Autocrine role of adrenomedullin in the human adrenal cortex

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Abstract

Previous studies from our laboratory have reported that adrenomedullin is synthesised in rat zona glomerulosa cells. In the present studies, it was found that the human adrenocortical cell line H295R expresses the gene encoding adrenomedullin, and that immunoreactive adrenomedullin is released into the culture medium. Furthermore, it was found that secretion of adrenomedullin is regulated by angiotensin II and forskolin.

Studies on the actions of adrenomedullin and calcitonin gene-related peptide (CGRP) revealed a stimulatory effect of adrenomedullin, but not of CGRP, on aldosterone and cortisol secretion. These data suggest that adrenomedullin is not acting by a CGRP receptor-mediated mechanism in the H295R cell line. Adrenomedullin was also found to increase cAMP production, suggesting that in the adrenal, as in other cell types, cAMP is a second messenger for adrenomedullin action. However, the effects of adrenomedullin were not fully mimicked by forskolin, possibly suggesting a role for an additional second messenger.

The presence of mRNA encoding both the putative adrenomedullin receptors, L1 and calcitonin receptor-like receptor/receptor-associated modulatory protein 2 (CRLR/RAMP-2), was demonstrated in H295R cells, but RAMP-1 was not detected, suggesting that these cells do not express the CGRPI receptor CRLR/RAMP-1.

Taken together, these data have demonstrated that adrenomedullin is synthesised and secreted by H295R cells. The observed rate of adrenomedullin synthesis suggests that this peptide exerts a paracrine/autocrine effect in this adrenocortical cell line, probably acting through a specific adrenomedullin receptor, to stimulate steroidogenesis and increase aldosterone synthase expression.

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Introduction

Since the discovery of adrenomedullin in extracts from a pheochromocytoma in 1993 (Kitamura et al. 1993), several studies have confirmed the presence of this peptide in the adrenal gland. There is now evidence that adrenomedullin is synthesised in the adrenal cortex as well as in the adrenal medulla. Previous studies from our laboratory have demonstrated the presence of both adrenomedullin mRNA and peptide in the rat adrenal zona glomerulosa (Kapas et al. 1998). In the human, adrenomedullin message is found in adrenocortical tumours and cultured cells (Liu et al. 1997, Takahashi et al. 1998). Furthermore, studies on the adrenocortical SW13 cell line have also found that immunoreactive adrenomedullin is produced and released into the culture medium (Takahashi et al. 1998), in common with many other human tumour cell lines (Miller et al. 1996). In some cases, however, for example the SW13 cell line, it appears that adrenomedullin release is constitutive and not regulated by hormones which alter adrenal function (Takahashi et al. 2000), in which case it is unlikely to be a significant regulator of adrenal function. In the human adrenal cortex, however, there is evidence that adrenomedullin gene expression is actively regulated (Liu et al. 1997), although regulation of peptide secretion has not been investigated. One of the aims of the present study was to determine whether the H295R cell line resembles human adrenal tissue in that adrenomedullin is actively regulated.

Several studies have addressed the question of the actions of adrenomedullin in the adrenal gland. Although an effect on medullary function has not been shown, it appears that adrenomedullin does affect cortical function, although the nature of this effect has proved controversial. Studies on the rat adrenal gland reported both stimulatory and inhibitory effects of adrenomedullin, which were initially thought to depend on the tissue preparation used (Mazzocchi et al. 1996a, b, 1996b). This interpretation now appears unlikely (for review see Hinson et al. 2000), and it now seems most likely that adrenomedullin inhibits aldosterone secretion when acting through the calcitonin gene-related peptide (CGRP) I receptor, an effect which

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is mimicked by CGRP itself (Mazzocchi et al. 1996). However, adrenomedullin appears to stimulate aldosterone secretion when acting through a specific adrenomedullin receptor (Hinson & Kapas 1998; Kapas et al. 1998). Studies on human adrenal cells have also demonstrated an inhibitory effect of adrenomedullin, mediated by the CGRPI receptor (Andreis et al. 1997). By the nature of their source, human adrenal glands are difficult to obtain and tend to yield variable results, with high basal levels of steroid release (Henville et al. 1989). In addition, we have previously found that angiotensin II is not specific for stimulation of aldosterone release in fresh human adrenal cells but also stimulates cortisol release (Henville et al. 1989). For these reasons we have chosen to use the human adrenocortical cell line, H295R.

The H295R adrenocortical cell line is a well-characterised cell line, derived from an adrenocortical tumour. In its basal state this cell line secretes the normal range of human adrenal steroids, but can be differentiated into more ‘glomerulosa-like’ cells by pretreatment with angiotensin II, or into ‘zona fasciculata-like’ cells by pretreatment with forskolin, an activator of adenylyl cyclase (Rainey et al. 1994, Bird et al. 1998). Although relatively unresponsive to adrenocorticotrophin (Mountjoy et al. 1994), this cell line possesses all the other characteristics of human adrenocortical cells, and is a powerful tool for the investigation of adrenocortical function.

The present study was designed to determine whether the H295R cell line secretes adrenomedullin and to find whether its production is actively regulated. The second part of the study concerns the actions of adrenomedullin on the H295R cells, and the nature of the receptor mediating these effects.

Materials and Methods

Human adrenocortical H295R cells were generously donated by Professor W E Rainey and Professor J I Mason. Additional cells were purchased from ATCC (Rockville, MD, USA). All tissue culture medium and supplements were purchased from Gibco Life Technologies (Paisley, Strathclyde, UK) with the exception of 2% Ultroser G, insulin (6·25 mg), transferrin (6·25 mg), selenium (6·25 mg), linoleic acid (5·35 mg) (+1ITS) medium supplement which was purchased from Universal Biologicals (Gloucester, Glos, UK). Ultraspec was purchased from AMS Biotechnology UK Ltd (Whitney, Oxon, UK). All reagents required for cDNA synthesis were purchased from Gibco Life Technologies. For PCR, all reagents were purchased from Helena Biosciences (Sunderland, Tyne and Wear, UK) with the exception of Taq DNA polymerase which was purchased from Flowgen (Lichfield, Staffs, UK), and the primers which were purchased from MWG Biotech Ltd (Milton Keynes, Bucks, UK).

Adrenomedullin (1–52, human) peptide and enzyme immunoassay kits and CGRPI peptide were purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). Cortisol antibody (ab 1002) was obtained from Bioclinical Services (Cardiff, S Glam, UK). Radiolabelled aldosterone and cortisol were obtained from Amersham UK (Aylesbury, Bucks, UK). Aldosterone antiserum was raised in house. All other reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK).

Culture of the H295R cell line

H295R cells were maintained in Dulbecco’s modified Eagle’s medium/F12 supplemented with +1ITS, 1% penicillin/streptomycin and 1% fungizone at 37 °C under an atmosphere of 95% air/5% CO2. All cells used for the experiments were between passages 10 and 30.

Pretreatment of cells

Cells were cultured in either six-well plates (for stimulation with peptides) or 80 cm² tissue culture dishes (for RNA extraction) and were then pretreated for 48 h in the above tissue culture medium containing either no agonist (control), angiotensin II (10 nM), forskolin (10 µM) or adrenomedullin (100 nM) as stated. The concentration of adrenomedullin was chosen as the maximally effective concentration for the stimulation of steroid secretion, the concentrations of angiotensin II and forskolin were chosen as the most effective concentrations previously reported (Rainey et al. 1994). The medium was replaced every 24 h and aliquots were stored at −20 °C for radioimmunoassay for aldosterone and cortisol. Control, angiotensin II and forskolin pretreatment medium was also assayed for adrenomedullin, following the kit manufacturer’s instructions and without pre-extraction.

Steroid secretion

To determine the effect of adrenomedullin and CGRP on steroid secretion, cells cultured in six-well plates which had not been pretreated were maintained overnight in +1ITS-free, Ultroser-free medium, then washed twice with phosphate-buffered saline before being incubated for 4 h at 37 °C with either no agonist (control), adrenomedullin (10 pM–1 µM) or CGRP (10 pM–1 µM). At the end of the incubation period the medium was removed to fresh tubes and stored at −20 °C for radioimmunoassay for aldosterone and cortisol. Steroids were assayed as previously described (Kapas et al. 1992). Cells were scraped off and the protein content determined by the method of Lowry et al. (1951). This experiment was carried out in triplicate and repeated four times.

cAMP release

To determine the response of cAMP to adrenomedullin and CGRP, H295R cells were cultured as for the steroid
experiments and incubated at 37 °C for 20 min with either no agonist (control), forskolin (10 µM), adrenomedullin (10 pM–1 µM) or CGRP (10 pM–1 µM). At the end of the 20-min incubation, medium was collected and stored at −20 °C until assayed. cAMP was measured by competitive protein-binding assay as previously described (Kapas et al. 1992). Cells were harvested and protein content assayed as described above. Experiments were carried out in triplicate and repeated four times.

**RNA extraction, cDNA synthesis and PCR**

Total RNA was isolated using Ultraspec solution following the manufacturer’s instructions. The purity of the RNA was estimated by measuring the optical density at 260/280 nm. Total RNA (5 µg) was subjected to first-strand cDNA synthesis in a 20 µl reaction volume containing 250 mM Tris–HCl (pH 8–3), 375 mM KCl, 15 mM MgCl₂, 1 mM of each dNTP, 1 µM of each primer and 200 U RNase inhibitor, in the presence of 1·5 µg oligo dT_{(12–18)} primer and 200 U superscriptase. After completion of first-strand cDNA synthesis the reaction was stopped by heat inactivation (95 °C for 5 min) and diluted to 50 ng/µl RNA equivalents with water. cDNA amounts equivalent to 100 ng total RNA were then subjected to PCR in a 50 µl reaction volume containing 10 mM Tris–HCl (pH 9), 1·5 mM MgCl₂, 200 µM of each dNTP, 1 µM of each primer and 0·2 U Taq DNA polymerase under the following conditions: one cycle of denaturation at 94 °C for 5 min, 35 cycles of denaturation for 1 min at 94 °C, 1-min primer annealing at the calculated temperature specific to the primer, 1-min primer extension at 72 °C, followed by one cycle of primer extension for 10 min at 72 °C. PCR products (10 µl) were electrophoresed through ethidium bromide-stained 1% agarose gels and viewed by UV illumination. The RNA extraction was carried out on three different cell cultures. Primers used were as listed in Table 1.

**Analysis of data**

Statistical means ± S.E.M. values were calculated. One-way analysis of variance (ANOVA) followed by a post-hoc Dunnett’s test was used to determine whether adrenomedullin had a significant effect on basal levels of aldosterone, cortisol or cAMP release, as appropriate. Student’s t-tests were used to determine whether angiotensin II significantly affected rates of adrenomedullin release.

**Results**

Immunoreactive adrenomedullin was detected in the culture medium from H295R cells and found to be produced at a basal rate of 1·4 nmol/mg cellular protein per 48 h (n=6). The rate of release of adrenomedullin was significantly increased by both angiotensin II and forskolin pretreatment (Fig. 1).

Incubation of H295R cells in the presence of increasing concentrations of adrenomedullin caused a dose-dependent increase in aldosterone secretion, although CGRP was without effect (Fig. 2). Similarly, adrenomedullin, but not CGRP, caused a dose-dependent increase in cortisol release (Fig. 3). Adrenomedullin was found to cause a dose-dependent increase in cAMP production (Fig. 4). The minimum effective concentration of adrenomedullin was 100 pM for cAMP and cortisol secretion and 1 nM for aldosterone. A maximum effect was seen with 100 nM adrenomedullin in all cases. CGRP at concentrations from 10 pM to 1 µM had no effect on either cortisol or aldosterone release. The effects of adrenomedullin on steroid secretion are compared with those of angiotensin II and forskolin in Fig. 5.
Investigation of the presence of the putative adrenomedullin receptors by mRNA analysis revealed the presence of message for L1, CRLR and the associated protein RAMP-2, but not RAMP-1 (Fig. 6).

Discussion

These studies have demonstrated that the adrenomedullin mRNA is present in extracts of H295R cells, and that the peptide is released into culture medium, suggesting that, in common with the rat adrenal cortex (Kapas et al. 1998), the SW13 adrenocortical cell line (Takahashi et al. 1998) and many other cell lines (Miller et al. 1996, Hinson et al. 2000), the H295R human adrenocortical cell line produces adrenomedullin. Moreover, the data showing an increase in adrenomedullin with angiotensin II treatment suggest that adrenomedullin gene expression is actively regulated in H295R cells. This increase was not simply due to an increase in cell number as the data are expressed per mg cellular protein, thus controlling for changes in cellular growth. Pretreatment of these cells with angiotensin II results in the differentiation of H295R cells into a zona glomerulosa-type phenotype, while treatment with forskolin differentiates the cells into a zona fasciculata cell.
The finding that both angiotensin II and forskolin caused an increase in adrenomedullin biosynthesis suggests that adrenomedullin production is not exclusively associated with either cell phenotype. This contrasts with our previous findings in the rat, demonstrating localisation of adrenomedullin peptide and message almost exclusively in the outer part of the adrenal cortex (Kapas et al. 1998).

It was found that adrenomedullin acts on H295R cells to stimulate both aldosterone and cortisol release. The finding of a dose-dependent increase in cAMP release, parallel to the increase in steroid secretion in response to adrenomedullin, suggests that this peptide exerts its effects on adrenocortical steroids by a cAMP-dependent mechanism. This accords well with our previous studies on rat adrenocortical cells, where a similar increase in cAMP was observed (Kapas et al. 1998). This observation is also congruous with the great majority of other studies on the intracellular actions of adrenomedullin (Hinson et al. 2000). However, the finding that the actions of adrenomedullin on aldosterone secretion were not effectively mimicked by forskolin suggests that adrenomedullin may

Figure 5 The effects of a maximally stimulating concentration of adrenomedullin (100 nM) on (a) aldosterone secretion and (b) cortisol secretion are compared with the effects of CGRP (100 nM), angiotensin II (All; 10 nM) and forskolin (FKS; 10 μM). Data are means ± S.E.M. of four separate experiments each carried out in triplicate. **P<0·01, ***P<0·001 compared with basal (ANOVA).
activate an alternative intracellular pathway in addition to increasing cAMP production.

Previous studies on freshly prepared human adrenocortical cells report no effect on either basal cortisol or aldosterone release and an inhibitory effect on angiotensin II-stimulated aldosterone release (Andreis et al. 1997). However, studies on human adrenal slices demonstrated a stimulatory effect of adrenomedullin on aldosterone release (Andreis et al. 1997). These studies have not investigated second messenger production in response to adrenomedullin, so it is not clear whether cAMP is generated in these cells. It is noteworthy that the effects of forskolin and angiotensin II were found to be specific for cortisol and aldosterone release respectively. Forskolin acts by the direct stimulation of adenyl cyclase, and thus it appears likely that adrenomedullin may act by another route in addition to stimulating cAMP.

From the data comparing the actions of adrenomedullin and CGRP on H295R cells, it is clear that adrenomedullin is not acting through a CGRP receptor-mediated mechanism: CGRP was entirely without effect on aldosterone or cortisol release by these cells, whereas adrenomedullin was stimulatory in both cases. This observation is in contrast to previous studies on human adrenocortical cells, where both adrenomedullin and CGRP appeared to inhibit angiotensin II-stimulated steroid secretion, acting through a common receptor, probably the CGRP1 receptor (Andreis et al. 1997). Autoradiography of human adrenals has also suggested that the adrenomedullin binding in this tissue is to the CGRP1 receptor, as binding was displaced by CGRP8–37 (Belloni et al. 1999). Unfortunately as yet there is no specific adrenomedullin receptor antagonist available, but the data we have obtained strongly suggest that, in the H295R cell line, the effects of adrenomedullin are not mediated by a CGRP receptor. It is not clear why there should be a difference between fresh human adrenal cells and the H295R cell line, but this may possibly be a defining characteristic of these cells.

There are additional difficulties in determining the nature of the receptor mediating the effects of adrenomedullin, as two putative adrenomedullin receptors, in addition to the CGRP1 receptor, have been identified. The first, characterised by Kapas et al. (1995), is termed L1, while the second, initially characterised by Njuki et al. (1993) is termed CRLR. It has been established that CRLR only functions as an adrenomedullin receptor in the presence of RAMP, with the presence of RAMP-1 conferring CGRP receptor activity, and RAMP-2 conferring adrenomedullin receptor activity (McLatchie et al. 1998). There has been considerable controversy surrounding the candidacy of L1 as an adrenomedullin receptor, but it is clear that the CRLR/RAMP combination does not account for all adrenomedullin binding (for review see Hinson et al. 2000). Analysis of mRNA extracted from H295R cells revealed the presence of message for both candidate adrenomedullin receptors, CRLR/RAMP-2 (McLatchie et al. 1998) and L1 (Kapas et al. 1995). However, RAMP-1 message was not detected, suggesting that these cells do not express the CRLR/RAMP-1 binding site conferring CGRP receptor activity (McLatchie et al. 1998). These data suggest that the H295R cells express an adrenomedullin receptor, but not a CGRP receptor, supporting the functional observations made of the effects of CGRP and adrenomedullin. The expression of the putative CGRP receptor RDC1 was not investigated in these studies for two reasons: first, there is no human sequence available and secondly, RDC1 was shown in expression studies not to bind adrenomedullin (Kapas & Clark 1995).

Taken together these data suggest that adrenomedullin acts as an autocrine regulator in the human adrenal as it secreted by these cells in a regulated manner and is able to act directly to stimulate steroidogenesis. The observed secretion rates of adrenomedullin suggest that this peptide is likely to exert a significant action on cellular function and may contribute to maintaining steroidogenesis. However, the role of adrenomedullin in the long-term regulation of adrenocortical function remains to be determined.

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