Cloning and Expression of cDNA Encoding a Rat Adrenomedullin Receptor*

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Adrenomedullin is a potent vasodilator peptide that exerts major effects on cardiovascular function. Its actions are mediated through an abundant class of specific binding sites that activate adenyl cyclase through a G protein-coupled mechanism. We report here the identification of a cDNA clone for the adrenomedullin receptor that was originally isolated as an orphan receptor from rat lung. The cDNA encodes a polypeptide of 395 residues that contains seven transmembrane domains and has a general structural resemblance to other members of the G protein-linked receptor superfamily. When expressed in COS-7 cells, this receptor mediates a CAMP response to adrenomedullin with an EC50 of \(7 \times 10^{-9}\) M, and binds \(125^{\text{I}}\)-adrenomedullin with a \(K_{D}\) of \(8.2 \times 10^{-9}\) M, properties that are consistent with those observed in cardiovascular and other target tissues. The receptor gene is expressed as several mRNA species of which the most prominent is a 1.8-kilobase transcript found in the lung, adrenal, heart, spleen, cerebellum, and other sites. Identification of this receptor cDNA should facilitate further investigation of the cellular actions of adrenomedullin and its regulatory effects in normal and disordered states of cardiovascular function.

The potent hypotensive peptide, adrenomedullin (AM), was originally isolated from a human adrenal phaeochromocytoma using a detection system based on its ability to elevate platelet cAMP levels (1). Subsequent work revealed that this peptide is produced by a wide variety of tissues, most notably the adrenal, lung, kidney, and heart (2–5). It is also synthesized and secreted in abundance by vascular endothelial cells (6) and is detectable in the circulation (1, 4, 5). The principal physiological action of AM appears to be as a potent vasodilator, and its properties that are consistent with those observed in cardiovascular and other target tissues. The receptor gene is expressed as several mRNA species of which the most prominent is a 1.8-kilobase transcript found in the lung, adrenal, heart, spleen, cerebellum, and other sites. Identification of this receptor cDNA should facilitate further investigation of the cellular actions of adrenomedullin and its regulatory effects in normal and disordered states of cardiovascular function.

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‡ The abbreviations used are: AM, adrenomedullin; AM-R, adrenomedullin receptor; CGRP, calcitonin gene-related peptide; PCR, polymerase chain reaction; MOPS, 3-[N-morpholino]propanesulfonic acid; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; kb, kilobase(s); kbp, kilobase pair(s); bp, base pair(s).

dosterone secretion (11), have been described.

There has been some difficulty in defining the receptor sites of AM, and several studies have suggested that its actions are mediated through calcitonin gene-related peptide (CGRP) receptors. There is modest sequence homology between these peptides, in particular the presence of a six residue intramolecular disulfide-linked ring structure that is also present in islet amyloid polypeptide. CGRP has similar vasodilator properties to AM, and this action of AM can be inhibited by the CGRP antagonist CGRP8–37 (12). However, while the specific AM binding sites in rat vascular smooth muscle cells exhibit a \(K_{D}\) of \(1.3 \times 10^{-8}\) M, the \(K_{D}\) for CGRP at these sites (3 \(\times 10^{-7}\) M) was significantly greater than that for binding to the CGRP receptor (13), suggesting that specific receptors for AM do exist and may have low affinity for CGRP. Identification of a cDNA done for such an AM receptor (AM-R) would enhance our understanding of the physiology of this peptide, and facilitate the development of therapeutically useful drugs that could stimulate or block these receptors. In this report we describe the cloning and expression of a rat lung cDNA encoding a functional AM-R.

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MATERIALS AND METHODS

Chemicals—Chemicals were purchased from Sigma (Poole, United Kingdom (UK)) or Aldrich (Gillingham, UK). Sequencing reagents and all radioactive isotopes were obtained from Amersham plc (Amersham, UK). The plasmid vectors pCR1000 and pcDNA1 were obtained from Invitrogen (San Diego, CA), COS-7 cells from ATCC (Rockville, MD), and rat adrenomedullin and CGRP from Peninsula Labs Inc. (St. Helens, UK).

Low Stringency Reverse Transcriptase Polymerase Chain Reaction—Degenerate polymerase chain reaction (PCR) primers (5′-TTGCTACCTGCTGGTCGACTCC-3′ and 5′-RGGTCHARAGGATGCGR-3′) based on conserved regions of the cDNAs encoding transmembrane domains VI and VII respectively of several G protein-linked receptors were synthesized. These were used in PCR for 27 cycles at 94°C for 1 min, 45°C for 1 min, 72°C for 1 min in buffer containing 2.5 mM MgCl2, random hexanucleotide primed cDNA synthesized from adult rat adrenal gland, and Taq polymerase. 10 μl of the 100 μl PCR product was subjected to another 27 cycles of PCR using the same primers, and products were separated by agarose gel electrophoresis. Several ethidium bromide staining bands were excised, purified by Genedean (Bio101, Vista, CA), and ligated into the pCR1000 vector. Individual colonies were picked and subjected to DNA sequence analysis as described below.

Northern Blot Analysis—Poly(A)+ mRNA was prepared from 21-day-old female and male rat tissues by a commercial oligo(dT) selection method (FastTrack, Invitrogen, San Diego, CA). Alligots (1–3 μg) of mRNA were subjected to electrophoresis on 1% MOPS/formaldehyde gels and transferred to nitrocellulose membranes. These membranes were then hybridized with probes derived from pAD6 or pGEM.L1 labeled with \([^{32}\text{P}]\)dCTP to a specific activity of \(10^{6}\) cpm/μg of DNA under conditions described elsewhere (15). Washed blots were exposed to Kodak X-AR-5 film (Eastman Kodak Co.).

cDNA Library Screening and DNA Sequencing—The insert from the plasmid pAD6 was labeled with \([^{32}\text{P}]\)dCTP (specific activity \(>6000\) Ci/mmol) by the random priming method (16) and used to screen approximately \(3 \times 10^{9}\) plaques of a rat lung oligo(dT)-primed λgt11 cDNA library using standard methods (17). Positive plaques on autoradiography were picked and subjected to two further rounds of purification by the same screening process. The cDNA insert from positive plaques was excised using EcoRI and ligated into pGEM7 (Promega, Madison, WI) to create pGEM.L1. This plasmid was used for double-stranded DNA sequencing by the dyeoxy chain termination method with \(3^{\text{5}}\)DATP using Sequenase (Amersham plc, Amersham, Bucks, UK). DNA sequences were aligned and analyzed using the PCGENE software package (IntelliGenetics, Mountain View, CA).
Expression in COS-7 Cells—The full-length cDNA was excised from pGEM.L1 and ligated into pcDNA1. This plasmid, pcDNA1.L1, was used in transient transfection studies with COS-7 cells. Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Paisley, UK) in 10% fetal calf serum, and were transfected using the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim GmbH, Germany). For functional studies cells were washed 48 h after transfection in serum-free medium and placed into medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and selected concentrations of rat AM or CGRP or AM and CGRP [8–37]. Cells were stimulated for 20 min at 37°C, and then placed on ice prior to removal of medium and cells for cAMP assay using an established protein binding assay method (18).

Ligand Binding Studies—48 h after transfection, cells were washed in serum-free medium and incubated at 22°C for 60 min with 125I-AM, iodinated by the chloramine-T method, and increasing concentrations of unlabeled AM in binding buffer (20 mM HEPES, pH 7.4, 5 mM MgCl₂, 10 mM NaCl, 4 mM KCl, 1 mM EDTA). Cells were then scraped from the dishes, and bound and unbound ligand were separated by centrifugation followed by washing twice in ice-cold binding buffer. Bound radioactivity was determined by γ-spectrometry and data were analyzed using the LIGAND software package (19).

RESULTS AND DISCUSSION
The DNA products derived from low stringency PCR with rat adrenal cDNA were excised from an agarose gel and ligated into pCR1000. After transformation, individual colonies were picked, plasmid DNA was prepared and subjected to DNA sequencing. Several of these contained inserts that bore no homology to the equivalent region of other seven transmembrane domain receptors. However one of these, denoted pAD6, contained an insert that appeared to encode a novel member of the seven transmembrane domain receptor family. When this was used to probe a Northern blot prepared from various rat tissues, high levels of expression were evident in the lung. An gt11 rat lung cDNA library was therefore screened with pAD6 and this led to the isolation of two plaques, one of which contained a 1.8-kbp cDNA insert. This was sequenced on both sides and indicated a single long open reading frame of 1185 bp, terminating in a TAG termination codon (underline) and three potential poly(A) additions (double underlines) in the 3′-untranslated region of the gene. There are two putative sites of N-linked glycosylation (●) and four consensus sites for phosphorylation by protein kinase C (●) (see Refs. 21 and 22).
Adrenomedullin Receptor cDNA

The principal transcripts of 1.8, 2.2, 3.0, and 5.0 kb can be seen in several lanes. The tissues examined include placenta (Pl), psoas muscle (Mu), ovary (O), testis (T), uterus (U), heart (H), lung (Lu), spleen (S), kidney (K), liver (L), adrenal capsule (Ad C), whole adrenal (Ad), cerebellum (Cb), pithy (Pit), hypothalamus (Hy), thalamus and hippocampus (Th), and cerebral cortex (CC). All lanes contained 3 μg of poly(A)⁺ RNA except for kidney, adrenal, and adrenal capsule, which contained only 1 μg of poly(A)⁺ RNA. The blot was exposed for 48 h with image intensifying screens at -70 °C.

The full-length cDNA was used to probe Northern blots (Fig. 2). The predominant mRNA is approximately 1.8 kb in size, with lesser species of 2.2, 3.0, and 5.0 kb. The lung is clearly the most abundant site of this mRNA, followed by the adrenal, ovary, heart, spleen, cerebellum, and cerebral cortex. It was recognized that this distribution was similar to the distribution of AM binding sites recently reported by Owji et al. (24); therefore, the hypothesis that this cDNA encoded the rat AM-R was tested. Although there is recent evidence to suggest that AM activates both cAMP and calcium mobilization pathways (25), studies in many different cell types have described a cAMP-stimulating action that can be abrogated by pretreatment with choleratoxin (26). Therefore, cAMP responses to AM were measured after transfection of this cDNA clone into heterologous cells.

The full-length cDNA was ligated into the expression vector pCDNA1, and this was used to transiently transf ect COS-7 cells cultured in six-well plates. 48 h after transfection, cells were washed with serum-free medium and stimulated with selected concentrations of rat AM or CGRP. As shown in Fig. 3a, there was a specific cAMP response to AM in transfected cells with a threshold of around 10⁻⁶ M, a peak response at 10⁻⁵ M, and an EC₅₀ of 7 × 10⁻⁹ M. No response was found in sham transfected cells or in cells stimulated with 10⁻⁶ M CGRP. CGRP(8–37) is an antagonist for CGRP receptors, and evidence suggests that it also antagonizes the actions of AM (13). When used at various concentrations in the presence of 10⁻⁹ M AM, this peptide appeared to have a weak antagonistic effect with a Kᵦ of 10⁻⁶ M.

Ligand binding studies lend further support to the identification of this cDNA clone as the AM-R (Fig. 3b). The calculated Kᵦ of this receptor when expressed in COS-7 cells was 8.2 × 10⁻⁹ M, a value similar to that reported for the wild type receptor in other studies (13, 24).

The technique of low stringency consensus primer PCR has been successfully used in the cloning of a number of G protein-coupled receptors, including the adenosine A1 and A2 receptors (23, 27, 28) and the MCL (α-MSH) and ACTH receptors (29). Moreover, the gene that we have now identified as the rAM-R has been cloned in this way and recognized as an orphan receptor by three independent groups. We have previously tested a large number of potential agonists for activation of this receptor without success, and it was the recent description of the prevalence and distribution of the AM-R that led to its identification as reported in this paper. It is not yet clear whether the widespread tissue distribution of AM-R transcripts is mainly a reflection of its vascular role, or whether it has other non-vascular functions in these tissues. The latter seems highly likely in view of recent reports proposing a role for AM as a bronchodilator (9), an inhibitor of drinking behavior (10), an aldosterone release inhibitory factor (11), and an ACTH inhibitory factor (30).

![Image](https://example.com/image.png)
The AM-R cDNA seems to be a relatively unique member of the G protein receptor superfamily, being most homologous (30%) to another orphan receptor known as RDC1. It remains to be seen whether this gene will define a new subfamily among the G protein-linked receptors.

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