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ADRENOMEDULLIN: A NOVEL HYPOTENSIVE PEPTIDE ISOLATED FROM HUMAN PHEOCHROMOCYTOMA

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Summary: A novel hypotensive peptide was discovered in human pheochromocytoma by monitoring the elevating activity of platelet cAMP. Since this peptide is abundant in normal adrenal medulla as well as in pheochromocytoma tissue arising from adrenal medulla, it was designated "adrenomedullin". The peptide, consisting of 52 amino acids, has one intramolecular disulfide bond and shows slight homology with calcitonin gene related peptide. It was found to elicit a potent and long lasting hypotensive effect. The peptide circulates in blood in a considerable concentration, but it was not found in brain. These data suggest that adrenomedullin is a new hormone participating in blood pressure control. Occurrence of adrenomedullin indicates the possible existence of a novel system for circulation control. © 1993 Academic Press, Inc.

Mammalian circulation is regulated by subtle mechanisms involving several neural and hormonal factors. Vasoactive peptides including atrial natriuretic polypeptide and endothelin are especially important regulators in the cardiovascular system. For clarifying the intricacies of circulation, it is important that still unidentified vasoactive peptides be discovered. In this context, we have been searching for peptides which may be relevant to circulation control, using an assay system which monitors the elevating activty of platelet cAMP (1,2). With this assay, we already isolated biologcally active peptides such as vasoactive intestinal polypeptide (VIP) and calcitonin gene related peptide (CGRP) from pheochromocytoma (PC) tissue (1) and peptide histidine isoleucine (PHI) from porcine intestine (2). These peptides are known to be potent vasorelaxants (3,4) and are thought to bind to the specific receptors on platelet membranes to increase intracellular cAMP. This assay seems to be a good tool for investigating biologically active peptides and raises the possibility that other vasoactive peptides remain to be identified, since several unidentified bioactive peaks which elevate platelet cAMP were observed in PC tissue extract (1). In the present study, by monitoring the elevating activity of platelet cAMP, we discovered in human PC arising from adrenal medulla a novel hypotensive peptide designated adrenomedullin.

Abbreviations: RIA, radioimmunoassay; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; RCM, reduced and S-carboxymethylated; BSA, bovine serum albumin; PTH, phenylthiohydantoin; VIP, vasoactive intestinal polypeptide; CGRP, calcitonine gene related peptide; PHI, peptide histidine isoleucine.

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MATERIALS AND METHODS

Peptides: Adrenomedullin [1-12], [3-12], [45-52]NH_2 and [45-52]COOH were synthesized by the solid phase method with a peptide synthesizer (430A, Applied Biosystems), and purified by reverse phase high performance liquid chromatography (HPLC). Human synthetic adrenomedullin prepared by solid phase methods in the Peptide Institute, Inc. (Osaka, Japan), will be described elsewhere.

Isolation: Preparation of peptide extract was performed in a method similar to that described (1,5). Human PC tissue was resected at surgery from a norepinephrine dominant PC patient. Fraction SP-III, containing strongly basic peptides, was separated from an acid extract of PC by batch-wise ion exchange chromatography on SP-Sephadex C-25 (H^+-form, 2 x 15 cm). The SP-III fraction, which contained 90% platelet cAMP elevating activity, was separated by Sephadex G-50 gel filtration. Fractions (Mr 4,000-6,000) showing activity were further separated by CM ion exchange HPLC on a column of TSK CM-2SW (8.0 x 300 mm, Tosoh). Adrenomedullin was finally purified from the arrowed peak (Fig. 1a) by reverse phase HPLC using phenyl (4.6 x 250 mm, Vydac) and μBondasphere C-18 (4.6 x 150 mm, 300A, Waters) columns.

Structural analysis: Two hundred pmol of purified adrenomedullin was reduced and S-carboxymethylated (RCM) by the described methods (5). The resulting RCM-adrenomedullin was purified by reverse phase HPLC. Half of the purified RCM-adrenomedullin (100 pmol) was subjected to a gas phase sequencer (Model 470A/120A, Applied Biosystems). The remaining half of the adrenomedullin was digested with 400 ng of arginylendopeptidase (Takara Shuzo, Kyoto, Japan) in 50 μl of 50 mM Tris-HCl (pH 8.0) containing 0.01% Triton-X 100 at 37℃ for 3 h. The peptide fragments were separated by reverse phase HPLC on a semi-micro column of Chemcosorb 3 ODS H (2.1 x 75 mm, Chemco, Osaka, Japan) and each fragment was sequenced by a gas phase sequencer (6).

Activity increasing platelet cAMP: Platelet cAMP elevating activity was assayed as described previously (1). Twenty-five microliters of sample dissolved in suspension medium containing 135 mM NaCl, 2 mM EDTA, 5 mM Glucose, 10 mM theophyllin and 15 mM Hepes (pH 7.5) was preincubated at 37℃ for 10 min. Reaction was initiated by adding 25 μl of washed rat platelets (4.0×10^5) and incubated for 30 sec. Reaction was stopped with 200 μl of ethanol containing 150 mM HCl and heated for 3 min. The sample was evaporated in a speedvac concentrator and was dissolved in 100 μl of 50 mM sodium acetate buffer (pH 6.2). Cyclic AMP in the solution was succinylated and analyzed by cAMP radioimmunoassay (RIA) (1).

Hypotensive effect: Depressor effect of adrenomedullin was examined by methods similar to those reported for rat brain natriuretic polypeptide (7). Ten-week old male Wistar rats (300 g) were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood pressure was monitored continuously from a right carotid artery catheter (PE-50) connected to a Statham pressure transducer (model P231D, Gould). A PE-10 catheter was inserted into the right jugular vein for administration of both maintenance solution and peptides. After equilibration for at least 60 min, CGRP or adrenomedullin was injected intravenously.

RIA for adrenomedullin [1-12]: Adrenomedullin [1-12] (10 mg) and bovine thyroglobulin (20 mg) were conjugated in 2 ml of 0.1 M sodium phosphate buffer (pH 7.4) by action of glutaraldehyde (8). The reaction mixture was dialyzed against 50 mM sodium phosphate buffer (pH 7.4)/0.08 M NaCl, and used for immunization as described (8). RIA sample was trypsinized with 1 μg trypsin (Worthington) in 100 μl of 0.1 M NH_4HCO_3 containing 20 μg of bovine serum albumin (BSA). RIA for adrenomedullin [1-12] was performed by a method similar to that reported for β-neo-endorphin (9). The RIA incubation mixture consisted of 100 μl of adrenomedullin [1-12] or unknown sample solution, 50 μl of antiserum at a dilution of 1 : 6,000 and 50 μl of ^{125}I-labelled ligand (18,000 cpm), which was prepared by the lactoperoxidase method (10). After incubation for 24 hr, free and bound tracers were separated by the polyethyleneglycol method. Radioactivity of the pellet was counted with a gamma counter (ARC-600, Aloka), and assay was performed in duplicate at 4℃.

Distribution of adrenomedullin: Each human tissue was boiled for 10 min in 5 vol of H_2O to inactivate intrinsic proteases. After cooling, glacial acetic acid was added to make 1 M, and the mixture was homogenized with a polytron mixer at 4℃. The supernatant of the extract, obtained after centrifugation at 24,000 x g for 30 min, was loaded onto a Sep-Pak C-18 cartridge (Waters) which was pre-equibrated with 1 M acetic acid, and the adsorbed materials were eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The immunoreactive adrenomedullin in the solution was analyzed by reverse phase HPLC using a column of TSK ODS 120A (4.6 x 150 mm, Tosoh), under the conditions as described (2).

RESULTS AND DISCUSSION

Adrenomedullin was isolated from acid extracts of human PC (140 g), by monitoring the activity increasing rat platelet cAMP. Low Mr extracts were subjected to cation exchange chromatography (SP-Sephadex C-25, H^+-form) as previously described (1), to collect an SP-III fraction containing basic peptides. The SP-III fraction was separated by gel filtration chromatography (Sephadex G-50, fine, 3 x 150 cm). A bioactive fraction (Mr 4,000-6,000) was further separated by cation exchange HPLC using a column of TSK CM-2SW, as shown in Fig. 1a. From the bioactive peak indicated by the arrow, adrenomedullin was isolated in a pure state by reverse phase HPLC with phenyl and μBondasphere C-18 columns. However, the amount of peptide thus obtained was so limited (20 pmol) that only the N-terminal 18th residue sequence was elucidated, with further information remaining unclear. In order to obtain enough of the peptide for complete analysis, we performed another purification to isolate 300 pmol of adrenomedullin from 40 g of PC tissue which contained a large amount of adrenomedullin. Fig. 1b shows the final purification of the peptide. The elution profiles of absorbance at 210 nm show exact agreement with that of percent increase of platelet cAMP, indicating that the peptide was homogeneous. Furthermore, immunoreactivity of adrenomedullin, described later, was also observed in the same fraction.

RCM-adrenomedullin (100 pmol) was subjected to a gas phase sequencer (Model 470A/120A, Applied Biosystems) and the amino acid sequence was determined up to the 47th residue. Arginylendopeptidase cleavage of RCM-adrenomedullin afforded six fragment peptides, RE1-6, which were separated by reverse phase HPLC using a column of Chemcosorb 3 ODS H. Each fragment was sequenced by a gas phase sequencer and the complete amino acid sequence of adrenomedullin was finally determined (Fig. 2a). Adrenomedullin, consisting of 52 amino acids, was found to have one intramolecular disulfide bond. The carboxy terminal Tyr was amidated, because RE6, native adrenomedullin [45-52], eluted at the same position as synthetic adrenomedullin [45-52]NH_2 on reverse phase HPLC (data not shown). The structure of adrenomedullin was confirmed by chromatographic comparison with native adrenomedullin as well as a synthetic specimen, which was prepared according to the determined sequence. A computer search (PRF-SEQDB, Protein Research Foundation, Osaka, Japan) indicated that identical peptide sequences have not been reported. Thus, we concluded that adrenomedullin is a new biologically active peptide. As shown in Fig. 2b, the sequence homology of adrenomedullin with human CGRP (11), CGRP II (12) and amylin (13), is not high, although they share a six residue ring structure formed by an intramolecular disulfide linkage and the C-terminal amide structure. Note that the 14 residue amino terminal extension in adrenomedullin is not found in CGRP and amylin.

Fig. 3a shows platelet cAMP elevating activity of adrenomedullin compared with that of CGRP and amylin. Adrenomedullin significantly increased rat platelet cAMP up to 4 fold at low concentrations in a dose dependent fashion, in a manner similar to that of CGRP. However, the ED_{50} value of adrenomedullin is 94 nM, a little higher than that of CGRP (35 nM). Interestingly, amylin, which shows higher sequence homology with CGRP, did not increase rat platelet

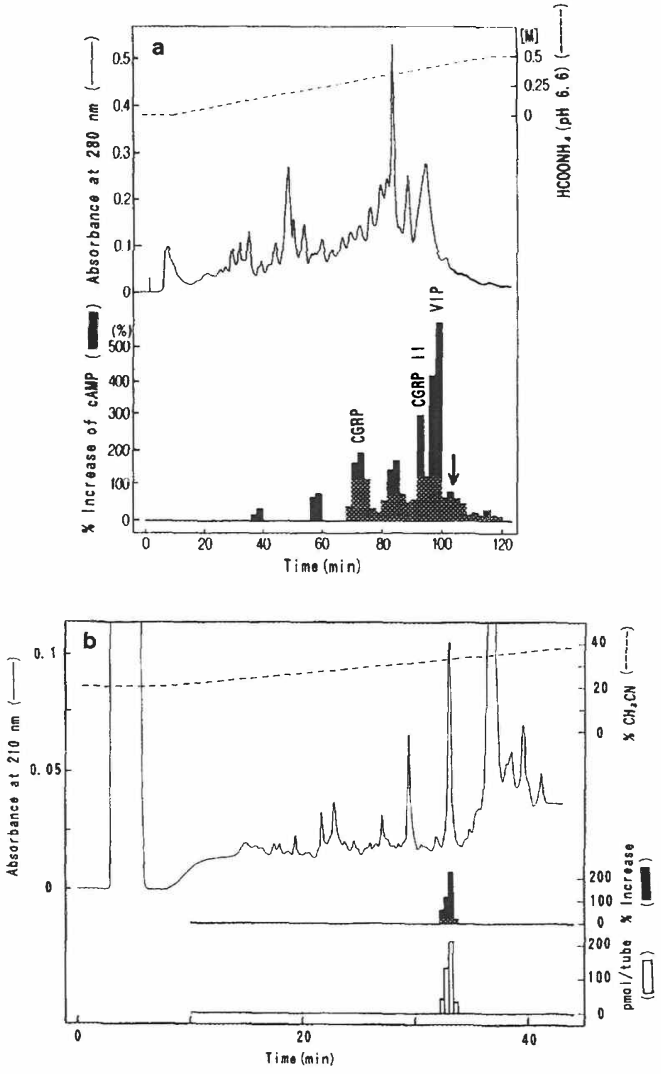

Fig. 1. Purification of adrenomedullin from PC extracts. **(a)** Ion exchange HPLC of peptide which elevates platelet cAMP. Sample, bioactive fraction in strong basic peptide fraction (SP-III) from PC; Column, TSK CM-2SW (4.6 x 250 mm, Tohso); flow rate, 1.0 ml/min; solvent system: linear gradient elution from A:B=100:0 to A:B=50:50 (120 min). (A), 10 mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v); (B), 1.0 M $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v). Three major peaks of activity and several minor peaks were observed. VIP, CGRP and CGRP II were isolated from major peaks (1). Adrenomedullin was initially isolated from the arrowed peak. **(b)** Final purification of adrenomedullin from PC. Sample, adrenomedullin fraction, obtained by CM-HPLC under the same conditions as in Fig. 1a; Column, phenyl (4.6 x 250 mm, Vydac); flow rate, 1.0 ml/min; solvent system, linear gradient elution from A:B=80:20 to A:B=0:100 (80 min). (A)H_2O:CH_3CN:10% TFA = 90:10:1 (v/v); (B)H_2O:CH_3CN:10% TFA = 40:60:1 (v/v). Absorbance at 210 nm (——) and % increase in rat platelet cAMP (■) as well as adrenomedullin immunoreactivity (□) were monitored.

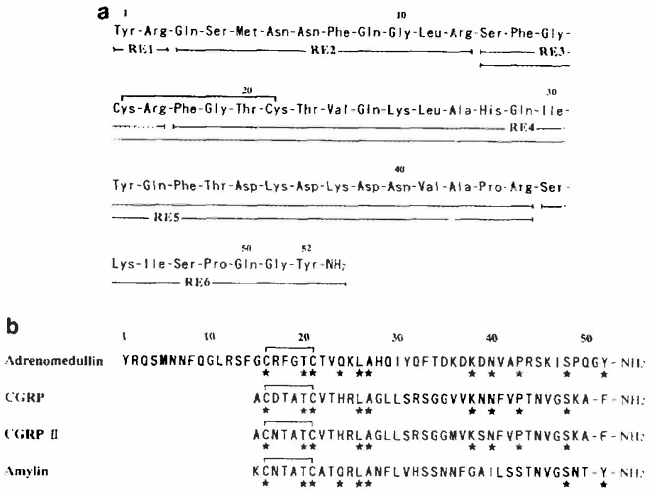

Fig. 2. (a) Complete amino acid sequence of adrenomedullin. Sequence analysis was performed with RCM-adrenomedullin and its fragments generated by arginylendopeptidase (designated RE1-6). (b) Comparison of amino acid sequence of adrenomedullin with human CGRP, CGRP II and amylin. Identical residues between adrenomedullin and other peptides are starred.

cAMP. The stimulation of platelet cAMP production by adrenomedullin is thought to be via a specific receptor on the platelet membrane.

In our previous study, we isolated VIP and CGRP from PC tissue by monitoring activity which increases rat platelet cAMP (1). In view of the potent vasorelaxant effect of these peptides (3,4), we examined the vasodepressor effect of adrenomedullin in anesthetized rats. Typical hypotensive profiles for anesthetized rat are shown in Fig. 3b. An intravenous bolus injection of adrenomedullin caused a rapid, strong and long lasting hypotensive effect in a dose dependent manner. When adrenomedullin at 3 nmol/kg was injected intravenously, the maximum decrease of mean blood pressure was 53 ± 5.0 mmHg (mean ± S.E.M., n=4). This significant hypotensive effect lasted for 30-60 min. As seen from Fig. 3b <3> and <4>, the hypotensive activity of adrenomedullin is comparable to that of CGRP which has been established as one of the strongest vasorelaxants (14). Consequently, it was concluded that adrenomedullin has a potent and long lasting hypotensive effect.

In concordance with the N-terminal sequence determined above, we raised an antibody against a fragment corresponding to the sequence [3-12], which is generated from adrenomedullin by trypsin digestion. As shown in Fig. 4, half maximal inhibition of radioiodinated ligand binding by adrenomedullin [1-12] and [3-12] was observed at 10 fmol/tube. The RIA recognized adrenomedullin with high affinity after trypsin digestion, but did not do so without such digestion. Using an RIA coupled with identification of the peptide by reverse phase HPLC, the distribution of adrenomedullin was investigated in human tissue (Table 1). PC tissue was found to be very rich in adrenomedullin, containing 1,900 ± 450 fmol/mg wet weight. The peptide was

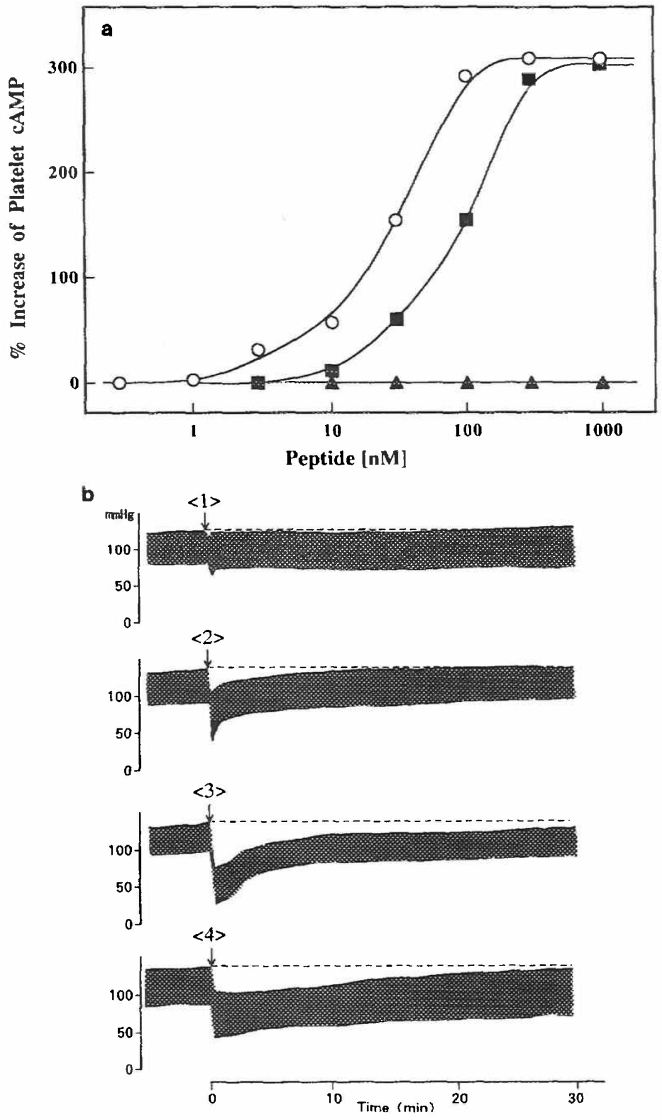

Fig. 3. (a) Elevation of rat platelet cAMP in response to various doses of human CGRP (—○—○—), amylin (—▲—▲—) and adrenomedullin (—■—■—). Half-maximal elevations of CGRP and adrenomedullin are 35 nM and 94nM, respectively. (b) Typical recordings for hypotensive responses in anesthetized rats to intravenous bolus injection of <1> adrenomedullin (0.3 nmol/kg); <2> adrenomedullin (1 nmol/kg); <3> adrenomedullin (3.0 nmol/kg); <4> CGRP (3.0 nmol/kg). The maximum decreases of mean blood pressure (mean ± S.E.M., n=4) were <1> 15 ± 5.0 mmHg; <2> 37 ± 4.8 mmHg; <3> 53 ± 5.0 mmHg; <4> 52 ± 6.5 mmHg .

also abundant in normal adrenal medulla at a concentration of 150 ± 24 fmol/mg. The concentration of adrenomedullin in lung and kidney is less than 1% of that of normal adrenal medulla. But the total amount of adrenomedullin in lung and kidney cannot be disregarded, because it is much

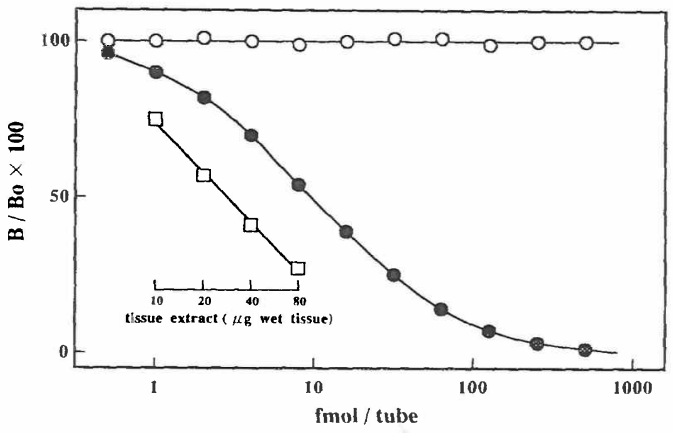

Fig. 4. Standard curve of radioimmunoassay for adrenomedullin [1-12] and [3-12] (—●—●—). This RIA has no crossreactivity with adrenomedullin without trypsin digestion (—○—○—). Inhibition of ^{125}I-adrenomedullin [1-12] binding to the antiserum by serial dilution of PC sample after trypsin digestion (— — —), which is roughly parallel to that of standard adrenomedullin [1-12] and [3-12].

larger than the total amount in adrenal medulla. It should be emphasized that adrenomedullin was not detectable in brain, even though CGRP localizes in brain and peripheral nerves where it functions as a neuropeptide (15). Furthermore, our preliminary experiments have shown that adrenomedullin exists in healthy human plasma in a considerable concentration (19 ± 5.4 fmol/ml, n=4). Thus, it is most likely that adrenomedullin, which is produced in peripheral tissue, adrenal medulla, lung and kidney, functions as a circulating hormone participating in blood pressure control. In addition, enhanced production of adrenomedullin in PC may be relevant to a wide variety of symptoms in PC patients, such as orthostatic hypotension (16).

Table 1. Distribution of immunoreactive adrenomedullin in human PC and normal human tissue

Tissue	Immunoreactive adrenomedullin (fmol/mg wet tissue)		
Pheochromocytoma	1,900	±	450
Adrenal medulla	150	±	24
Lung	1.2	±	0.16
Kidney	0.15	±	0.012
Brain cortex	< 0.1		
Intestine	< 0.1		
Ventricle	< 0.1		

Data are mean ± S.E.M. (n=3). PC tissue was surgically removed from the patient. Normal human tissue was obtained from cadavers.

In conclusion, we have identified a novel hypotensive peptide, adrenomedullin, from human PC. Adrenomedullin is a candidate as an important circulating hormone participating in blood pressure control. The identification of this peptide may open new horizons in cardiovascular research.

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