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Atrial natriuretic factor mRNA and binding sites in the adrenal gland

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The factor inhibiting aldosterone secretion produced by the adrenal medulla may be atrial natriuretic factor (ANF), since the latter abolishes aldosterone release in response to a number of secretagogues, including angiotensin II and K^+. In this study we have shown that cells in the adrenal medulla contain ANF mRNA and therefore have the potential to synthesize this peptide. The presence of binding sites for ANF predominantly in the adrenal zona glomerulosa suggests that, if ANF is synthesized in the medulla and transferred to the cortex, it may affect mineralocorticoid status.

INTRODUCTION

Atrial natriuretic factor (ANF) is a circulating 28-residue peptide [1–4] with potent pharmacological effects on the kidney [5–7]. It also alters plasma levels of adrenal steroids and their release from cultured adrenal cortical cells [8–14]. Immunoreactive ANF has been found in the adrenal medulla [15,16]. However, this does not necessarily indicate local synthesis, and it is possible that the antisera cross-reacted with brain natriuretic peptide (BNP), a recently isolated peptide which has a striking sequence similarity to ANF [17] and binds to ANF receptors [18,19]. More recently, bovine adrenal chromaffin granules and cultured adrenal chromaffin cells have been shown to contain and release ANF into the culture medium [20,21], but phenotypic alteration may have occurred in culture. The pharmacological profile of the adrenal medullary aldosterone-secretion-inhibitory factor identified by De Lean and colleagues suggests that it may be ANF or BNP [22,23]. The present study was designed to study the distribution of ANF binding sites and mRNA within the adrenal gland as part of the investigation of the hypothesis that ANF, synthesized locally within the adrenal medulla, acts on specific receptors to control steroid secretion from the adrenal cortex.

METHODS

Fresh bovine adrenal glands were collected from the local abattoir and rapidly frozen on solid CO_2 before being mounted on to microtome chucks or homogenized for RNA preparation. Adrenals, kidneys, livers and hearts were rapidly removed from adult male Porton–Wistar rats (approx. 300 g) and were processed similarly.

For receptor autoradiography *in vitro* and '*in situ*' hybridization, 10 μm frozen tissue sections were thaw-mounted on to gelatin-coated slides.

Receptor autoradiography *in vitro*

Receptor autoradiography *in vitro* was performed as described by Mantyh *et al.* [24]. Non-specific binding was assessed by co-incubating adjacent control sections with ^{125}I-labelled rat ANF and 1 μM unlabelled rat ANF-(3–28). The dry sections were exposed to autoradiography film [X-Omat XS1 (Kodak) or Hyperfilm βmax (Amersham International)].

'*In situ*' hybridization

'*In situ*' hybridization was performed as previously described using a 42-mer synthetic ^{35}S-5'-end-labelled oligodeoxynucleotide complementary to the mRNA coding for ANF-(103–116) (5' CTGGGCTCCAATCCTGTCCATCCTGCCCCCGAAGCAG-CTGGA) [25]. After overnight hybridization at 37 °C in 50% formamide, the sections were washed with increasing stringency (down to 20 mM-NaCl + 14 mM-β-mercaptoethanol at 37 °C for 1 h), progressively dehydrated in ethanol solutions, air-dried and co-exposed to Hyperfilm βmax with tissue paste standards containing increasing amounts of ^{35}S [26]. The autoradiographical images were processed using a Quantimet 970 computer-assisted image analysis system (Cambridge Instruments, Cambridge, U.K.) [27]. The images of the ^{35}S-labelled standards were utilized to create a standard curve of $\log_e$(photographic optical density) versus $\log_e$ (radioactivity) from which the computer calculated the amount of ^{35}S-labelled ANF mRNA probe bound to any part of the tissue section by using the specific radioactivity of the labelled probe. Adjacent control sections were pre-digested with RNAase A as we have described before [25]. The non-specific binding to RNAase-treated sections was digitally subtracted from the total bound.

Northern analysis

Total cellular RNA was extracted from bovine and rat adrenal glands and rat kidneys, liver, and cardiac ventricles and right atria by a modified guanidinium isothiocyanate method [28]. Total RNA (20 μg) was denatured, separated on a 1% agarose/formaldehyde gel and transferred to a Hybond N membrane (Amersham) using a Vacugene blotter (LKB) as directed by the manufacturers. The membrane was then hybridized with the ^{35}S-labelled 42-mer ANF anti-sense oligonucleotide and washed under the same conditions as those used for '*in situ*' hybridization, as we have reported before [25].

The relative amounts of RNA present in each lane were assessed by the ethidium fluorescence of the 28 S and 18 S RNA bands.

cDNA amplification

Total RNA (20 μg) from bovine and rat adrenals and rat right atria and kidneys were reverse-transcribed with oligo(dT) as we have reported before [29]. One-twentieth of the cDNA was

Abbreviations used: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; PCR, polymerase chain reaction.

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amplified for 40 cycles (93 °C for 30 s, 55 °C for 30 s and 73 °C for 3 min; final extension 73 °C for 15 min) by the polymerase chain reaction (PCR) using 3 units of Taq DNA polymerase (Cambio, Cambridge, U.K.), the supplier's reaction buffer and 1 μM each of specific oligodeoxynucleotide primers. For the detection of ANF cDNA, one of the two primers was the 42-mer anti-sense oligonucleotide used for '*in situ*' hybridization. The other (5′ TACAGTGCGGTGTCCAACACAGATCTGATGG ATTTCAAG) was designed from published ANF cDNA and genomic sequences [30–32] to span an intron and therefore differentiate the amplification of cDNA (predicted length 340 bp) from that of genomic DNA (predicted size 460 bp). It also permitted amplification of rat and bovine cDNAs because of substantial inter-species sequence similarity. To provide control amplification products for the Southern analysis, rat kidney cDNA was amplified with pairs of primers designed from the sequence of the rat ANF 'A' receptor (5′ AAGAGCCTG-ATAATCCTGAGTACT and 5′ TTGCAGGCTGGGTCCTC-ATTGTCA) [33], the human 'B' receptor (5′ CACCAGGT-GTATGCCCGAGAGCCA and 5′ CCGTCCCGTCCACCAA-ATCTGCTT) [19] and the bovine 'C' receptor (5′ ATCG-TGCGCCACATCCAGGCCAGT and 5′ TCCAAAGATATC-ACCAATAACCTCCTGGGTACCCGC) [34]. All of the PCR products were separated by agarose gel electrophoresis, blotted on to Hybond N membranes and probed with a ^{35}S-3′-end-labelled oligonucleotide (5′ GAGGGCAGATCTATCGGAG-GAGTCCCAGGG) complementary to a consensus ANF sequence occurring between the amplification primers which was designed to detect rat, bovine and human ANF cDNA. After stringent washing (down to 20 mM-NaCl + 14 mM-β-mercapto-ethanol + 0.1 % SDS at 42 °C), the blots were dried and exposed to autoradiography film.

RESULTS AND DISCUSSION

After receptor autoradiography *in vitro*, the greatest density of specific ANF binding sites in the rat and bovine adrenal glands was found in the zona glomerulosa, with lower levels in the zona fasciculata and zona reticularis (Fig. 1). The levels of binding detectable in the medulla were much lower. These results are in agreement with previous autoradiographical studies [24,35,36]. Although only a single concentration of ^{125}I-ANF was used in these studies, the binding characteristics reported for ANF receptors [19,33,34] indicate that all three receptor types would be detected. We have not attempted to assess competition using unlabelled BNP in addition to ANF, since BNP and ANF appear to interact very similarly with the binding sites defined by ^{125}I-BNP and ^{125}I-ANF [18].

We have previously validated the use of this oligonucleotide ANF probe for '*in situ*' hybridization in the rat heart [25], and have continued to use synthetic oligomers for quantification because we have found that large cDNAs always produce weaker '*in situ*' hybridization signals [25]. Also, because of their size, large probes are more prone to non-specific hybridization to partially related sequences which cannot be overcome by high-temperature hybridization and washing due to disruption of the tissue section. Following digital subtraction of background RNAase A-resistant binding of the oligonucleotide ANF probe, ANF mRNA was localized within the bovine adrenal medulla, with much lower levels in the cortical zones (Fig. 2). A clear decrease in the binding of the probe was seen after treatment of adjacent control sections with RNAase A, which digests single-stranded RNA, including mRNA, and permits assessment of non-specific probe binding. Indeed, it was only after digital subtraction of this background binding from the total that the hybridization was shown to be predominantly medullary, sup-

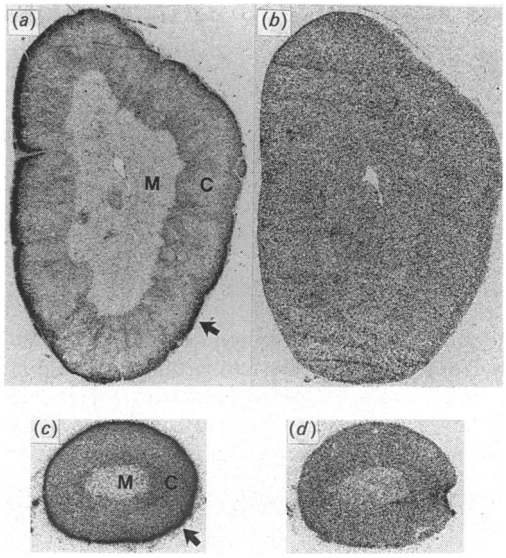

Fig. 1. ANF binding sites in the adrenal gland

Autoradiographical images of bovine (*a*) and rat (*c*) adrenal sections labelled with ^{125}I-ANF; non-specific binding in the presence of 1 μM-ANF is shown in (*b*) and (*d*) respectively. The highest density of binding sites was present in the zona glomerulosa (arrow) of the cortex (C). The lowest levels of binding were present in the medulla (M).

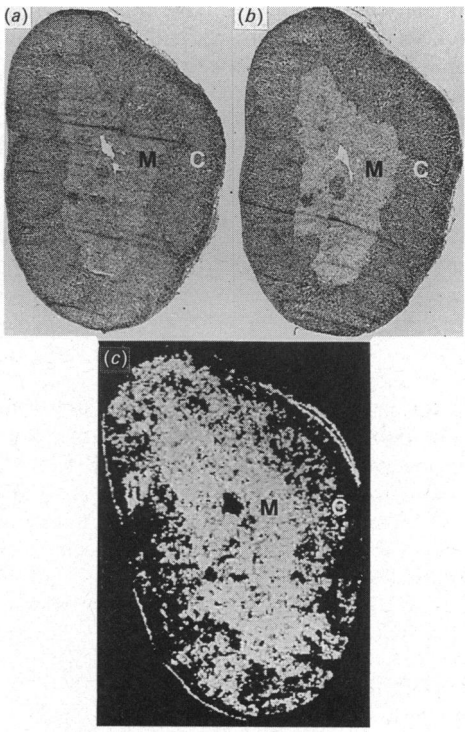

Fig. 2. Localization of ANF mRNA in the adrenal gland by '*in situ*' hybridization

Autoradiographical images of bovine adrenal sections after '*in situ*' hybridization with a specific 42-mer ^{35}S-labelled ANF probe. The non-specific binding image (*b*) obtained after RNAase A digestion was digitally subtracted from the 'total' (*a*) to produce a specific image (*c*), which is shown here as a monochrome photograph of the colour-coded computer-generated image. Specific probe hybridization was greater in the medulla (M) than in the cortex (C).

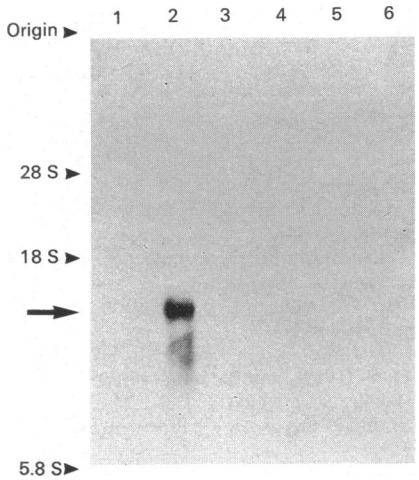

Fig. 3. Northern analysis of rat and bovine RNAs with an ANF mRNA probe

Identification of ANF mRNA by Northern analysis of rat ventricular (1), rat right atrial (2), rat adrenal (3), bovine adrenal (4), rat liver (5) and rat kidney (6) total RNA. After electrophoresis under denaturing conditions, the RNA was transferred to Hybond N membranes and hybridized with the 42-mer ^{35}S-labelled oligonucleotide used for '*in situ*' hybridization. The autoradiogram shows a major band of radioactivity (large arrow) in lane 2 (right atrium) corresponding to the expected position of ANF mRNA (relative to the positions of the rRNA bands). No bands were seen in the other lanes. There were no significant differences in the ethidium fluorescence of the 28 S and 18 S rRNA bands in lanes 1–6 (results not shown). The positions of 28 S, 18 S and 5.8 S rRNA are indicated.

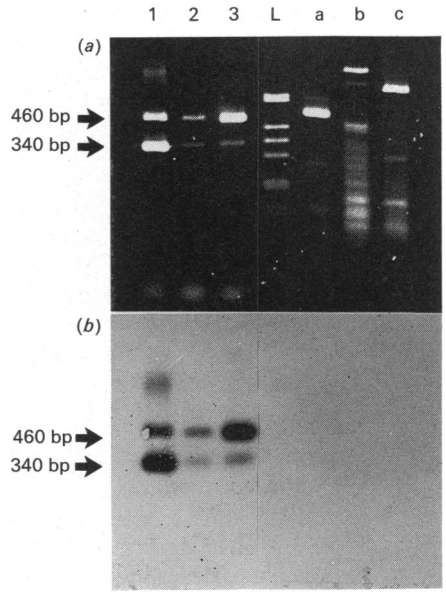

Fig. 4. Detection of ANF mRNA by cDNA amplification

The products of cDNA amplification were separated by electrophoresis on a 1 % agarose gel and visualized using ethidium bromide (*a*). In lanes 1–3, the 340 bp and 460 bp bands, derived from cDNA and genomic DNA respectively, hybridize with a 30-mer ^{35}S-labelled intervening-sequence probe specific for ANF cDNA (*b*). The larger band in lane 1 which hybridizes to the probe is at present unidentified. Lanes a–c show the amplification products obtained from rat kidney cDNA with primer pairs designed from the sequence of the rat ANF 'A' receptor (predicted size ~ 450 bp), the human 'B' receptor (predicted size ~ 670 bp) and the bovine 'C' receptor (predicted size ~ 570 bp) respectively. No hybridization is seen to the bands in the DNA ladder or to amplification products in lanes a–c. L, 1 kb DNA ladder (from BRL); 1, rat right atrium; 2, rat adrenal gland; 3, bovine adrenal gland.

porting the localization and synthesis of ANF in adrenal chromaffin cells, but not cortical cells [15,16,20,21]. In the rat adrenal gland the level of RNAase A-resistant non-specific binding did not permit visualization of specific hybridization after digital subtraction of the images (results not shown). We have seen this degree of non-specific binding in the adrenal gland when we have used certain anti-sense oligomer probes to detect mRNAs other than that for ANF.

Northern analysis of total RNA with the 42-mer ANF antisense probe (Fig. 3) clearly reveals the presence of ANF mRNA in the right atrium, but not in the rat adrenal gland, ventricle, liver or kidney or the bovine adrenal gland. Therefore it could not be used to assess the specificity of '*in situ*' hybridization in the adrenal gland. Nevertheless, the absence of binding of the ^{35}S-labelled probe to the non-atrial lanes suggests that, in a tissue section, non-specific hybridization to ribosomal and transfer RNAs is insignificant.

To provide independent confirmation of the presence of ANF mRNA, we utilized the PCR to amplify adrenal cDNA with a pair of specific ANF primers, one of which was the oligonucleotide used for '*in situ*' hybridization. Fig. 4 demonstrates the sizes of the amplification products after electrophoretic separation. A band approx. 340 bp in length is present in the rat right atrial and in the rat and bovine adrenal lanes. It is indistinguishable in size from that predicted from published sequence data [30–32], and its existence indicates the presence of ANF mRNA. The larger 460 bp band seen in the rat and bovine samples represents amplification of contaminating genomic DNA. The specific nature of these bands was confirmed by hybridization with an ^{35}S-labelled intervening-sequence ANF probe. The 340 bp and 460 bp amplification bands hybridized to the probe, but no binding was seen to the 1 kb DNA ladder bands or to the PCR products obtained by amplification with the ANF receptor primers, despite the presence of intensely fluorescent DNA bands. Restriction fragment analysis after digestion with the endonuclease *Hin*cII provided a further test of specificity. The 340 bp and 460 bp bands were cleaved into two fragments of the sizes predicted from the position of the *Hin*cII restriction site in the ANF cDNA (results not shown).

The distribution of the ANF mRNA transcripts suggests that ANF may be synthesized predominantly in the adrenal medulla, but no clear-cut effects of ANF on medullary cells have been reported. The localization of ^{125}I-ANF-binding sites indicates that its principal sites of action are in the cortex, as shown by the effects of ANF on adrenal cortical steroid production. Basal and stimulated aldosterone synthesis and secretion are inhibited by ANF [8–10], but the effects on glucocorticoids are variable [11–14].

ANF synthesis in the adrenal medulla raises the question of its release characteristics and its site of action, whether in the immediate vicinity of the synthetic cells or further afield. The high concentrations of steroids reaching the medulla from the cortex may be partly responsible for the activation of the ANF gene, as seen in the heart ([25] and references therein). Effects on steroidogenesis by ANF released from the medulla into the systemic circulation would be very unlikely, because the small amounts produced should be very rapidly degraded [37]. Although our quantitative studies (D. J. R. Nunez & A. P. Davenport, unpublished work) suggest that the levels of ANF mRNA in the medulla are low relative to those in normal rat atria [25], local synthesis of this peptide may produce high concentrations in close proximity to cortical receptors and may be a confounding factor affecting the correlation between changes in circulating ANF and the observed biological effects.

How could medullary ANF reach the cortex? Convention assumes that in the adrenal gland blood flows from cortex to medulla [38,39]. However, as pointed out by Weinkove & Anderson [40], the wall of the central vein contains bundles of mainly longitudinal smooth muscle fibres which are pierced by medullary veins joining the central vein. Contraction of these muscle bundles may occlude the medullary veins and lead to damming up of blood in the medulla and adjacent cortex with reversal of blood flow towards the cortex [39,40]. An alternative suggested by Nguyen *et al.* [21] is that the chromaffin cells themselves may be scattered within the cortex. There is still a further possibility that ANF is transferred by nerves having their cell bodies in the medulla and their terminals in the cortex [41]. Already there is evidence for the presence of immunoreactive ANF in sympathetic ganglia [21,42], but it may not be localized to sympathetic neurones.

The pharmacological effects of ANF on steroidogenesis suggest that it may have a physiological role in producing the cortical 'zonation' of steroid production by selectively inhibiting aldosterone formation from cells in the zona fasciculata and reticulosa [38]. Dilution or degradation of the peptide in the zona glomerulosa would then permit synthesis and secretion of the mineralocorticoid in this zone. There is some evidence supporting this hypothesis. First, neutral endopeptidase (EC 3.4.24.11), the principal enzyme degrading ANF, has been localized selectively to the zona glomerulosa using a specific affinity-purified polyclonal antibody [43]. Secondly, the hypertension described by Skelton [44] after adrenal enucleation is dependent on salt retention and may be due to high aldosterone secretion. This was confirmed by Jungmann and colleagues [45], who found that adrenal demedullation results in elevated aldosterone levels. Hornsby [46] has argued that all cortical cells are pluripotent and exhibit only temporary functional and morphological differences according to their location in the cortex. Thus the removal by enucleation of a tonic inhibitory effect of medullary ANF may allow all cortical cells to secrete aldosterone, producing excessive salt retention and hypertension.

In conclusion, we have demonstrated that the rat and bovine adrenal gland contains a differential distribution of ANF binding sites and mRNA. Changes in circulating ANF can clearly affect adrenal steroid, and particularly aldosterone, secretion, but the question remains of whether ANF, which is synthesized locally in the adrenal, plays any role in the physiological regulation of adrenal steroid secretion.

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