has a neutral pH optimum [14, 20] (6.0 for insulin B-chain and 7.0 for [Leu^5]enkephalin) with a bell-shaped pH curve, the ANF-degrading activity was perfectly resistant to basic pH, up to the limit of the chemical stability of the peptide (pH 10). Below pH 7.0, little ANF degrading activity could be detected.

A similar difference in sensitivity was seen with three covalent blockers of EP 24.11, butanedione, phenylglyoxal and diethylpyrocarbonate, to which the ANF-degrading activity was quite resistant. Since

these compounds are believed to interact with amino acids in the active site of EP 24.11 [15], these findings might suggest that a peptidase endowed with a different active site is involved in the inactivation of ANF. These results are all the more surprising in light of the fact that nearly all metalloendopeptidases studied to date contain amino acids sensitive to these reagents in their active sites.

A third difference concerns the tissue distribution of the ANF-degrading activity. Inactivation of ANF was not observed with membranes from other tissues known to be rich in EP 24.11, such as the striatum and testicle, and in which, indeed, enkephalin degradation could be demonstrated.

Three studies have appeared recently in which the degradation of ANF by pig [11], rabbit [20] and rat [21] kidney membranes was examined. In the rat, Koehn *et al.* [21] have demonstrated an enzyme active at basic pH, which cleaves ANF at the Cys_{105}–Phe_{106} bond. The present results thus confirm these findings and extend the investigation to the effects of selective inhibitors and covalent blockers.

Stephenson and Kenny [11] have shown that the properties of the ANF-degrading activity of pig microvillar membranes appear to be very similar to those of purified pig kidney EP 24.11, and they suggest that the degradation of ANF is indeed initiated by this enzyme. However, the present study clearly demonstrates that rat kidney membranes are capable of inactivating ANF under conditions where the degradation of enkephalins by EP 24.11 is abolished (notably, basic pH and following pretreatment with diethylpyrocarbonate). The simplest explanation for this would be that another thiorphan-sensitive peptidase is present in our membrane preparation. The discrepancy between this conclusion and that of Stephenson and Kenny may stem from the difference in species or that the crude membrane preparation used here contains additional peptidases to those in the brush border. Finally, it cannot be excluded that the differences observed between ANF and enkephalin degradation arise from differences not in the enzyme but in the substrate.

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REFERENCES

1. Anderson JV and Bloom SR, Atrial natriuretic peptide: what is the excitement all about? *J Endocr* **110**: 7–17, 1986.
2. De Bold AJ, Atrial natriuretic factor: a hormone produced by the heart. *Science* **230**: 767–770, 1985.
3. Ackerman U, Structure and function of atrial natriuretic peptides. *Clin Chem* **32**: 241–247, 1986.
4. Waldman SA, Rapoport RM and Murad F, Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J Biol Chem* **259**: 14332–14334, 1984.
5. Ohlstein EH and Berkowitz BA, Cyclic guanosine monophosphate mediates vascular relaxation induced by atrial natriuretic factor. *Hypertension* **7**: 306–310, 1985.
6. Luft FC, Lang RE, Aronoff GR, Ruskoaho H, Toth M, Ganten D, Sterzel RB and Unger T, Atriopeptin III kinetics and pharmacodynamics in normal and in anephric rats. *J Pharmacol Exp Therap* **236**, 416–418, 1986.
7. Murthy KK, Thibault G, Garcia R, Gutkowska J, Genest J and Cantin M, Degradation of atrial natriuretic factor in the rat. *Biochem J* **240**: 461–469, 1986.
8. Yandle TG, Richards AM, Nicholls MG, Cuneo R, Espiner EA and Livesey JH, Metabolic clearance rate and plasma half life of alpha-human atrial natriuretic peptide in man. *Life Sci* **38**: 1827–1833, 1986.
9. Tang J, Webber RJ, Chang D, Chang JK, Kiang J and Wei ET, Depressor and natriuretic activities of several atrial peptides. *Regulatory Peptides* **9**: 53–59, 1984.
10. Briggs JP, Marin-Grez M, Steipe B, Schubert G and Schnermann J, Inactivation of atrial natriuretic substance by kallikrein. *Am J Physiol* **247**: F480–F484, 1984.
11. Stephenson SL and Kenny AJ, The hydrolysis of alpha-human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase-24.11. *Biochem J* **243**: 183–187, 1987.
12. Malfroy B and Schwartz JC, Properties of "enkephalinase" from rat kidney: comparison of dipeptidylcarboxypeptidase and endopeptidase activities. *Biochem Biophys Res Commun* **106**: 276–285, 1982.
13. Kimura H, Mittal CK and Murad F, Activation of guanylate cyclase from rat liver and other tissues by sodium azide. *J Biol Chem* **250**: 8016–8022, 1975.
14. Llorens C, Malfroy B, Schwartz JC, Gacel G, Roques BP, Roy J, Morgat JL, Javoy-Agid F and Agid Y, Enkephalin dipeptidtyl carboxypeptidase (enkephalinase) activity: selective radioassay, properties and regional distribution in human brain. *J Neurochem* **39**: 1081–1089, 1982.
15. Beaumont A and Roques BP, Presence of a histidine at the active site of the neutral endopeptidase-24.11. *Biochem Biophys Res Commun* **139**: 733–739, 1986.
16. Lowry OH, Rosebrough NG, Farr RL and Randell RG, Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
17. Lynch DR, Braas KM and Snyder SH, Atrial natriuretic factor receptors in rat kidney, adrenal gland, and brain: autoradiographic localization and fluid balance dependence changes. *Proc Natl Acad Sci USA* **83**: 3357–3361, 1986.
18. Napier MA, Arcuri KE and Vandlen RL, Binding and internalization of atrial natriuretic factor by high-affinity receptors in A10 smooth muscle cells. *Arch Biochem Biophys* **248**: 516–522, 1986.
19. Roques BP, Fournie-Zaluski MC, Soroca E, Lecomte JM, Malfroy B, Llorens C and Schwartz JC, The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* **288**: 286–288, 1980.
20. Olins GM, Spear KL, Siegel NR and Zurcher-Neely HA, Inactivation of atrial natriuretic factor by the renal brush border. *Biochem Biophys Acta* **901**: 97–100, 1987.
21. Koehn JA, Norman JA, Jones BN, LeSueur L, Sakane Y and Ghai RD, Degradation of atrial natriuretic factor by kidney cortex membranes: isolation and characterization of the primary proteolytic product. *J Biol Chem* **262**: 11623–11627, 1987.