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Degradation of [^{125}I]-Atrial Natriuretic Peptide by a Soluble Metallopeptidase Isolated from Rat Ventricular Myocytes

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SUMMARY: Atrial natriuretic peptide is rapidly degraded by a soluble, heat labile peptidase isolated from ventricular myocytes. Degradation of [^{125}I]-ANP is antagonized by unlabelled ANP, bradykinin, glucagon, 1,10-phenanthroline, PCMB, EDTA and the bacterial antibiotic bacitracin, but not by phenylmethylsulphonyl fluoride, aprotinin, phosphoramidon, E-64, amastatin or the ACE inhibitor SQ 20881 and bradykinin potentiator C. In addition neither bovine serum albumin nor caesin afforded any protection against degradation. Peptidase activity was optimal at pH values above 8.5. The peptidase is likely to be of intracellular origin and may contribute to the extensive ANP degradative activity found in various ventricular muscle preparations.

Atrial natriuretic peptide (ANP) is a hormone with potent diuretic, natriuretic and vasorelaxant actions which is released from atrial tissue in response to increases in blood volume [1,2,3]. Most information about hormone function has dealt with the mechanisms of action on target tissues where specific receptors have been described in the kidney and aorta [4], vascular smooth muscle cells [5,6], adrenal cortex [7], brain [8], pituitary [9] and the ventricular myocardium [10]. It is possible that receptor subclasses with different functions may exists for the peptide [6,7]. ANP binding to these receptors results in a stimulation of guanylate cyclase activity and/or an inhibition of adenylate cyclase activity indicating that the cyclic nucleotides and in particular cGMP, are likely to be involved in the hormone response.

As a result of the short half life of ANP in the blood (1-3min) [3,11], many of the physiological actions of the peptide are transient in nature [12,13,14]. The mechanisms operating to account for this rapid removal of ANP from the circulation still remain to be characterised. Despite the inherent instability of the peptide both in vivo and in vitro, very little information is available about the endogenous factors responsible for its degradation.

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In this paper we report that a soluble peptidase isolated from rat heart rapidly degrades ANP. This factor may account for part of the rapid degradation found when ANP is incubated with ventricular muscle extracts.

MATERIALS AND METHODS

Materials:

Unless otherwise stated ANP, bradykinin potentiator C, glucagon and all inhibitors, biochemicals and reagents were obtained from Sigma Chemicals, London. General chemicals were from BDH, Poole, Dorset. The ACE inhibitor SQ 20881 was a gift from Squibb, Hounslow, Middlesex and PCMB was obtained from Fluorchem Ltd., Glossop, Derbyshire. Radiolabelled [^{125}I]ANP (2200 Ci/mmol) was supplied by Amersham International plc.

Methods:

Isolation of Ventricular Myocytes:

Ventricular myocytes were isolated from 250g male Wistar rats by the method described by Powell et al. [15]. Cells were resuspended at a final concentration of 10^6 cells/ml in a HEPES buffer Krebs solution, pH 7.4 containing 118mM NaCl, 2.6mM KCl, 1.18mM KH_2PO_4, 1.18mM $MgSO_4$, 11.0mM glucose, 25mM HEPES, 0.1mM $CaCl_2$ and 1% bovine serum albumin.

Preparation of soluble fraction:

Myocyte suspensions were centrifuged at 60g for 1 min, pellets resuspended to 10^5 cells/ml with 10mM Tris HCl, pH 7.4 and then homogenized using a Polytron PT-10, 3 x 20 s. setting 4. The homogenate was centrifuged at 2000g for 15min and the resulting supernatant was then recentrifuged at 100,000g for 60min. The final supernatant was stored in 1ml aliquots at -20°C until use. Freezing did not alter the proteolytic activity of the fractions.

Measurement of [^{125}I]ANP Degradation:

[^{125}I]ANP degradation was measured as the percentage of radiolabel not precipitated by 6% trichloroacetic acid (TCA). In a typical experiment 50 µl soluble fraction (extract from 5 x 10^3 cells) was incubated for 15 min at 37°C with 25 µl [^{125}I]ANP (100-400 pM) and either 25 µl buffer (50 mM Tris-HCl, pH 7.4) or buffer containing the compound under investigation. Incubations were terminated by the addition of 100µl of an ice cold bovine serum albumin solution (10%) followed by rapid removal of 50µl aliquots into 250µl 6% TCA. After standing on ice for 15 min, non-degraded [^{125}I]ANP (precipitable radioactivity) was separated from degraded radiolabel by centrifuging for 2 min in a Beckman microfuge. Radioactivity in both the pellet and supernatant was measured using a Packard gamma scintillation counter. As myocyte homogenates contained variable amounts of BSA which was carried over from the initial myocyte suspensions, degradative activity was reported as fmol ANP degraded/ min./ soluble extract from 10^6 cells.

Statistics:

Errors indicated in figures and tables reflect standard error of the means of at least three separate experiments.

RESULTS AND DISCUSSION

The incubation of myocyte soluble fraction with radiolabel at 37°C resulted in the rapid breakdown of [^{125}I]ANP as measured by TCA

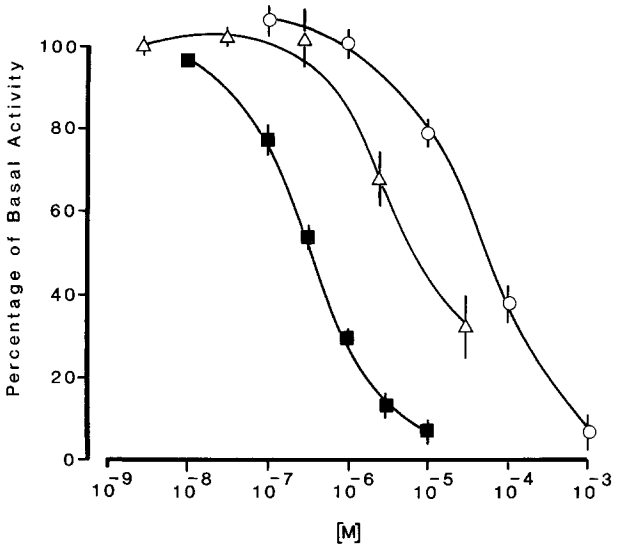

Fig. 1: Inhibition of [^{125}I]-ANP degradation by ANP (■), glucagon (△) or bradykinin (○). See methods for details.

precipitation. In the presence of 100pM [^{125}I]-ANP, the basal rate of degradation was 50.9 ± 2.2 fmols ANP/min/10^6cells. Degradation of [^{125}I]ANP was inhibited by unlabelled ANP, glucagon and bradykinin (Fig. 1). Half-maximal inhibition of degradation was found at 0.3 x 10^{-6}M ANP whereas 10-100 fold higher concentrations of the peptides glucagon and bradykinin were required for similar inhibition (Table 1). Preliminary analysis of degradation products by HPLC have concluded that unlabelled and radiolabelled ANP are both substrates for hydrolysis (results not shown) however it is not known whether bradykinin and glucagon are also degraded during the incubation period. Although ventricular myocytes are known to have receptors for ANP it is unlikely, due to the nature of the preparation, that receptor binding is involved in the degradation observed. Inhibition constants are 3 to 4 orders of magnitude higher than values reported

Table 1: Concentration of ANP, glucagon or bradykinin required for half maximum inhibition (IC_{50}) of [^{125}I]-ANP degradation

PEPTIDE	IC_{50} (M)
ANP	3.33 ± 0.72 x 10^{-7}
GLUCAGON	1.25 ± 0.65 x 10^{-5}
BRADYKININ	9.62 ± 0.34 x 10^{-5}

Values derived from Fig. 1.

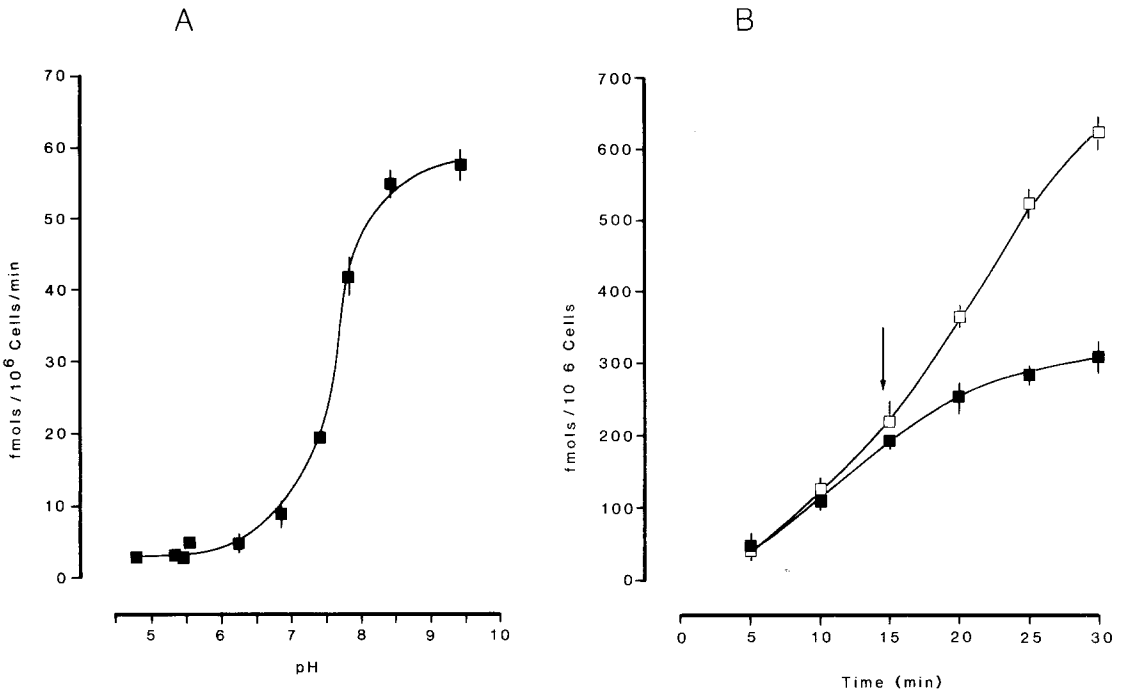

Fig. 2: Effect of pH on the rate of [^{125}I]-ANP degradation by myocyte soluble fraction. (a). pH profile of degradative activity; [^{125}I]-ANP was incubated for 15 min. at 37°C with soluble fraction at the pH indicated. (b). Time course of [^{125}I]-ANP degradation at pH 6.1 and 7.5; degradative activity was monitored either at pH 6.1 for all time points (■) or initially at pH 6.1 and then after adjustment to pH 7.5 (□) as indicated by the arrow. See methods for other details.

for half maximal ANP-receptor binding or action in a number of tissues [4-10,16-19]. The degradative activity of this soluble extract is heat-labile (15 min at 60°C completely inhibits activity) and exhibits an alkaline pH optimum with a 30-fold stimulation in activity at pH values greater than 8.5 (Fig. 2a). Low levels of proteolytic activity are enhanced by increasing the pH from 6.1 to 7.5 (Fig. 2b), indicating that small changes in pH around the physiological range can have marked effects on the stability of ANP in these extracts.

To investigate the specificity of the peptidase in degrading ANP, incubations were carried out in the presence of various peptides and protease inhibitors. In addition to unlabelled ANP, bradykinin and glucagon, the bacterial antibiotic, bacitracin, and the metallo-proteinase inhibitor 1,10-phenanthroline (1mM) also inhibited the breakdown of [^{125}I]-ANP (Fig. 3). Preincubation of the extract with the cysteine protease inhibitor 4-chloromercuribenzoate (PCMB) or EDTA also resulted in inhibition of [^{125}I]-ANP degradation whereas bovine serum albumin, caesin or the general protease inhibitors,

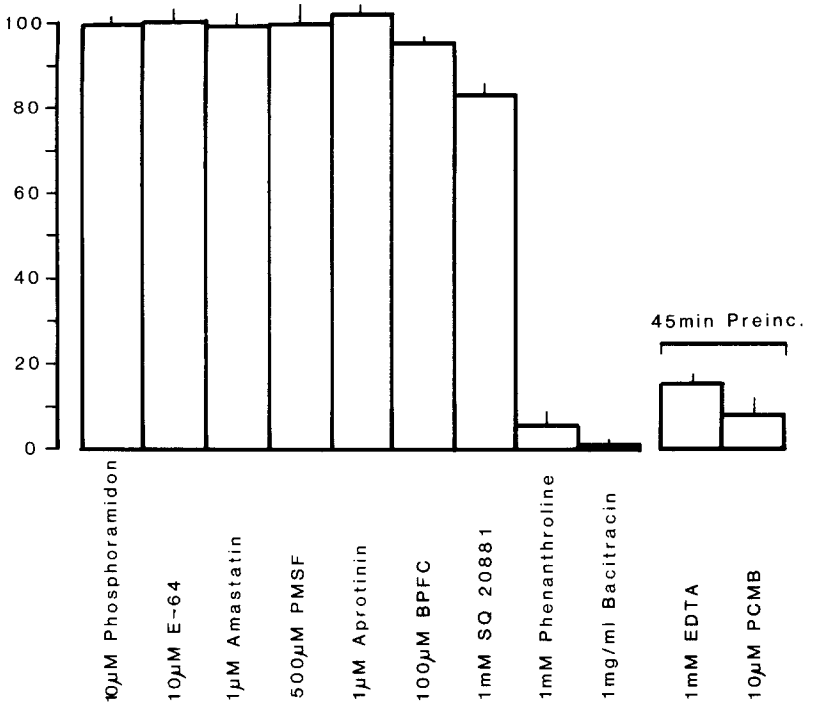

Fig. 3: Effects of various protease and peptidase inhibitors on degradation of [^{125}I]-ANP by myocyte soluble fraction. Preincubation of soluble fraction with EDTA and PCMB at 37°C was required for maximum inhibition. Similar preincubations did not affect degradative activity or increase the actions of the other inhibitors.

phenylmethylsulphonyl fluoride (PMSF) and aprotinin, were without effect (Fig. 3). A specific inhibitor of bradykinin degradation, bradykinin potentiator C, and the angiotensin converting enzyme inhibitor (ACE) SQ 20881 also failed to prevent ANP breakdown, as did phosphoramidon, E-64 or amastatin (Fig. 3). Comparison of the amino acid composition of ANP and bradykinin reveals the presence of the dipeptide sequences phenylalanine-arginine, at the carboxy-termini of both peptides. This bond is known to be hydrolysed in bradykinin by kininase II (E.C. 3.4.15.1) and both bradykinin potentiator C and SQ 20881 are potent inhibitors of this hydrolysis [20]. The lack of effect of these inhibitors on ANP degradation suggests that this dipeptide sequence is not cleaved by the peptidase. These observations are in agreement with previous reports which show that ACE inhibitors do alter the extensive ANP degradation found in perfused rabbit lung [21] or pig kidney [22]. We conclude that kininase II is not involved in the degradation of ANP in these preparations but activity is due to a novel metallopeptidase. The alkaline pH optimum of the peptidase and its insensitivity to

phosphoramidon also suggests that this peptidase is distinct from kidney membrane endopeptidase-24.11 which has recently been shown to degrade ANP and other peptides [22]. Inhibition by PCMB suggest the presence of an active cysteine sulphydryl group at the catalytic site. The inhibitor E-64 which has been shown to inhibit certain cysteine proteases has no effect on the peptidase activity. These results indicate that degradative activity is the result of a cysteine metallopeptidase which has substrate specificities and inhibition profiles similar to, but not identical with those reported for insulin-glucagon proteinase [23], collagenase-like peptidase [24] and both brain [25] and kidney cortex [26] metalloendopeptidase.

The activity of this soluble peptidase shows similar characteristics to the proteolytic activity released from suspensions of intact ventricular myocytes [27]. This indicates that this soluble peptidase may be released from intact cells and account for the degradation of ANP found in myocyte suspensions and also may explain the inability to detect ANP receptors using steady state kinetics [26].

The antibiotic bacitracin, which is often included in incubations as a potent inhibitor of glucagon degradation, is an effective inhibitor of ANP degradation, indicating that degradation of both hormones may be the result of the action of a common or related peptidase. We are currently attempting to isolate and characterize both the soluble peptidase and the hydrolysis products resulting from its action on ANP. However it is suggested that all bioassays involving ANP, especially in cardiac muscle, should include bacitracin in the incubations to prevent ANP degradation by this peptidase.

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