ADRENOMEDULLIN (ADM) is a 52-amino acid peptide that was recently discovered in human pheochromocytoma extracts (14). Under physiological conditions ADM is mainly produced in the adrenal medulla, lung, heart and kidney (14, 22). Rich cellular sources of ADM peptide release are adrenal chromaffin cells, vascular smooth muscle cells, and endothelial cells, which also contain specific binding sites for ADM (4, 13, 31, 32). Similar to other vasoactive peptides, ADM is found in picomolar concentrations in plasma (14), but no single organ source of circulating ADM has been identified. Since ADM production is found at, or close to, sites of action, an autocrine/paracrine role of ADM is believed to be more important than that of a classic hormone. An emerging body of evidence suggests that ADM influences blood pressure directly at the level of the resistance vessels (14) and indirectly by significant effects on salt and water homeostasis. Thus ADM is present in the hypothalamus and is colocalized with vasopressin and oxytocin in neurons (33). Functionally, all levels of the hypothalamus-hypophysis-adrenal axis are affected by ADM (17, 24, 34), and ADM also decreases atrial natriuretic peptide (25) and increases renin (9) mRNA and secretion. Direct effects on salt metabolism are accomplished through combined actions on central nervous control of salt appetite and water drinking (20, 23) and by effects on major renal parameters (2, 9, 11, 16).

ADM mRNA is abundantly expressed in whole kidneys (22), and ADM immunoreactivity has been detected in various segments of the nephron (11). Consequently, ADM of renal origin could potentially play a physiological role in the control of renal salt and water handling. It appears reasonable therefore to consider the intrarenal relation between ADM production and action both from the view of localization as well as of a possible regulation. In the present study, we localized ADM production and ADM receptor (ADM-R) expression by detection of their mRNAs by RT-PCR in microdissected nephron segments. Second, the abundances of mRNAs for ADM and ADM-R were semiquantified by ribonuclease protection in kidney zones. From localizing the intrarenal sites of ADM and ADM-R gene expression, we were interested to find out whether the expression level is subject to physiological regulation. Since ADM has profound effect on salt excretion, it was important to determine whether the intrarenal expressions of the ADM and the ADM-R genes change during renal adaptation to different dietary salt loads.

We found localized expression of ADM and ADM-R in defined nephron segments, and overall abundance of ADM mRNA was about four times higher in cortex than in the papilla, whereas ADM-R mRNA was predominantly expressed in the papilla. Neither ADM nor ADM-R gene expression in any kidney zone was influenced by changes of salt intake.

METHODS

Animals

Male Sprague-Dawley rats weighing 140–160 g served as controls and were fed standard rodent diet (Altromin, NaCl content 0.5% wt/wt). Two age-matched groups of rats (n = 8 each group) were maintained for 10 days on a high-salt diet (HS; 4% NaCl wt/wt) or on a low-salt diet (LS; 0.04% NaCl wt/wt). All rats were allowed free access to tap water. Animals were killed by decapitation, and blood was sampled in EDTA-pretreated vials. Whole organs were rapidly extirpated, weighed, and frozen in liquid nitrogen. Alternatively, kidneys were placed in physiological salt solution on ice, and for each kidney the cortex was carefully dissected from the outer medulla with a scalpel blade. Finally, outer and inner medulla with the papilla were separated. No attempt was made to separate outer and inner stripe of the outer medulla. To obtain sufficient amounts of RNA, the outer medullas from two rats were pooled, and the inner medullas from four rats were pooled. On completion of dissection the different tissue samples were frozen in liquid nitrogen.

Glomeruli from four additional control rats were obtained by a mechanical sieving technique (18). Cortex was passed through a polyamide screen with pore size of 150 µm onto a screen of 50 µm pore size. Glomeruli were collected from this screen, washed once with physiological salt solution and...
resuspended in 5 ml of RNA extraction solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) and quick frozen in liquid nitrogen until RNA extraction. Organs and zones were stored at −80°C until RNA extraction.

**Plasma Renin Activity Measurements**

Plasma samples incubated for 1.5 h at 37°C. The generated angiotensin I was determined by a radioimmunoassay kit (Sorin-Biomedica, Düsseldorf, Germany).

**Microdissection of Rat Renal Nephron Structures**

The left kidney was perfused with ice cold Hanks’ modified microdissection solution (in mM: 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.34 H₃PO₄, 1 MgCl₂, 1 CaCl₂, 4 NaHCO₃, 10 sodium acetate, 5 d-glucose, 20 HEPEs, and 1 mg/ml BSA), and was subsequently perfused with collagenase (1 mg/ml) dissolved in microdissection solution. Thin pyramids cut along the corticomedullary axis were incubated at 35°C for 25 min (45 min for microdissection of inner medulla) in aerated microdissection solution containing 1 mg/ml collagenase, then thoroughly rinsed in ice-cold microdissection solution and kept at 4°C. Microdissection was performed as described (5) to isolate arcuate and cortical radial arteries (these vessels were washed), glomeruli, proximal convoluted tubule, proximal straight tubule (PS), thin limb of Henle loop (TL), thick ascending limb (TAL), distal convoluted tubule (DC), and cortical (CCD) and inner medullary (IMCD) collecting ducts. Pools consisting of identical types of tubules (10–40 mm) or 50–100 glomeruli were washed free of contaminating cells or debris and transferred into denaturing solution for RNA isolation.

**Extraction of RNA**

RNA was extracted from whole organs and dissected kidney zones basically according to the acid-guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi (1). Final RNA pellets were dissolved in diethyl pyrocarbonate-treated water, the yield of RNA was quantified by spectrophotometry at 260 nm, and samples were aliquoted and stored at −80°C until further processing. The quality of extracted RNA was confirmed by the observation of intact 18S and 28S bands after gel electrophoresis in an ethidium bromide-stained agarose gel.

RNA extraction from isolated nephron segments. Isolated nephron segments were transferred into 400 µl of denaturing solution with addition of 15 µg of yeast tRNA. Total RNA was extracted using a microadaptation of (5) the method of Chomczynski and Sacchi (1).

**RT-PCR: Nephron Segments**

RT was performed using a kit from Life Technologies (GIBCO-BRL) with oligo(dT) priming. For cDNA amplification, the primers used were 5′ TGG ATG CCG GCT TGG GGA CCT G G′ (sense, position 468–489 nt) and 5′ AAT GCT GCC ACC CGC ACC TAT 3′ (antisense, position 708–729 nt) for rat ADM (12); 5′ CCT ACT GCC TTT TCC TCT CAT 3′ (sense, position 902–923 nt) and 5′ GCA GAG TGA CGA CGA TAG 3′ (antisense, position 1051–1072 nt) for rat ADM-R (12); and 5′ CGG GAA CTA GAA CCG CAC AGT CA 3′ (sense, position 224–246 nt) and 5′ GGT TTC TCC AGG CGG CAT GTC A 3′ (antisense, position 799–820 nt) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA was submitted to 35 PCR cycles (94°C, 40 s; 60°C for ADM, 57°C for ADM-R, and 54°C for GAPDH, 40 s; and 72°C, 1 min), followed by final elongation for 10 min. As a control of cDNA contamination, tubes without RT or cDNA were run in parallel for all RNA samples.

**Cloning of ADM-R cDNA Sequence**

cDNA specific for ADM-R was positional cloned after RT-PCR for in vitro generation of a labeled cRNA probe for ribonuclease protection assay. Rat lung cDNA was used as template for PCR

RT reaction. One microgram of total RNA, 1.5 µg yeast tRNA, and 0.5 µg oligo(dT) primer (GIBCO) were heated at 94°C for 3 min in a volume of 8 µl (Perkin-Elmer Cetus Thermocycler). Then samples were cooled on ice, and each of the following components (in µl) were added for reverse transcription: 4 deoxyribonucleotides (2.5 mmol/l); 4 RT buffer (supplied with the reverse transcriptase kit), 2 dithiothreitol (100 mmol/l), 0.5 RNasin (40 U/µl, Promega), 0.5 BSA (20 µg, Boehringer), and 1 reverse transcriptase (200 U/µl; GIBCO-BRL). Samples were then incubated for 1 h at 37°C, and the reaction was stopped by heating the samples to 95°C for 2 min.

PCR. To facilitate cloning, primers were synthesized with restriction sites for BamH I and EcoR I in the 5′ direction. Sense 5′ CAT ATC CAG CTG CTG GAT 3′ and antisense 5′ CGG GAG AAG ATT GTA GTA 3′ amplified a 405-bp sequence of rat ADM-R cDNA (12). PCR was performed with 3 µl undiluted cDNA. To the cDNA was added (in µl) 1 of each primer (10 pmol), 2 deoxyribonucleotides (2.5 mmol/l), and 2 PCR buffer (supplied with the Taq polymerase), as well as water to a final volume of 20 µl. The mixture was overlaid with one drop of mineral oil, and the samples were denatured at 94°C for 5 min, followed by annealing at 65°C for 5 min, during which 1 U of Taq polymerase (Boehringer, Mannheim, Germany) was added. PCR was performed for 36 cycles consisting of 1 min denaturation at 95°C, 1 min annealing at 60°C, and polymerization at 72°C for 30 s.

Cloning. Amplified products were verified on a 2% agarose gel, pooled, purified, digested with BamH I/EcoR I (5 U, Pharmacia Biotech) for 2 h, separated on 1% low-melting point agarose gels, excised, purified by phenol/chloroform extraction, and ligated for 16 h at 14°C into BamEco polylinker sites of vector psp73 (Promega) for heat-shock uptake into Escherichia coli (DH5 α, GIBCO). Positive clones were grown, and plasmids were isolated for sequencing and in vitro transcription by a plasmid purification kit (Maxi-kit, Qiagen). Inserts were sequenced by the dideoxy chain termination method by the use of SP6 and T7 polymerases (Sequiseve, Dæsenhofen, Germany). To achieve specific ADM-R probes with different sizes, the plasmid was cut with Hind III (cuts in the polylinker that result in a probe size of 405 bp) or with Hinc II (cuts in the cloned sequence that result in a probe size of 93 bp). The shorter probe allowed simultaneous measurement of ADM and ADM-R in one RNA sample.

**Ribonuclease Protection Assay for mRNAs for ADM, ADM-R, Renin, and GAPDH**

Specific mRNA levels were measured by ribonuclease protection assay as previously described for renin and GAPDH (6, 27). An ADM-specific 420-bp cDNA sequence has previously been cloned for in vitro transcription (9). Plasmids yielded radiolabeled antisense cRNA transcripts by incubation with SP6 polymerase (Promega) and [α-32P]GTP (Amersham) according to the Promega riboprobe in vitro transcription protocol. A quantity of 5 × 10⁶ cpm of the cRNA probes solution hybridized with total RNA at 60°C for 16–18 h, then digested with RNase A/T1 (RT for 30 min) and proteinase K (37°C for 30 min). After phenol/chloroform extraction and...
ethanol precipitation, protected fragments were separated on a 8% polyacrylamide gel, the gel was dried for 2 h, signals were quantitated in a Phosphorimager (Instant Imager, Packard), and autoradiography was performed at \(-80^\circ\text{C}\) for 1–3 days.

Validation of Ribonuclease Protection Assay for ADM and ADM-R mRNA

The range where yield for ADM and ADM-R RNA hybrids (cpm) is a linear function of the amount of assayed total RNA was determined. Figure 1 shows the radioactivity incorporated into ADM and ADM-R cRNA-mRNA hybrids as a function of the assayed amount of kidney total RNA. Linearity between the amount of total RNA assayed and the activity from the hybrids was observed in the tested range. All further ADM and ADM-R assays were therefore performed with 20 µg of total RNA.

Statistics

Levels of significance between groups were calculated using the unpaired Student’s \(t\)-test. \(P < 0.05\) was considered significant.

RESULTS

Distribution of ADM mRNA and ADM-R mRNA in the Kidney

Initial RT-PCR experiments revealed significant expression of ADM and ADM-R mRNA in all renal zones and in arcuate and cortical radial arteries (Fig. 2A). There was a clear trend toward a decrease of ADM mRNA in the corticopapillary direction, whereas ADM-R mRNA increased. ADM mRNA was at the limit of detection in the vessels, but the receptor was abundantly expressed in this preparation (Fig. 2A).

In the next experiments, ADM and ADM-R gene expression along rat renal nephron segments was determined by RT-PCR (Fig. 2B). No products were detected in the absence of RT or cDNA. A single band of the expected size for ADM (261 bp) was detected in glomeruli, in the PC and PS tubules, and in the IMCD. ADM cDNA was not observed in DC tubules, CCD, TL, or TAL. A single 170-bp amplification product for ADM-R was detected in glomeruli, in DC tubules, and in IMCD. (Fig. 2B). No ADM-R transcripts were found in the other nephron segments tested. GAPDH mRNA could be amplified in all nephron segments (597 bp). Thus ADM and ADM-R mRNAs were colocalized in vessels, glomeruli, and in IMCD.
To semiquantify ADM and ADM-R mRNA in renal zones, ribonuclease protection assays were performed with total RNA from the renal cortex, outer medulla, and papilla. The autoradiograph in Fig. 3A illustrates the uneven distribution of ADM and ADM-R mRNAs in renal zones. Because there was no significant difference between GAPDH mRNA levels in the three zones (cortex 908 ± 33; outer medulla 906 ± 82; and papilla 892 ± 93 cpm/µg total RNA; values are averages ± SE of 10, 5, and 3 measurements for each zone), GAPDH

![Image of autoradiograph ribonuclease protection assay for ADM and ADM-R mRNAs in kidney zones.](image)

**Fig. 3.** A: autoradiograph ribonuclease protection assay for ADM and ADM-R mRNAs in kidney zones. Single RNA hybrid bands for ADM and ADM-R are seen in all the following zones: K, whole kidney; C, cortex; O, outer medulla; and P, papilla. Most intensive labeling for ADM is seen with cortex RNA. Intensity of ADM-R hybrids increases in the medullary zones compared with cortex. B: autoradiograph ribonuclease protection assays for ADM and ADM-R mRNAs in glomeruli and cortex. Single cRNA-mRNA hybrid bands for ADM and ADM-R are seen in all lanes. C: quantification of ADM and ADM-R mRNAs in kidney zones. Values are means ± SE obtained from 10 RNA samples for cortex and outer medulla and from 3 samples for the papilla. Data are shown as the ratio between cyclooxygenase (COX)/GAPDH mRNA × 10⁻³. *P < 0.05 cortex vs. medullary zones.
was used as a standard internal control for normalization of zonal mRNAs. Quantification done in this way revealed heterogeneous mRNA levels along the corticopapillary axis. ADM mRNA was distributed with ratio of cortex/outer medulla/papilla of 1.3:0.3:0.3. On the contrary, ADM-R was more abundant in the renal medulla with ratio of 1:3:4 for cortex/outer medulla/papilla (Fig. 3C). We also compared the abundance of ADM and ADM-R mRNAs in glomeruli with that of cortex by ribonuclease protection assay (Fig. 3B). When GAPDH-corrected values were compared by this method (GAPDH: cortex 908 ± 33; glomeruli, 852 ± 55 cpm/µg total RNA; average ± SE of 10 and 4 determinations, respectively), ADM mRNA was 3-fold more abundant in glomeruli versus cortex, but ADM-R mRNA was 10-fold lower in glomeruli compared with whole cortex. Thus ADM mRNA is primarily expressed in cortex with lower levels in medulla, whereas ADM-R expression increases in the corticopapillary direction.

**Influence of Dietary Salt Intake on Abundance of ADM and ADM-R mRNAs**

The next series of experiments was designed to investigate the possible regulation of renal ADM and ADM-R mRNA by dietary salt load. For that purpose, RNA was isolated from kidney cortex, outer medulla, and papilla from rats that were kept 10 days on three different levels of salt intake. To control for the efficiency of the diet, plasma renin activity was measured in the three groups of rats after 10 days (n = 8, each group). In control rats, plasma renin activity was 6.5 ± 0.98 ng ANG I·h⁻¹·ml⁻¹. In rats on a high-sodium intake plasma renin was suppressed to 2.1 ± 0.29 ANG I·h⁻¹·ml⁻¹, and in sodium-deprived rats the value was 15.9 ± 1.4 ANG I·h⁻¹·ml⁻¹. GAPDH mRNA abundance was not influenced by dietary salt intake in any kidney zone (not shown). We therefore used GAPDH as an internal standard for RNA quality. Quantification done this way did not reveal any differences in zonal ADM or ADM-R mRNA abundance in response to dietary salt load (Fig. 4). We conclude that dietary salt intake has no influence on renal ADM and ADM-R mRNA levels.

**DISCUSSION**

In the present study we aimed to characterize the renal cellular localization of mRNAs for ADM and ADM-R and their potential regulation by salt intake. To study the expression pattern of ADM and ADM-R along the rat nephron, we used combined ribonuclease protection assays and RT-PCR on microdissected, defined nephron segments. ADM mRNA was predominantly localized in the renal cortex. In cortex, ADM expression was detected in vessels, glomeruli, PC, and PS tubules. In the medulla, only the IMCD was identified as a tubular segment with significant ADM expression. Thus our findings on the presence of ADM mRNA in glomeruli and IMCD confirm previous data about ADM production (11, 19). In addition, our results suggest that proximal tubules are also relevant sites of ADM expression, whereas

![Fig. 4](http://ajprenal.physiology.org/) Influence of dietary salt intake on zonal expression of ADM and ADM-R mRNA. Influence of dietary salt intake on cortical (A), outer medullary (B), and papillary (C) ADM and ADM-R mRNA levels. No difference in GAPDH-normalized specific mRNA quantity was observed. For each diet type, values are means ± SE obtained from 8, 5, and 3 RNA samples from cortex, outer medulla, and inner medulla, respectively.

Jougasaki et al. (11) detected ADM immunoreactivity in the DC tubule, a segment in which we were unable to amplify ADM transcripts. Since this segment is a major target site for ADM, the immunohistochemical signals could therefore represent peptide bound to receptors or internalized by the tubular cells. We also found ADM mRNA in renal resistance vessels (arcuate and cortical radial arteries), although no ADM immunoreactivity was detected in the renal vasculature (11). ADM mRNA has previously been discovered in small and large caliber renal vessels in adult animals and in embryos (9, 19, 28). These apparently discrepant findings could be caused by a rapid release of ADM peptide. Indeed, cultured smooth muscle and endothelial cells do not contain ADM peptide stored in secretory granules but release large amounts of ADM constitutively (31). Alternatively, the discrepancy could simply reflect different sensitivities of the methods used.
ADM-R mRNA was mainly found in the tubular system, namely in DC tubule and in the IMCD. This matches a previous report in which ADM significantly raised cAMP in DC tubule (3). In contrast to that study, however, we obtained evidence for the expression of the ADM-R mRNA in the medullary collecting duct rather than in the cortical TAL (3). Moreover, we found ADM-R mRNA expression in renal resistance vessels and a relative low expression in glomeruli. In accordance, ADM induces an increase in cAMP production in isolated glomeruli (3) and in mesangial (15) and juxtaglomerular cells (9), which suggests functional ADM-R at juxtaglomerular sites. The relatively low expression level of ADM-R mRNA in glomeruli when compared with whole cortex is indeed compatible with functional studies in which ADM peptide stimulated cAMP formation fourfold stronger in DC tubules than in glomeruli (3). The DC tubule is therefore likely to be the cortical structure with the highest level of functional ADM-R expression. Also in agreement with a widespread renal vascular expression of the ADM-R is the fact that most renovascular segments dilate in response to ADM (7, 16, 28).

Altogether, our findings suggest that ADM production and ADM-R expression colocalize in renal vessels (including glomeruli) and in the IMCD, a characteristic that has already been found in cultured smooth muscle (4, 31, 32) and in various tissues in vivo (19). ADM could therefore exert an autocrine control of cellular function in these structures. Moreover, in view of the predominant expression of ADM in the renal cortex and the predominant expression of ADM-R in the papilla, it is conceivable that cortical ADM could influence the function of the renal papilla by acting on IMCD cells. Possible transport pathways for ADM peptide could include the tubular fluid but also the bloodstream via the vasa recta. ADM acting on DC tubule cells could be derived from the glomeruli but also from proximal tubules.

In view of this potential intrarenal control of renovascular resistance and salt excretion by ADM, it was of interest to find out whether the intrarenal ADM system is regulated by salt intake such as other systems involved in the renal salt handling, such as renin (8), angiotensin II receptors (26), NO synthases (30), or cyclooxygenases (10). However, our findings indicate that renal mRNAs for ADM and ADM-R are not regulated by dietary salt load in any renal zone. Certainly, our molecular biology approach would not detect any posttranslational influence of salt intake on ADM production or ADM-R. Up to now, however, no posttranslational control of ADM production has become obvious. It has been found that ADM is not stored, but is quickly released (31), and therefore the ADM secretory capacity depends on a constant transcription and translation of the ADM gene. Thus ADM mRNA and ADM secretion correlate directly (29, 31, 32), and it can be assumed that this holds also for the kidney. Interestingly, plasma ADM concentrations have been found to increase during salt load, and indeed in the heart, ADM mRNA abundance correlates directly with salt intake (29).

Therefore, the increase in plasma ADM during salt loading could be of cardiac origin. Whether these elevated plasma levels of ADM are of relevance for kidney function in vivo, in particular for the physiological control of salt excretion, remains to be demonstrated.

While this article was in review, a study by Owada et al. (21) was published that demonstrated the existence of ADM-mRNA in glomeruli and IMCD cells and provided evidence for a stimulation of cAMP formation by ADM in glomeruli and in IMCD cells. These data are consistent with the profile of the expression of ADM and ADM-R found in the present study.

We gratefully acknowledge the expert technical and graphical assistance of Karl-Heinz Götz and Marlies Hamann and the secretarial help provided by Hannelore Trommer. We thank Peter Sandner for assistance with cloning ADM.

This study was supported by grants from the University of Copenhagen, by the Danish Health Science Research Council, by Danske Laegers Forsikring under Codan Forsikring (to B. L. Jensen), and by Deutsche Forschungsgemeinschaft Ku/859 2–3 (to A. Kurtz). Address for reprint requests: B. L. Jensen, Institut für Physiologie I, Universität Regensburg, Postfach 101042, D-93040 Regensburg, Germany.

Received 1 October 1997; accepted in final form 4 March 1998.

REFERENCES


