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Complete amino acid sequence of porcine adrenomedullin and cloning of cDNA encoding its precursor

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Abstract

Porcine adrenomedullin was isolated from adrenal medulla extract and its amino acid sequence was determined The peptide is identical to human adrenomedullin with a single replacement of Gly for Asn at position 40 The cDNA clone encoding the porcine adrenomedullin precursor was isolated and sequenced The precursor for adrenomedullin (preproadrenomedullin) is 188 amino acids in length, including the adrenomedullin sequence, followed by a glycine (the amide donor) In addition to adrenomedullin, proadrenomedullin (proAM) contains a candidate for a unique 20-residue peptide, proAM-N20, whose carboxy-terminus may be amidated By RNA blot analysis, porcine adrenomedullin mRNA was found to be highly expressed in several porcine tissues including lung and kidney as well as adrenal medulla

Key words Adrenomedullin, Peptide purification, Amino acid sequence, cDNA cloning, Precursor structure

1. Introduction

Adrenomedullin (AM) is a novel hypotensive peptide found in human pheochromocytoma [1]. The peptide, consisting of 52 amino acids, has one intramolecular disulfide bond and shows slight homology with calcitonin gene related peptide (CGRP) AM elicits a potent and long-lasting hypotensive effect in anesthetized rat The hypotensive activity of AM is comparable to that of CGRP which has been established as one of the strongest vasorelaxants [2]. In studies of the regional distribution of immunoreactive AM in human tissue, it was abundant in normal adrenal medulla as well as pheochromocytoma tissue, but was not detectable in brain AM is thought to be a candidate for an important new hormone participating in circulation control because it circulates in blood in a considerable concentration [1].

To further understand the physiological implications and biosynthesis of AM, it is essential to analyze the cDNA encoding AM. As a first step, we have determined the complete amino acid sequence of porcine AM and have succeeded in cloning the cDNA encoding the precursor of porcine AM.

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2. Materials and methods

2 1 *Peptides*

AM (1–12), AM (3–12), AM (45–52)NH_2 and AM (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 AM was prepared by solid-phase methods by Peptide Inc (Osaka, Japan)

2 2 *Radioimmunoassay (RIA) for AM*

RIA for AM was performed as described [1] An RIA sample containing 20 μg of bovine serum albumin (BSA) was incubated with 1 μg trypsin (Worthington) in 100 μl of 0 1 M NH_4HCO_3 for 2 h at 37°C The RIA incubation mixture consisted of 100 μl of AM (1–12) or unknown sample solution, 50 μl of antiserum against AM (1–12) at a dilution of 1 16,000 and 50 μl of ^{125}I-labelled AM (1–12) (18,000 cpm), which was prepared by the lactoperoxidase method [3] After incubation for 24 h, free and bound tracers were separated by the polyethyleneglycol method Radioactivity in the pellet was counted with a gamma counter (ARC-600, Aloka), and assays were performed in duplicate at 4°C

2 3 *Preparation of peptide extract*

Diced pieces of porcine adrenal medulla (18 g) were boiled for 10 min in 10 vols of 1 M acetic acid to inactivate intrinsic proteases After cooling, the mixture was homogenized at 4°C with a polytron homogenizer The supernatant of the extracts, obtained after 30 min centrifugation at 22,000 × *g*, was applied to a Sep-Pak C-18 cartridge column (20 ml, Waters) After washing the column with 0 1% trifluoroacetic acid (TFA), the materials adsorbed onto the column were eluted with 60% CH_3CN containing 0 1% TFA The eluate was concentrated and was used as the starting material in the present study

2 4 *Structural analysis*

Reduced and S-carboxymethylated (RCM) porcine AM was prepared by reduction with 50 mM dithiothreitol in 0 5 M Tris-HCl buffer (pH 8 5) for 4 h at 37°C, followed by treatment with 100 mM sodium monoiodoacetate for 20 min [4] RCM-porcine AM was purified by

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reverse phase HPLC on a μ-Bondasphere C-18 (4.6 × 150 mm, 300A, Waters) column. RCM-AM (100 pmol) was digested with 500 ng of trypsin or chymotrypsin (Worthington) in 0.1 M NH_4HCO_3 containing 0.01% Triton X-100 at 37°C for 3 h. Peptides generated by each digestion were purified by reverse-phase HPLC on a Chemcosorb 30DS-H (2.1 × 75 mm, Chemco, Osaka, Japan) column. RCM-AM and the purified fragments were sequenced with a gas-phase sequencer (470A/120A Applied Biosystems).

2.5. cDNA library construction

Total RNA was extracted from porcine adrenal medulla (4 g) by the guanidine thiocyanate method [5]. Poly(A)$^+$RNA was isolated by oligo(dT)-Latex (Takara Shuzo Co., Ltd., Kyoto, Japan) [6]. Double-stranded cDNA was synthesized from 5 μg of porcine adrenal medulla poly(A)$^+$RNA by the method of Gubler and Hoffman [7]. The cDNA was ligated to *Eco*RI adaptors and was ligated to phage λgt10 arms (Bethesda Research Laboratory) and packaged in vitro by using Gigapack Gold (Stratagene).

2.6. Amplification of the AM cDNA probe by polymerase chain reaction

Two six-amino acid regions, located at positions 3–8 and 35–41 of porcine AM, were chosen for the synthesis of degenerate oligonucleotide sense primers (pAM-S [1] and [2]) and an antisense primer (pAM-A) respectively.

pAM-S [1]
CA(A/G)TCNATGAA(C/T)AA(C/T)TT(C/T)CA(A/G)GG

pAM-S [2]
CA(A/G)AG(C/T)ATGAA(C/T)AA(C/T)TT(C/T)CA(A/G)GG

pAM-A ACNCC(A/G)TC(C/T)TT(A/G)TC(C/T)TT(A/G)TC

Amplification was carried out with 5% of the reverse transcript prepared from adrenal medulla poly(A)$^+$RNA and 5 μM of each of two mixed primers (PAM-S [1] and PAM-A, PAM-S [2] and PAM-A) in a 50 μl reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. After heating at 94°C for 10 min, 0.5 μl (2.5 U) Taq DNA (Perkin Elmer-Cetus, Norwalk, CT) polymerase was added to the reaction. The reaction mixture was overlaid with mineral oil and subjected to 30 cycles of amplification in a DNA cycler (94°C for 50 s, 48°C for 50 s, 72°C for 1 min).

2.7. cDNA cloning and sequencing

The amplified cDNA fragment was labeled by the random-prime method and was used to screen the porcine adrenal medulla cDNA library as previously described [8]. Positive clones were purified, and cDNAs were excised to subclone into Bluescript plasmids. The clone (pPAM-2), which carried the longest cDNA insert, was used for sequencing. Restriction fragments generated from the cDNA insert by digesting with relevant restriction endonucleases (*Sma*I, *Nae*I and *Rsa*I) were subcloned into BlueScript plasmids and were sequenced by the dyeprimer cycle sequencing method using an automated DNA sequencer (373A, Applied Biosystems) as described in the manufacturer's manual.

2.8. RNA blot analysis

Porcine tissue was obtained from a local slaughter house. Total RNA was extracted from each tissue (1 g) by the guanidine thiocyanate method [5]. Poly(A)$^+$RNA (5 μg), isolated by oligo(dT)-Latex, was denatured using glyoxal and dimethylsulfoxide, and was electrophoresed on a 1.0% agarose gel, then transferred to a nylon membrane (Zeta Probe, Bio-Rad). A cDNA insert of pPAM-2 was labeled by the random-primed method and used as a probe for hybridization under the conditions described [8].

3. Results and discussion

We have already established a sensitive RIA system monitoring human AM (3–12) which is generated from adrenomedullin by trypsin digestion [1]. Using this RIA coupled with trypsin digestion, we started purification of porcine AM from adrenal medulla. Porcine adrenal medulla peptide extract, prepared as described in Section 2, was separated by gel filtration chromatography (Sephadex G-50, fine, 3 × 150 cm). One major immunoreactive AM was observed in M_r 5,000–6,000 daltons. The peptides in the fraction were further separated by CM ion exchange HPLC on a column of TSK CM-2SW (8.0 × 300 mm, Tosoh). One major immunoreactive AM was observed at the same position as human AM, and was purified by reverse-phase HPLC using a phenyl column (4.6 × 250 mm, Vydac). The recovery of this peptide was 800 pmol, starting from 18 g of porcine adrenal medulla. As shown in Fig. 1, reverse-phase HPLC of purified AM on a C-18 column gave a single peptide peak showing AM immunoreactivity, confirming the homogeneity of the peptide.

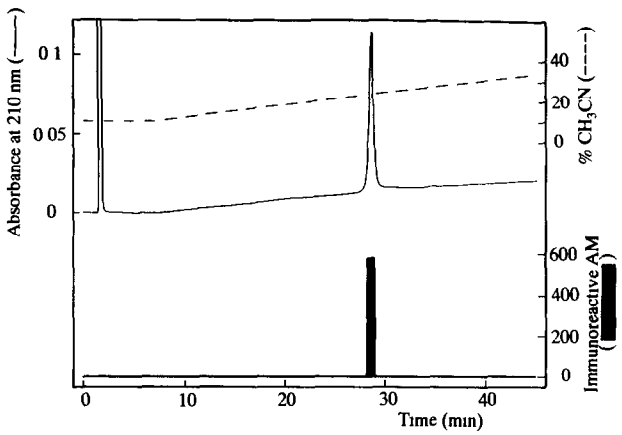

Fig. 1. Reverse-phase HPLC of a portion of purified porcine AM. Sample: 50 pmol of purified AM as described in the text. Column: μ-Bondasphere C18 300A (2.1 × 150 mm, Waters). Flow rate: 0.3 ml/min. Solvent system: linear gradient elution from A:B = 80:20 to A:B = 0:100 (80 min) (A) H_2O/CH_3CN/10% TFA = 90:10:1 (by volume), (B) H_2O/CH_3CN/10% TFA = 40:60:1 (by volume). Absorbance at 210 nm (—) and ir-AM (■) were monitored.

One hundred pmol of RCM-AM was subjected to a gas-phase sequencer and the amino acid sequence was determined up to the 37th residue. By sequence analyses of all fragment peptides generated by trypsin or chymotrypsin digestion, the complete amino acid sequence of porcine AM was finally determined, as shown in Fig. 2. The carboxy-terminal Tyr was amidated, because T-6, corresponding to native AM (47–52), eluted at the exactly same position as synthetic AM (47–52)NH_2 on reverse phase HPLC (data not shown). The amino acid sequence of porcine AM is identical with that of human AM except that Gly at position 40 in porcine AM has been substituted for Asn in human AM. These results indicate that the amino acid sequence of human and porcine AM is well conserved.

In our previous study, we demonstrated that ir-AM was highly localized in human adrenal medulla [1]. The

present study indicated that porcine adrenal medulla is very rich in AM. Therefore, the cDNA library for isolation of clones encoding an AM precursor was constructed with poly(A)$^+$RNA of porcine adrenal medulla.

According to the established amino acid sequence of porcine AM, primer mixtures representing all possible complementary sequences were synthesized for amplifying cDNA of porcine AM When oligo(dT)-primed porcine adrenal medulla cDNA was amplified in a PCR using pAM-S [2] and pAM-A primers, a band of the predicted size (116 base pairs) was visible after electrophoresis on a 2 0% agarose gel containing ethidium bromide. After cloning into pT7Blue T-vector (Novagen, WI), the recombinant clones containing the AM sequence were identified by sequence analysis This amplified cDNA was used as a probe to screen the porcine adrenal medulla cDNA

A portion of the porcine adrenal medulla cDNA library (approximately 2×10^5 p f u) was subjected to a first screening with the amplified probe to isolate 6 positive clones By a second screening of these clones, 4 positive clones were finally isolated. A clone designated pAM-2, which harbored the longest insert of approximately 1,500 base pairs, was used for sequencing According to the strategy indicated in Fig 3, the complete nucleotide sequence of the AM precursor cDNA, which was 1,493 bp long, was determined from both strands Fig 4 shows the complete nucleotide sequence and the deduced amino acid sequence encoded in the open reading frame. A putative initiation codon ATG is located at nucleotides 1–3, preceded by the consensus sequence for the initiation, while a termination codon TAG is found 188 codons later at nucleotides 565–567 A typical polyadenylation signal, AATAAA, is found at nucleotides 1,330–1,335

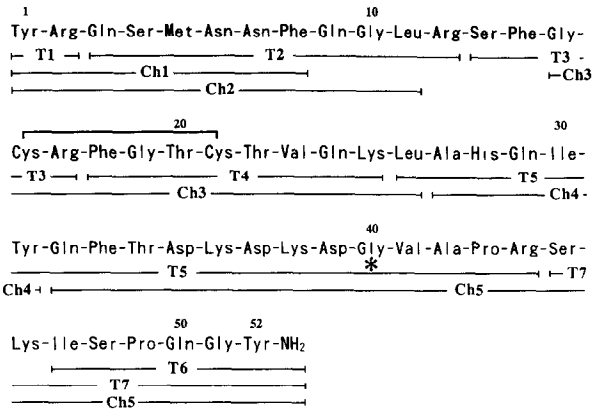

Fig 2 Complete amino acid sequence of porcine AM Sequence analysis was performed with RCM-AM and its fragments generated by trypsin and chymotrypsin (designated T1-6 and Ch1-4) Carboxy-terminal Tyr was amidated Amino acid sequence of porcine AM is identical to human AM [1] with a single replacement of Gly for Asn at position 40 (marked with *)

The deduced amino acid sequence, shown in Fig 4, indicates that porcine AM mRNA encodes a 188-residue protein. The nucleotide sequence of bases 283–438 corresponds exactly to the amino acid sequence of porcine AM isolated from the adrenal medulla Nucleotides 439–441 encode Gly, which is likely to contribute the amide structure [9] to the C-terminal Tyr residue of AM The first 21-residue peptide starting from the initial Met is thought to be a signal peptide, as predicted by its characteristic hydrophobic feature and the weight-matrix method [10] Therefore, it is likely that the first processing of the precursor takes place between Thr^{21} and Ala^{22} to generate the 167-residue proadrenomedullin (pro-AM)

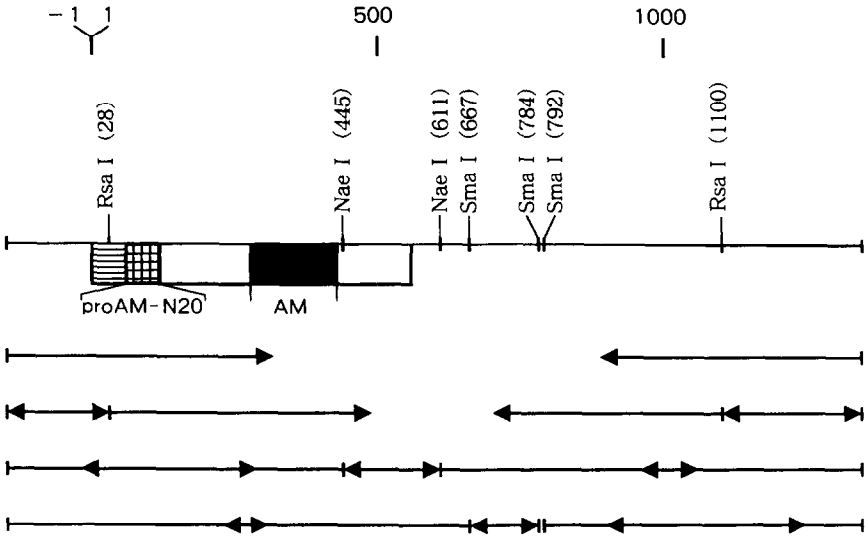

Fig 3 Strategy of sequencing the cDNA insert in clone pPAM-2 The restriction map displays only relevant restriction sites (▤) signal peptide, (▦) proAM-N20, (■) AM Arrows show the direction and extent of sequence determination

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-147                                                GCG GAA CAG CTC GAG CCT TGC CAC CTC   -121
-120 TAG TTT CTT ACC ACA GCT TGG ACG TCG GGG TTT TGC CAC TGC CAG AGG GAC GTC TCA GAC    -61
 -60 TTC ATC TTC CCA AAT CTT GGC AGA TCA CCC CCT TAG CAG GGT CTG CAC ATC TCA GCC GGG     -1

   1 Met Lys Leu Val Pro Val Ala Leu Met Tyr Leu Gly Ser Leu Ala Phe Leu Gly Ala Asp     20
   1 ATG AAG CTG GTT CCC GTA GCC CTC ATG TAC CTG GGC TCG CTC GCC TTC CTG GGC GCT GAC     60
                 ↓
  21 Thr Ala Arg Leu Asp Val Ala Ala Glu Phe Arg Lys Lys Trp Asn Lys Trp Ala Leu Ser     40
  61 ACA GCT CGG CTC GAC GTG GCG GCA GAG TTC CGA AAG AAA TGG AAT AAG TGG GCT CTA AGT    120

  41 Arg Gly Lys Arg Glu Leu Arg Leu Ser Ser Ser Tyr Pro Thr Gly Ile Ala Asp Leu Lys     60
 121 CGT GGA AAA AGA GAA CTT CGG CTG TCC AGC AGC TAC CCC ACC GGG ATC GCC GAC TTG AAG    180

  61 Ala Gly Pro Ala Gln Thr Val Ile Arg Pro Gln Asp Val Lys Gly Ser Ser Arg Ser Pro     80
 181 GCC GGG CCT GCC CAG ACT GTC ATT CGG CCC CAG GAT GTG AAG GGC TCC TCT CGC AGC CCC    240

  81 Gln Ala Ser Ile Pro Asp Ala Ala Arg Ile Arg Val Lys Arg Tyr Arg Gln Ser Met Asn    100
 241 CAG GCC AGC ATT CCG GAT GCA GCC CGC ATC CGA GTC AAG CGC TAC CGC CAG AGT ATG AAC    300

 101 Asn Phe Gln Gly Leu Arg Ser Phe Gly Cys Arg Phe Gly Thr Cys Thr Val Gln Lys Leu    120
 301 AAC TTC CAG GGC CTG CGG AGC TTC GGC TGT CGC TTT GGG ACG TGC ACC GTG CAG AAG CTG    360

 121 Ala His Gln Ile Tyr Gln Phe Thr Asp Lys Asp Lys Asp Gly Val Ala Pro Arg Ser Lys    140
 361 GCG CAC CAG ATC TAC CAG TTC ACG GAC AAA GAC AAG GAC GGC GTC GCC CCC CGG AGC AAG    420

 141 Ile Ser Pro Gln Gly Tyr Gly Arg Arg Arg Arg Arg Ser Leu Pro Glu Ala Ser Leu Gly    160
 421 ATC AGC CCC CAG GGC TAC GGC CGC CGG CGC CGA CGC TCT CTG CCC GAA GCC AGC CTG GGC    480

 161 Arg Thr Leu Arg Ser Gln Glu Pro Gln Ala His Gly Ala Pro Ala Ser Pro Ala His Gln    180
 481 CGG ACT CTG AGG TCC CAG GAG CCA CAG GCG CAC GGG GCC CCG GCC TCC CCG GCG CAT CAA    540

 181 Val Leu Ala Thr Leu Phe Arg Ile ***                                              188
 541 GTG CTC GCC ACT CTC TTT AGG ATT TAG GCG CCT ACT GTG GCA GCA GCG AAC AGT CGC GCA    600

 601 TGC ATC ATG CCG GCG CTT CCT GGG GCG GGG GGC TTC CCG GAG CCG AGC CCC TCA GCG GCT    660
 661 GGG GCC CGG GCA GAG ACA GCA TTG AGA GAC CGA GAG TCC GGG AGG CAC AGA CCA GCG GCG    720
 721 AGC CCT GCA TTT TCA GGA ACC CGT CCT GCT TGG AGG CAG TGT TCT CTT CGG CTT AAT CCA    780
 781 GCC CGG GTC CCC GGG TGG GGG TGG AGG GTG CAG AGG AAT CCA AAG GAG TGT CAT CTG CCA    840
 841 GGC TCA CGG AGA GGA GAA ACT GCG AAG TAA ATG CTT AGA CCC CCA GGG GCA AGG GTC TGA    900
 901 GCC ACT GCC GTG CCG CCC ACA AAC TGA TTT CTG AAG GGG AAT AAC CCC AAC AGG GCG CAA    960
 961 GCC TCA CTA TTA CTT GAA CTT TCC AAA ACC TAG AGA GGA AAA GTG CAA TGT ATG TTG TAT   1020
1021 ATA AAG AGG TAA CTA TCA ATA TTT AAG TTT GTT GCT GTC AAG ATT TTT TTT TGT AAC TTC   1080
1081 AAA TAT AGA GAT ATT TTT GTA CGT TAT ATA TTG TAT TAA GGG CAT TTT AAA ACA ATT GTA   1140
1141 TTG TTC CCC TCC CCT CTA TTT TAA TAT GTG AAT GTC TCA GCG AGG TGT AAC ATT GTT TGC   1200
1201 TGC GCG AAA TGT GAG AGT GTG TGT GTG TGT GTG CGT GAA AGA GAG TCT GGA TGC CTC TTG   1260
1261 GGG AAG AAG AAA ACA CCA TAT CTG TAT AAT CTA TTT ACA TAA AAT GGG TGA TAT GCG AAG   1320
1321 TAG CAA ACC AAT AAA CTG TCT CAA TG                                              1346
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Fig 4 Nucleotide sequence of the cDNA insert in pPAM-2 with predicted amino acid residues Nucleotide residues are numbered beginning with the first residue of ATG triplet encoding a putative initiating methionine, and those on the 5'-site of nucleotide 1 are indicated by negative numbers The termination codon is marked with three consecutive asterisks The AATAAA sequence is double underlined The arrow indicates the potential signal peptide cleavage site to generate the 167 residue proAM Typical sites for proteolytic cleavage to mature peptides are underlined AM and proAM-N20 sequences are boxed Gly residues which contribute to C-terminal amide structure are dotted boxed

It is well known that many peptide hormones and neuropeptides are produced from larger, biologically inactive, precursors through cleavage at pairs of basic amino acids, primarily Lys-Arg and Arg-Arg [11,12]. As shown in Fig. 4, three typical pairs of basic amino acids, which represent sites for the proteolytic processing signal, are found in the predicted mature precursor protein The last two flank the AM peptide and represent sites for proteolytic processing to release AM The first pair of basic amino acids, Lys^{43}-Arg^{44}, is a representative site for proteolytic cleavage, and follows an Arg^{41}-Gly^{42} residue These sequences indicate the possibility that the site converts to Arg-NH_2 as the carboxy-terminus, because the Gly followed by the paired basic amino acid will contribute the amide structure to the C-terminus [9] Therefore, it is possible that a novel 20 residue peptide, termed 'proadrenomedullin N-terminal 20 peptide' (proAM-N20), whose carboxy-terminus may be Arg-NH_2, is processed from the amino-terminal region of proAM. This predicted peptide shows no significant homology with other biologically active peptides. However, a computer search (PRF-SEQDB, Protein Research Foundation, Osaka, Japan) indicated that the amino acid sequence of a glucagon-like peptide 1 receptor (405–424) has significant homology to proAM-N20 (45% identity) [13]. The sequence is not flanked on either side by the typical processing signal to release biologically active peptides. We cannot explain at present the physiological implications of this sequence homology. Although the biological activity of proAM-N20 is obscure, the peptide may have a profound biological function, because the carboxy-terminal amide structure is often observed in many other biologically active peptides An investigation of the biological activity of proAM-N20 is now in progress

Fig 5 shows RNA blot analyses of porcine AM. When the cDNA insert of pPAM-2 was used as an AM probe, an intense AM signal was detected in adrenal medulla at 1 6 kb, and a faint band is also seen at 2 4 kb High levels of AM mRNA were observed in lung and kidney, and lower levels in thyroid, spleen, intestine and ventricle. A very slight mRNA expression in brain is consistent with the fact that immunoreactive AM was not detectable in human brain [1] It is not expected that AM mRNA expression in lung and kidney is comparable to that in adrenal medulla, because as we have already reported

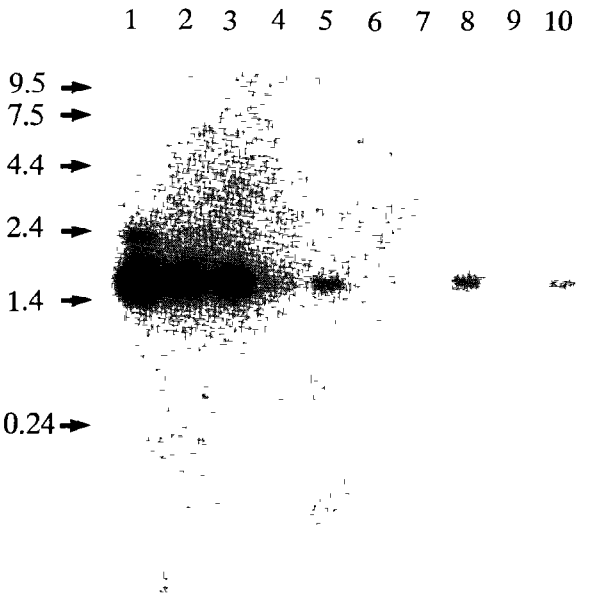

Fig 5 RNA blot analysis of porcine AM transcript in porcine tissues Each lane contained 5 μg of poly(A)$^+$RNA Numbers on the left indicate kb as determined from RNA size markers Lanes (1) adrenal medulla, (2) lung, (3) kidney, (4) ventricle, (5) intestine, (6) brain, (7) liver, (8) spleen, (9) pancreas, (10) thyroid Exposure was at −80°C for 24 h

that the concentration of immunoreactive AM in lung and kidney is less than 1% of that in adrenal medulla [1] This discrepancy may be explained by the possibility that the AM biosynthesized in lung and kidney is rapidly released into the blood In contrast, AM synthesized in adrenal medulla is thought to be stored in granules. The total production of AM in lung and kidney must be much larger than that in the adrenal medulla and it may contribute to the rather high concentration of AM in plasma [1]

In conclusion, porcine AM and its precursor structure was determined The AM precursor contains not only the AM sequence, but a candidate for a novel 20-residue biologically active peptide, proAM-N20, whose carboxy-terminus may be amidated While porcine AM was originally found in adrenal medulla, AM mRNA is highly expressed in several peripheral tissues including lung and kidney as well as adrenal medulla

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