

Rat-2 fibroblasts express specific adrenomedullin receptors, but not calcitonin-gene-related-peptide receptors, which mediate increased intracellular cAMP and inhibit mitogen-activated protein kinase activity

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Rat-2 fibroblasts demonstrate specific binding of ¹²⁵I-labelled rat adrenomedullin ($K_D = 0.43$ nM; $B_{max} = 50$ fmol/mg of protein) in the absence of ¹²⁵I-labelled calcitonin-gene-related peptide (CGRP) binding. Therefore Rat-2 cells were used to examine the pharmacology and signal transduction pathways of adrenomedullin receptors. We examined the effects of adrenomedullin, the CGRP receptor antagonist CGRP-(8–37) and the amylin antagonists AC187 and AC253 on receptor binding and cAMP production. AC253, AC187 and CGRP-(8–37) inhibited ¹²⁵I-adrenomedullin binding, with respective IC_{50} values of 25 ± 8 , 129 ± 39 and 214 ± 56 nM. Adrenomedullin dose-dependently increased intracellular cAMP (approximate $EC_{50} = 1.0$ nM). CGRP-(8–37), AC253 and AC187 antagonized adrenomedullin-stimulated cAMP production at micromolar concentrations. Using kinase-substrate assays, Mono Q FPLC and 'phospho-specific' Western blotting, we found that adrenomedullin alone abolished basal mitogen-activated protein kinase (MAPK) ac-

tivity and dose-dependently inhibited platelet-derived-growth-factor-stimulated MAPK activity. Radioimmunoassay for adrenomedullin of media from Rat-2 cells showed a linear release of adrenomedullin-like immunoreactivity of 3.1 fmol/h per 2×10^6 cells. Gel-filtration chromatography showed that this adrenomedullin-like immunoreactivity co-eluted with synthetic rat adrenomedullin. Northern blotting with a rat adrenomedullin cDNA probe was used to confirm the presence of adrenomedullin mRNA. However, neither Northern blotting nor reverse transcriptase-PCR showed the presence of the cloned adrenomedullin receptor (L1). We conclude that the Rat-2 cell line expresses a specific adrenomedullin receptor (coupled to cAMP production and regulation of MAPK) and secretes adrenomedullin, which may participate in a regulatory control loop.

Key words: calcitonin family receptors, signal transduction.

INTRODUCTION

Adrenomedullin is a novel and abundant hypotensive peptide originally isolated from human pheochromocytomas [1]. Rat adrenomedullin is a 50-amino-acid peptide with two deletions and six substitutions compared with the human peptide [2]. Both peptides have an N-terminal ring (formed by an intramolecular disulphide bridge) and are amidated at the C-terminal residue. These structures place adrenomedullin in the calcitonin family of peptides, along with calcitonin, calcitonin-gene-related peptide (CGRP) and amylin [3]. Adrenomedullin appears to have many important roles, including actions as a powerful vasodilator [1,4,5], a growth factor [6,7], a regulator of embryogenesis and differentiation [8], a bronchodilator [9] and an inhibitor of peptide hormone secretion [10].

Originally it was thought that the biological effects of adrenomedullin are mediated through CGRP receptors [11], as these effects could be inhibited by the CGRP receptor antagonist fragment CGRP-(8–37), and adrenomedullin shows a high (nanomolar) affinity at these receptors [12,13]. However, some effects of adrenomedullin are not inhibited by CGRP-(8–37), e.g.

the *in vivo* hypotensive effect in the rat [4,14]. More recently, specific adrenomedullin-binding sites that do not bind CGRP have been demonstrated by us and others in rat tissues, endothelial cells, vascular smooth muscle cells and cell lines [6,13,15–17] using ¹²⁵I-labelled adrenomedullin. Adrenomedullin can also compete for the amylin-binding sites in rat lung with a higher affinity than amylin [13,18]. Thus adrenomedullin is capable of acting via CGRP receptors, lung amylin-binding sites and specific adrenomedullin receptors. This has made the interpretation of the effects and physiology of adrenomedullin very difficult, as most tissues and cells investigated express more than one of these receptors.

A putative adrenomedullin receptor (L1) has been cloned by Kapas et al. [19] which has low affinities for CGRP and CGRP-(8–37). Moreover, the pattern of expression of the cloned L1 receptor mRNA matches very closely the distribution of ¹²⁵I-adrenomedullin-binding sites in rat tissues [13]. Thus the L1 receptor may represent at least one subtype of adrenomedullin receptor [19]. However, attempts to replicate adrenomedullin binding to the L1 receptor in COS-7 cells by other groups [20] have been unsuccessful, leading to some doubts about the

Abbreviations used: AM-IR, adrenomedullin-like immunoreactivity; BSOE, bis-[2-(succinimido-oxycarbonyloxy)ethyl] sulphone; CGRP, calcitonin-gene-related peptide; CRLR, calcitonin-receptor-like receptor; IBMX, isobutylmethylxanthine; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PDGF, platelet-derived growth factor; RAMP, receptor-activity-modifying protein; RT-PCR, reverse transcriptase-PCR.

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authenticity of L1 as an adrenomedullin receptor. Additionally, adrenomedullin binding has been shown in the uterus in the absence of L1 mRNA [21], indicating the possibility of more than one type of adrenomedullin receptor. Recently, a further complication has been proposed, with the cloned 'CGRP receptor', calcitonin-receptor-like receptor (CRLR) [22], acting as a specific adrenomedullin receptor when expressed together with receptor-activity-modifying protein 2 (RAMP2) [23].

The exact signal transduction pathways activated by adrenomedullin are uncertain. However, stimulation of adenylate cyclase to increase intracellular cAMP has been demonstrated in rat vascular smooth muscle cells [24], bovine aortic endothelial cells [25], rat skeletal muscle cell lines [17] and cells transfected with the cloned L1 receptor [19]. In bovine adrenal medullary cells [26] and bovine aortic endothelial cells [25], adrenomedullin also elevates intracellular calcium. Adrenomedullin has been shown to suppress cell growth and activation of mitogen-activated protein kinase (MAPK) in cell lines [27]. Effects of adrenomedullin on growth and MAPK remain controversial, with adrenomedullin eliciting a powerful cAMP-mediated increase in DNA synthesis in Swiss 3T3 cells without any effect on MAPK activity [6]. Adrenomedullin has also been proposed to form an autocrine growth-stimulatory loop in human cancer cell lines, with expression of both peptide and receptor in these cells [7].

In most of the tissues and cell lines mentioned above, it has been difficult to examine the action of adrenomedullin at specific adrenomedullin receptors due to cross-reactivity with the CGRP receptor [12,17]. We have also shown ^{125}I -adrenomedullin-binding sites in a rat skeletal muscle cell line (L6) which are not coupled to increased cAMP [17]. It has therefore become important to identify a simple model system with ^{125}I -adrenomedullin binding, but not ^{125}I -CGRP binding, and a corresponding biological effect. Therefore we investigated the pharmacology and the signal transduction pathways of adrenomedullin in the Rat-2 fibroblast cell line.

EXPERIMENTAL

Materials

Rat-2 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). All media for cell culture were supplied by Life Technologies (Paisley, Renfrewshire, U.K.). Rat adrenomedullin was obtained from Peptide Institute Inc. (Osaka, Japan), CGRP-(8–37) from Bachem (Saffron Walden, Essex, U.K.) and rat $[\text{Tyr}^0]\alpha\text{CGRP}$ from Peninsula (St. Helens, Merseyside, U.K.). Rat αCGRP was synthesized and purified to homogeneity by HPLC on C_8 columns (Aquapore RP300; Anachem, Luton, U.K.) as described previously [17]. The fidelity of synthesis was confirmed by amino acid analysis and MS. AC187 and AC253 were a gift from Amylin Pharmaceuticals (San Diego, CA, U.S.A.). Na^{125}I and ^{32}P -labelled nucleotides were from Amersham International (Little Chalfont, Bucks., U.K.). BSOCOES {bis-[2-(succinimidoxycarbonyloxy)ethyl] sulphone} and Iodogen reagent (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) were supplied by Pierce and Warriner (Chester, Cheshire, U.K.). Chemicals were from Sigma (Poole, Dorset, U.K.) or Merck (Poole, Dorset, U.K.).

Peptide iodination for receptor assay

Rat adrenomedullin was iodinated by the Iodogen method as previously described [13]. Briefly, 12.5 μg (2 nmol) of peptide in 10 μl of 0.2 M sodium phosphate buffer, pH 7.2, was reacted with 37 MBq of Na^{125}I and 10 μg of Iodogen reagent for 4 min

at 22 °C. The ^{125}I -labelled peptide was purified by reverse-phase HPLC (C_{18} Novapak; Waters Chromatography, Watford, Herts., U.K.) using a gradient of 15–40% (v/v) acetonitrile in water/0.05% trifluoroacetic acid. Fractions showing binding were divided into aliquots, freeze-dried and stored at -80°C . The specific radioactivity of the label was 10 Bq/fmol. Iodination of rat $[\text{Tyr}^0]\alpha\text{CGRP}$ was by the Iodogen method as previously described [28]. The specific radioactivity of the CGRP label was 36 Bq/fmol.

Cell culture and receptor binding assays

Rat-2 fibroblasts were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 100 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. For binding studies and cAMP experiments, cells were plated out at a density of 15000 cells per well into 24-well culture plates and grown to confluence before use. For adrenomedullin binding assays, cells were grown in poly(L-lysine)-coated 24-well plates. Cells were incubated for 60 min at 4 °C in 0.5 ml of binding buffer (20 mM Hepes, pH 7.4, 5 mM MgCl_2 , 10 mM NaCl, 4 mM KCl, 1 mM EDTA, 1 μM phosphoramidon, 0.25 mg/ml bacitracin and 0.3% BSA) containing 1000 Bq (200 pM) of ^{125}I -labelled rat adrenomedullin. Non-specific binding was measured in the presence of excess (500 nM) unlabelled rat adrenomedullin. After the incubation, binding buffer was removed by aspiration and cells were washed twice with 0.5 ml of ice-cold assay buffer, then dissolved in 1 M NaOH for counting bound ^{125}I -adrenomedullin. For ^{125}I -CGRP binding assays, ^{125}I -CGRP (1000 Bq; 55 pM) was incubated for 60 min at 22 °C and unbound label was removed as described above. For equilibrium competition experiments, the concentration of unlabelled peptides was varied from 0 to 1.0 μM . Binding data were analysed by non-linear regression using Receptor-Fit (Lundon Software, Cleveland, OH, U.S.A.) to calculate the dissociation constant (K_D), IC_{50} and receptor concentration (B_{max}).

Membrane preparation and chemical cross-linking

For membrane preparation, cells were washed with ice-cold PBS and scraped into 50 mM Hepes, pH 7.6, containing 0.25 M sucrose, 15 $\mu\text{g}/\text{ml}$ aprotinin, 0.5 $\mu\text{g}/\text{ml}$ pepstatin, 0.25 $\mu\text{g}/\text{ml}$ leupeptin, 0.25 $\mu\text{g}/\text{ml}$ antipain, 0.1 mg/ml benzamidin and 0.1 mg/ml bacitracin. Cells were homogenized and centrifuged at 1000 g for 15 min at 4 °C. The supernatants were centrifuged at 100000 g for 1 h at 4 °C. The final pellets were resuspended in sucrose-free homogenization buffer and protein concentration was measured by the biuret assay. Chemical cross-linking with BSOCOES followed by SDS/PAGE was performed as previously described [13,17]. Briefly, membranes were incubated with 12000 Bq (1.9 nM) of ^{125}I -labelled rat adrenomedullin, as for binding assays, and autoradiographs were exposed to Kodak X-Omat film (IBI Ltd., Cambridge, U.K.) at -80°C for 1–10 days before developing.

cAMP assays

Cells were plated out as described above. Confluent cells were serum-starved for 3 h, then treated with peptides in serum-free medium containing 50 μM 3-isobutyl-1-methylxanthine (IBMX) for 15 min. After treatment, cells were extracted in 250 μl of acid ethanol (75% ethanol/16 mM HCl) overnight at -20°C . Extracts were dried and assayed using a radioimmunoassay kit (DuPont, Stevenage, Herts., U.K.).

MAPK assays

Cultured fibroblasts (75 cm² flasks, or 35 mm dishes for dose-response experiments) were grown to confluence and then serum-starved overnight. Cells were exposed to agonists in serum-free medium for 0–15 min. Platelet-derived growth factor (PDGF)-BB (25 ng/ml; Sigma) was used as a known stimulator of MAPK in rat fibroblast cells [29]. After treatment, medium was removed and cells were scraped into ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml anti-pain) [30]. Cellular debris was removed by centrifugation at 15000 g and 4 °C. Supernatants were assayed immediately for MAPK activity by phosphorylation of myelin basic protein (MBP), or stored at –80 °C prior to Western blotting.

Cell extracts were assayed for MAPK activity using a kit (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Briefly, tubes were set up containing 10 µl of cell extract, 10 µl of inhibitor cocktail (inhibitors of protein kinase A, protein kinase C and cyclin-dependent kinase) and 10 µl of substrate [MBP at 2 mg/ml or assay dilution buffer (20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na₃VO₄, 1 mM dithiothreitol)]. The reaction was started by the addition of 10 µl of magnesium/ATP cocktail (75 mM MgCl₂/500 µM ATP) containing approx. 37 kBq of [γ-³²P]ATP. Tubes were incubated at 30 °C for 10 min and the reaction was stopped by the addition of 10 µl of 1 M HCl. Samples of 25 µl from each tube were spotted on to phosphocellulose squares and washed three times in 0.75% phosphoric acid and once in acetone before liquid scintillation counting.

The specificity of the kit for measurement of MAPK activity was verified by assaying samples of cell extracts, which had been purified by ion-exchange FPLC using the method of Bogoyevitch et al. [31]. Briefly, cell extracts were loaded on to a Mono Q HR5/5 column (Pharmacia Biotechnology Ltd., St. Albans, Herts., U.K.) equilibrated with buffer A [50 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.5% (v/v) glycerol, 0.05% (w/v) Brij 35, 0.3 mM Na₃VO₄, 1 mM benzamidine and 4 µg/ml leupeptin (pH 7.3)]; the flow rate was 1 ml/min. After an initial 5 min isocratic stage using buffer A, MAPK activities were eluted by a linear gradient of NaCl (0–0.3 M) in buffer A. Fractions (0.5 ml) were assayed for MBP kinase activity using the kit as described above.

Samples of cell extracts from the above experiments were also examined by Western blotting using a PhosphoPlus MAPK antibody kit (New England Biolabs, Beverly, MA, U.S.A.). Extracts containing 1.5 mg of protein were boiled for 2 min in Laemmli sample buffer, centrifuged at 15000 g and 4 °C for 2 min and then separated by SDS/PAGE on 4–20% (w/v) acrylamide/Tris/HCl gradient gels (Bio-Rad, Hemel Hempstead, Herts., U.K.). Proteins were transferred to nitrocellulose and probed with phosphospecific antibodies against p42^{MAPK} and p44^{MAPK} according to the manufacturer's instructions.

RNA extraction and Northern analysis

Total RNA was extracted from 175 cm² flasks of confluent cells or from Wistar rat (body wt. 200–250 g; Bantin and Kingman, Hull, U.K.) tissues using the guanidinium isothiocyanate/phenol/chloroform extraction method [32]. Poly(A)⁺ RNA was prepared from total RNA using oligo(dT)-cellulose affinity chromatography (Pharmacia). Total RNA (50 µg) or poly(A)⁺ RNA (derived from 500 µg of total RNA) was size-separated by electrophoresis on denaturing Mops/formaldehyde/1% agarose gels and transferred to Hybond-N (Amersham International) nylon membranes, followed by baking for 2 h at 80 °C.

Membranes were probed for rat adrenomedullin mRNA, using a 150 bp cDNA probe containing the entire coding region of rat adrenomedullin (bases 283–432) [2], or for rat adrenomedullin (L1) receptor mRNA, using a 416 bp cDNA probe corresponding to nucleotides 467–882 of the rat adrenomedullin receptor cDNA [19]. Probes were labelled and the prehybridization, hybridization and washing conditions were as described previously [21].

Reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared and its integrity checked on denaturing Mops/formaldehyde/agarose gels as above. First-strand cDNA was synthesized from 40 µg of total RNA using 9 units of avian myeloblastosis virus reverse transcriptase (Promega, Southampton, Hants., U.K.) and 200 ng of oligo(dT)_{12–18} primer as previously described [33]. Single-stranded cDNA was added to a solution containing *Taq* polymerase buffer [20 mM Tris/HCl (pH 8.4), 50 mM KCl and 1.5 mM MgCl₂], 200 µM dNTP, 25 pmol of each primer and 1 unit of *Taq* polymerase. The cDNA was amplified using a program cycle as follows: 95 °C for 4 min, followed by addition of enzyme; then three cycles of denaturing (95 °C; 1 min) and annealing (65 °C; 90 s), with the annealing temperature lowered by 2 °C per cycle; then four cycles starting with an annealing temperature of 60 °C for 2 min, with the temperature lowered by 1 °C per cycle; and finally, 24 cycles of 95 °C for 1 min, 55 °C for 50 s and 72 °C for 1 min, followed by a final step at 72 °C for 5 min. The primers used were based on the rat adrenomedullin receptor (L1) cDNA sequence (GenBank accession number L09249): the fourth transmembrane segment (5'-CGCGGATCCTGAGGTGGTACATAT-3') and the 3'-untranslated region (5'-CTCGTTCGACTTGGTGGAAGAAGCT-3'). Underlined sequences represent restriction sites for *Bam*HI and *Sal*I respectively, and these are followed by sequences corresponding to bases 782–796 and 1440–1454 respectively of the L1 receptor. The expected product is 678 bp in size. The reaction products, a control PCR without cDNA and a 1 kb ladder (Gibco-BRL) were size-separated by electrophoresis on a 1 × TAE (40 mM Tris/acetate and 1 mM EDTA, pH 8.0)/1% agarose gel containing 0.5 µg/ml ethidium bromide, and viewed by UV illumination.

Release of adrenomedullin-like immunoreactivity (AM-IR) from Rat-2 cells

To ascertain the rate of release of AM-IR from Rat-2 fibroblasts, cells were plated out into 6-well plates at 200000 cells per well. Cell cultures were allowed to stabilize for 24 h and then washed twice with serum-free medium. Medium was then replaced with serum-free Dulbecco's modified Eagle's medium and samples were removed for radioimmunoassay at timed intervals. For adrenomedullin radioimmunoassay, radioactive tracer was prepared with a synthetic adrenomedullin-(22–52) fragment using the Iodogen method, and the iodinated peptide was purified by reverse-phase HPLC as previously described [34]. The specific radioactivity of the tracer was 22.1 Bq/fmol. The assay was performed as previously described [21].

To verify that the AM-IR represented intact adrenomedullin, 20 ml of medium was added to a flask of confluent cells and incubated for 6 h. Medium was removed, then acidified using acetic acid (1 M final concentration) and boiled for 15 min prior to loading on to a prepared reverse-phase C₁₈ Sep-Pak cartridge (Millipore, Milford, MA, U.S.A.). Peptides were eluted with 60% (v/v) acetonitrile/40% (v/v) water/0.1% (v/v) trifluoroacetic acid and dried by rotary evaporation. The pellet was dissolved in G-50 column buffer [0.06 M sodium phosphate buffer, pH 7.2, 10 mM EDTA, 7 mM NaN₃, 0.2 M NaCl, 0.3%

(w/v) BSA] and loaded on to the column. The sample, along with standards, was eluted using the same buffer at a flow rate of 3–4 ml/h [35]. The elution coefficient of immunoreactive peaks (K_{av}) was calculated using the method of Laurent and Killander [36].

Statistical analysis

Results are shown as mean values \pm S.E.M. For the cAMP assays, data were compared by repeated-measures ANOVA with subsequent *post hoc* Tukey's tests (Systat, Evanston, IL, U.S.A.) between control and experimental groups, with $P < 0.05$ considered to be statistically significant. For binding data, analysis of one-site versus two-site competition curves was by *F*-test, with two-component fits considered significant at $P < 0.05$.

RESULTS

Rat-2 cell receptor binding and chemical cross-linking of 125 I-adrenomedullin to Rat-2 membranes

Specific 125 I-adrenomedullin binding was observed in Rat-2 cells ($K_D = 0.43 \pm 0.01$ nM; $B_{max} = 50 \pm 10$ fmol/mg of protein; 6022 ± 1200 receptors per cell, $n = 3$) (Figure 1, upper panel). Non-

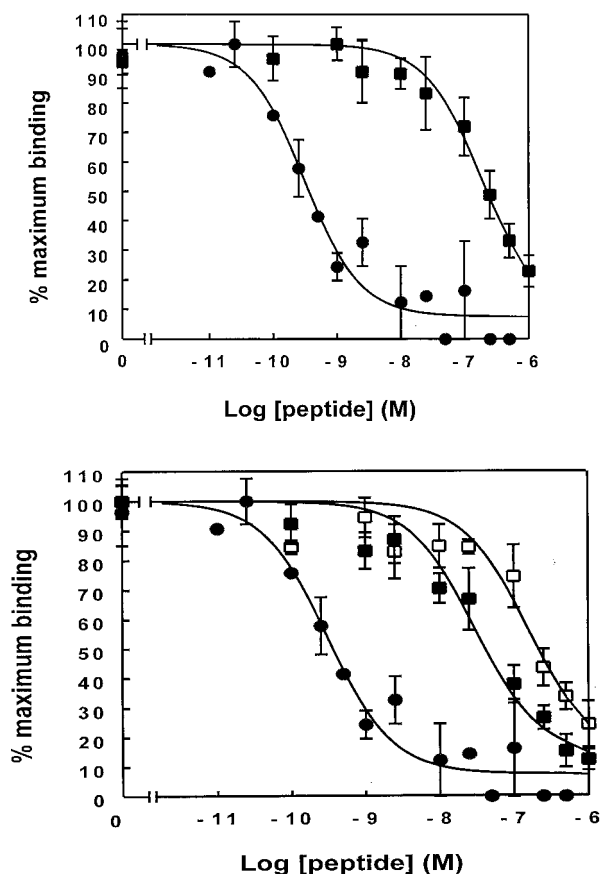


Figure 1 Competition curves for 125 I-adrenomedullin binding in Rat-2 cells

Equilibrium competition binding for 125 I-adrenomedullin with the competitors adrenomedullin (●) and CGRP-(8–37) (■) (upper panel), and adrenomedullin (●), AC187 (□) and AC253 (■) (lower panel) in Rat-2 cells. The maximum specific binding of 125 I-adrenomedullin was 33.7 ± 1.7 Bq/ 4×10^5 cells, which represented 68% of the total binding. Data are expressed as a percentage of the maximum specific binding, and are means \pm S.E.M. for three experiments with assays in triplicate.

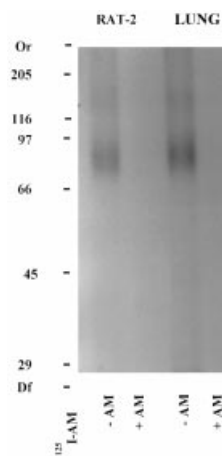


Figure 2 Chemical cross-linking of 125 I-adrenomedullin to Rat-2 membranes

The autoradiograph shown is representative of three experiments showing chemical cross-linking of 125 I-adrenomedullin-binding site complexes in Rat-2 membranes, using rat lung as a positive control. Cross-linking was performed using BSOCOES as the cross-linking reagent, as described in the Experimental section. The specificity of binding was verified by addition of excess unlabelled peptide at $1 \mu\text{M}$ in the initial binding assay (+AM). Total binding is indicated in the columns labelled —AM. Molecular masses (kDa) of the protein standards are shown at the left. Protein standards are: myosin, 205 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. Abbreviations: Or, origin; Df, dye front; AM, adrenomedullin. All lanes are from the same experiment and were exposed for 7 days.

specific binding in the presence of 500 nM unlabelled adrenomedullin was $35 \pm 2\%$ ($n = 3$). There was no specific 125 I-CGRP binding to the same cells (total label bound, 2 ± 0.5 Bq/ 4×10^5 cells; non-specific binding, 2 ± 0.2 Bq/ 4×10^5 cells; $n = 4$). In addition, there was no specific 125 I-amylin binding to Rat-2 cells (total label bound, 15 ± 3 Bq/ 4×10^5 cells; non-specific binding, 12 ± 1 Bq/ 4×10^5 cells; $n = 4$). CGRP-(8–37) (IC_{50} 214 ± 56 nM) and the amylin antagonists AC187 (IC_{50} 129 ± 39 nM) and AC253 (IC_{50} 25 ± 8 nM) were able to compete with 125 I-adrenomedullin at the specific adrenomedullin-binding sites, as shown in Figure 1 (lower panel). CGRP did not compete with 125 I-adrenomedullin binding at concentrations up to $1 \mu\text{M}$ (results not shown). Analysis of all the competition binding data for one or two sites by *F*-test supported the existence of only a single adrenomedullin-binding site.

Chemical cross-linking using BSOCOES in Rat-2 membranes showed that, in this cell line, specific adrenomedullin-binding site complexes have a mean molecular mass of 83 kDa (Figure 2). After subtracting the mass of the peptide, this gives a molecular mass of 77 kDa for the adrenomedullin-binding site, which is similar to that in rat lung (Figure 2).

Release of AM-IR from Rat-2 cells

Rat-2 cells released AM-IR in a constitutive manner over a 48 h period (results not shown). Release was below detectable levels during the first 2 h, but linear thereafter. The rate of release was approx. 37 fmol/24 h per 10^6 cells. Analysis of AM-IR by G-50 gel-filtration chromatography showed that the AM-IR eluted as a single peak in the same position as synthetic rat adrenomedullin (results not shown), indicating that Rat-2 cells probably release authentic adrenomedullin.

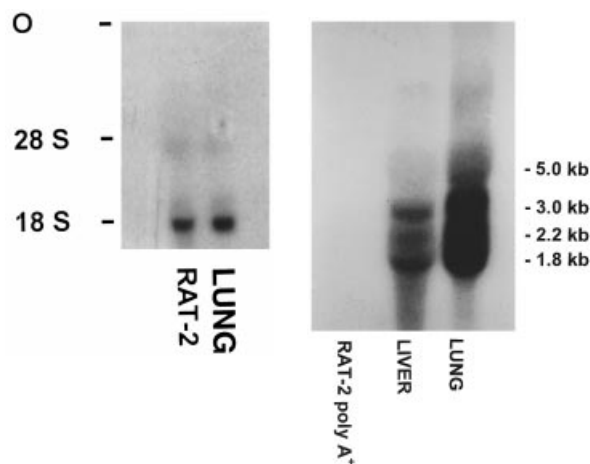


Figure 3 Representative Northern blot analysis of rat adrenomedullin mRNA and adrenomedullin receptor mRNA

Left panel: adrenomedullin mRNA. Lanes contained 50 μg of total RNA from Rat-2 cells or rat lung. Exposure to film was for 3 days. The positions of the 28S and 18S rRNA bands are shown on the left of the Figure. O is the origin. The probe used was a 150 bp cDNA probe corresponding to the entire coding sequence of rat adrenomedullin. Adrenomedullin mRNA appears as a 1.6 kb band, as expected. The blot shown is representative of three separate experiments. Right panel: adrenomedullin receptor mRNA. Lanes contained 50 μg of total RNA from lung or liver, or poly(A)⁺ corresponding to 500 μg of total RNA from Rat-2 cells. Exposure to film was for 8 days. The probe used was a random-primer-labelled *Bgl*I fragment of the rat adrenomedullin (L1) receptor cDNA. The expected bands at 1.8, 2.2, 3 and 5 kb were present in lung and liver, but no bands could be observed in Rat-2 cell mRNA. The blot is representative of three separate experiments.

Northern analysis of adrenomedullin and adrenomedullin receptor (L1) mRNAs in Rat-2 cells

Total RNA (50 μg) from Rat-2 cells showed a strong hybridization with the adrenomedullin probe (Figure 3, left panel). The hybridizing band in Rat-2 cells and rat lung was approx. 1.6 kb in size, as expected. Although we did not specifically quantify these blots, the amount of adrenomedullin mRNA in Rat-2 cells seemed to be about the same as in rat lung, which is considered to be a major site of adrenomedullin production. In contrast with the findings for adrenomedullin mRNA, no mRNA corresponding to the adrenomedullin receptor (L1) could be found, even when poly(A)⁺ RNA from 500 μg of total RNA was probed (Figure 3, right panel). Despite this, the same experiments clearly showed hybridization of the probe with rat lung total RNA (50 μg), with bands present at 1.8, 2.2, 3.0 and 5.0 kb, as expected [19].

RT-PCR for adrenomedullin receptor (L1) using Rat-2 cell RNA

No mRNA for the adrenomedullin (L1) receptor could be detected by Northern blotting of Rat-2 poly(A)⁺ RNA. This may have been due either to a true lack of adrenomedullin receptor mRNA or to the relatively low sensitivity of Northern blotting. Since the adrenomedullin receptor concentration is not high in Rat-2 cells, it is possible that a very low level of mRNA expression could account for the observed binding. We therefore used RT-PCR to determine the presence of low levels of adrenomedullin receptor mRNA. Figure 4 shows a clear lack of an amplified band after 24 cycles of PCR using specific primers in Rat-2 cDNA, even though a band of the correct size (678 bp) was present in rat lung cDNA.

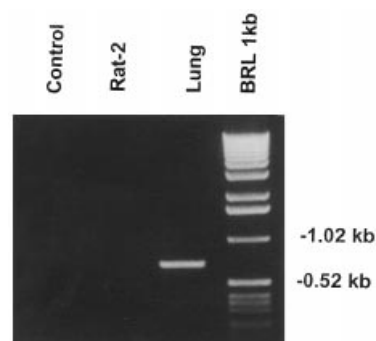


Figure 4 RT-PCR of mRNA from Rat-2 cells using specific rat adrenomedullin receptor primers

The lanes contained: no cDNA in PCR (control); reverse-transcribed Rat-2 cDNA; reverse-transcribed rat lung cDNA; 1 kb ladder. RT-PCR of rat adrenomedullin receptor cDNA was performed as described in the Experimental section. After PCR, a distinct band in ethidium bromide-stained gels illuminated by UV light, corresponding to the adrenomedullin receptor cDNA (expected size 678 bp), could be visualized in lung cDNA, but not in control or Rat-2 cDNA. On the right hand side of the Figure, the sizes of the two bands in the 1 kb ladder either side of the amplified band are shown. The figure shown is representative of three separate experiments.

Effect of adrenomedullin on cAMP production

Rat-2 cells produced cAMP in a dose-dependent manner when stimulated with increasing concentrations of adrenomedullin. Concentrations of 200 pM–1 μM gave a 5-fold increase in cAMP compared with basal levels in serum-free medium, with an approximate EC_{50} of 1 nM (Figure 5). The absence of CGRP receptors in these cells was confirmed, as CGRP, even at 1 μM , did not significantly increase cAMP (Figure 5). Each of the peptides AC187, AC253 and CGRP-(8–37), which competed with ¹²⁵I-adrenomedullin binding, were tested for their effects on cAMP production at concentrations of 500 nM and 1 μM . None of these peptides alone increased cAMP above basal levels (results not shown). We then tested whether increasing concentrations of AC187, AC253 and CGRP-(8–37) were able to inhibit the elevation of cAMP by 50 nM adrenomedullin. At the

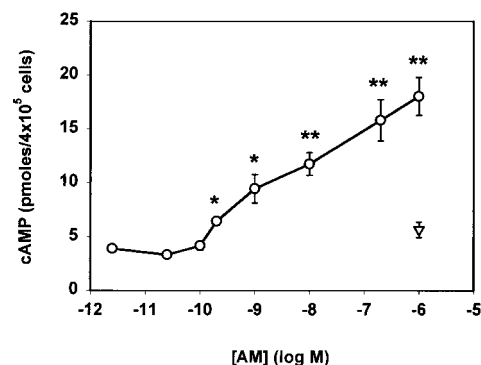


Figure 5 Stimulation of cAMP production by adrenomedullin

Intracellular cAMP was measured as described in the Experimental section in the presence of 50 μM IBMX, with increasing concentrations of rat adrenomedullin (AM; \circ). Control or basal cAMP was measured using serum-free medium instead of adrenomedullin. CGRP (∇) at 1 μM did not significantly increase intracellular cAMP. Results are expressed as means \pm S.E.M. ($n = 6$, with all assays performed in triplicate). * $P < 0.05$; ** $P < 0.005$ with respect to control cells.

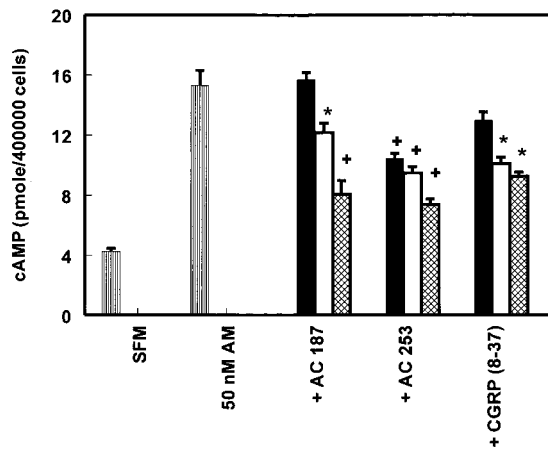


Figure 6 Inhibition of adrenomedullin-stimulated cAMP production by AC187, AC253 and CGRP(8–37)

Intracellular cAMP was measured as described in the Experimental section, in the presence of 50 μ M IBMX. Cells were stimulated with 50 nM rat adrenomedullin (AM) and increasing concentrations of the amylin antagonist AC187 or AC253, or of the CGRP antagonist CGRP(8–37); control or basal cAMP was measured using serum-free medium (SFM). Concentrations used were 5 μ M (solid bar), 10 μ M (open bar) and 20 μ M (cross-hatched bar). Results are expressed as means \pm S.E.M. ($n = 6$, with all assays performed in triplicate). * $P < 0.05$; * $P < 0.01$ with respect to 50 nM adrenomedullin-treated cells.

concentrations used, all peptides resulted in a dose-dependent decrease in adrenomedullin-stimulated cAMP production; AC253 was the most potent, causing a 50% decrease in adrenomedullin-stimulated cAMP production at 20 μ M (Figure 6). None of the peptides tested achieved complete inhibition of stimulated cAMP production at the concentrations used.

Effect of adrenomedullin on MAPK activity

To determine the optimal assay period for activation of MAPK, we performed a time course for MAPK activity in Rat-2 cells stimulated with 25 ng/ml PDGF-BB. The activity of MAPK increased rapidly from the resting level (1.79 ± 0.05 pmol/min per mg of protein) to a maximum of 5.55 ± 0.09 pmol/min per mg over 5 min, after which the activity slowly decreased (results not shown). To demonstrate that the MBP kinase activity observed using the assay kit was true MAPK activity, Rat-2 cell extracts were purified by Mono Q FPLC and the resulting fractions were assayed for MAPK activity. Two peaks of activity eluted from the column at 0.19 and 0.24 M NaCl (Figure 7, upper panel), representing p42^{MAPK} and p44^{MAPK} respectively, as shown previously [31]. Figure 7 (lower panel) shows a corresponding Western blot using a primary antibody specific for the phosphorylated (and therefore activated) forms of p42^{MAPK} and p44^{MAPK}, with phosphorylated MAPK clearly present in lanes B1 and B3 for unstimulated cells, and at greater levels in lanes P1 and P3 for PDGF-stimulated cells. This confirms that the kinase activity measured was due to activated MAPK.

Adrenomedullin alone almost totally inhibited basal MAPK activity (Figure 7, lanes A1 and A3), and was able to reduce PDGF-stimulated activity to below basal levels (Figure 7, lanes PA1 and PA3). Figure 8 shows that adrenomedullin caused a dose-dependent inhibition of PDGF-stimulated MAPK activation, with concentrations as low as 0.05 nM reducing MAPK activity to control levels. Increasing concentrations of adrenomedullin (10 nM or greater) reduced PDGF-stimulated MAPK activity to below control levels. Thus adrenomedullin potently inhibits basal and PDGF-stimulated MAPK activity.

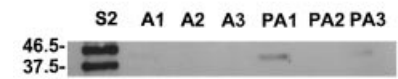
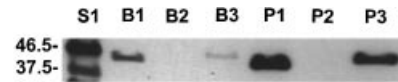
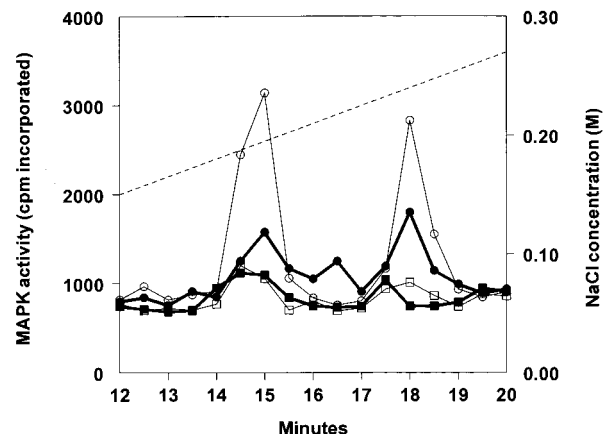


Figure 7 Purification of MAPK by Mono Q FPLC

Upper panel: MBP kinase assay. After treatment with 25 ng/ml PDGF and/or 100 nM adrenomedullin, cells were extracted, purified and assayed as described in the Experimental section. Symbols: ●, unstimulated cells; ○, 25 ng/ml PDGF; ■, 100 nM adrenomedullin; □, 25 ng/ml PDGF + 100 nM adrenomedullin. Lower panels: Western blotting. Western blotting using a phospho-specific MAPK antibody was performed as described in the Experimental section. Lanes S1 and S2 contain biotinylated molecular mass markers; the markers shown are maltose-binding protein 2 (46.5 kDa) and lactate dehydrogenase M (37.5 kDa). Lanes B1 and B3 contain the two peak MAPK activities for unstimulated cells. The first peak of activity (B1) corresponds to p42^{MAPK}, and the second peak (B3) to p44^{MAPK}. Lanes P1 and P3 contain the two peak MAPK activities for PDGF-stimulated cells; lanes A1 and A3 contain the two peak MAPK activities for adrenomedullin-treated cells; and lanes PA1 and PA3 contain the two peak MAPK activities for cells treated with PDGF and adrenomedullin. Samples in lanes B2, P2, A2 and PA2 are from fractions between the peaks showing no MBP kinase activity when assayed as described for the upper panel.

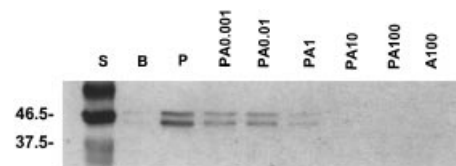


Figure 8 Dose-dependent inhibition of PDGF-stimulated MAPK by adrenomedullin

Cells were stimulated with PDGF (25 ng/ml) in the presence of concentrations of adrenomedullin from 10 pM to 100 nM. Cells were extracted and Western blotting was performed as described in the Experimental section. Shown is a representative (one of three) Western blot using a phosphospecific antibody to detect activated MAPK. Lanes are labelled as follows: S, molecular mass markers; B, basal activity; P, 25 ng/ml PDGF-BB; PA0.001, PDGF + 0.001 nM adrenomedullin; PA0.01, PDGF + 0.01 nM adrenomedullin; PA1, PDGF + 1 nM adrenomedullin; PA10, PDGF + 10 nM adrenomedullin; PA100, PDGF + 100 nM adrenomedullin; A100, 100 nM adrenomedullin alone.

DISCUSSION

Preliminary experiments with the Rat-2 fibroblast cell line showed it to be a model system for studying the specific actions of adrenomedullin, as these cells have no demonstrable specific ¹²⁵I-CGRP binding. ¹²⁵I-Adrenomedullin binding showed compe-

tion with adrenomedullin, the specific CGRP receptor antagonist fragment CGRP-(8–37) and two amylin antagonists, AC187 and AC253 [order of potency: adrenomedullin > AC253 > AC187 > CGRP-(8–37)]. As previously demonstrated in the L6 [17] and Swiss 3T3 [6] cell lines and in oral keratinocytes [37], CGRP did not compete with adrenomedullin at ^{125}I -adrenomedullin-binding sites. The Rat-2 adrenomedullin receptor has a similar high-affinity K_D to that demonstrated in both L6 cells and tissues [13,17], and a much lower affinity for CGRP and amylin. Although amylin is unable to compete with ^{125}I -adrenomedullin at adrenomedullin receptors, AC253 and AC187 are able to compete with moderate affinities, despite limited sequence identity with adrenomedullin. One possible explanation of this phenomenon is that Rat-2 cells are expressing amylin receptors, as adrenomedullin binds to lung amylin sites with a higher affinity than amylin itself [13]. However, we were unable to detect any ^{125}I -amylin binding or any elevation of cAMP by amylin in these cells (results not shown). Chemical cross-linking of ^{125}I -adrenomedullin to membranes prepared from Rat-2 cells showed the receptor to have a molecular mass of 77 kDa, which corresponds well with the molecular mass of the receptor demonstrated in lung (77 kDa) [13] and L6 cells (76 kDa) [17].

Adrenomedullin dose-dependently increased cAMP, as shown previously in various cell and tissue types [1,6,17,38]. As expected, none of the antagonist peptides stimulated cAMP production at micromolar concentrations. The antagonist peptides were tested for their ability to inhibit an increase in cAMP stimulated by 50 nM adrenomedullin. AC253 appeared to be the most effective, with concentrations of 5–20 μM causing a statistically significant decrease in stimulated cAMP levels. This fits with the observation that AC187, AC253 and CGRP-(8–37) are weak competitors at the adrenomedullin receptor, with a 50–400-fold lower affinity than adrenomedullin, and thus can only inhibit adrenomedullin-stimulated cAMP increases at micromolar concentrations. There is some discrepancy between the binding affinity of the antagonists and their ability to inhibit adrenomedullin-stimulated cAMP levels, which may be explained by the differences in incubation temperature and buffer conditions in the two assays. While it is conceivable that CGRP-(8–37), AC187 and AC253 could be used as models for the further development of a specific adrenomedullin antagonist, clearly both affinity and specificity must be improved.

It has been hypothesized that adrenomedullin exerts its effects in many systems via the CGRP receptor [11,12,17,39,40], as demonstrated by inhibition with the specific CGRP receptor antagonist CGRP-(8–37). Thus CGRP-(8–37) at nanomolar concentrations can be used to differentiate between effects mediated by occupancy of CGRP [17] and adrenomedullin receptors. As micromolar concentrations of CGRP-(8–37) are needed to inhibit the effects of adrenomedullin at adrenomedullin receptors in Rat-2 cells, it can be safely surmised that, at least in Rat-2 cells, adrenomedullin acts via a specific adrenomedullin receptor to elevate intracellular cAMP. As well as mechanisms involving cAMP, it has been suggested that, in bovine aortic endothelial cells, adrenomedullin may also elevate intracellular calcium [25]. However, in Swiss 3T3 cells, adrenomedullin had no effect on intracellular calcium [6]. The effects of adrenomedullin on inositol phosphate turnover, and hence intracellular Ca^{2+} concentration, were examined in Rat-2 cells, but no effect was seen compared with endothelin-1 (used as a positive control; results not shown). Therefore we conclude that adrenomedullin signals via the cAMP pathway in Rat-2 cells.

mRNA for the cloned L1 adrenomedullin receptor [19] was not detected in Rat-2 cells, even when RT-PCR was used. This suggests not only that the Rat-2 receptor is not L1, but also that

L1 may only be one of several (at least four: L1 adrenomedullin, Rat-2 adrenomedullin, CRLR/RAMP2 combination and 'non-CRLR' CGRP receptors) receptors capable of binding adrenomedullin that are differentially expressed in different tissues. We have shown that CRLR, when expressed in HEK 293 cells, is capable of binding adrenomedullin, but with a CGRP receptor ligand-binding profile [41]. When probes for RAMP2 become available, it will be interesting to determine whether Rat-2 cells express this protein along with CRLR.

Rat-2 cells constitutively released AM-IR and expressed adrenomedullin mRNA. The presence of adrenomedullin has been detected in many tissues and cell types in which adrenomedullin-binding sites and cAMP responses have been demonstrated previously [17,42–44]. This would suggest that adrenomedullin may have autocrine (Rat-2 cells) or paracrine (tissues) actions, as shown in several human tumour cell lines [7]. The effects of adrenomedullin on cell proliferation have been examined in various cell types, where it has been shown to both stimulate [6,7] and inhibit [27,45] proliferation. In at least one of these cases, the effects of adrenomedullin appeared to be mediated via CGRP receptors, as shown by inhibition by CGRP-(8–37) [45]. Increases in intracellular cAMP have been shown to correspond with an increase [6,7] or a decrease [27,46,47] in cell proliferation. Thus adrenomedullin may increase or decrease proliferation by the same mechanism, depending on the cell type concerned. Previous work has shown adrenomedullin to have either no effect [6] or an inhibitory effect [27] on the growth-regulatory enzyme MAPK. Therefore we investigated the effect of adrenomedullin on MAPK activity in Rat-2 cells, and showed a dose-dependent inhibition of PDGF-stimulated MAPK activity. Adrenomedullin alone was able to inhibit unstimulated MAPK activity to below basal levels. This agrees with published findings showing that adrenomedullin decreases PDGF-stimulated MAPK activity in mesangial cells [27]. However, mesangial cells express CGRP receptors [48] and, as adrenomedullin is capable of acting via CGRP receptors to increase cAMP [17], this cannot be discounted as a mechanism in mesangial cells. In support of this hypothesis, CGRP is able to inhibit thymocyte [49] and splenocyte [50] cell growth. Rat-2 cells, however, do not express CGRP receptors, and it can be concluded that, in these cells, adrenomedullin is acting via specific receptors to increase intracellular cAMP and inhibit MAPK activation. This result conflicts with those obtained with Swiss 3T3 cells, in which adrenomedullin is a potent mitogen [6]. In Swiss 3T3 cells adrenomedullin acts via a specific adrenomedullin receptor, stimulating an increase in cAMP, but with no corresponding effect on MAPK or any other intracellular kinases. cAMP is an established mitogen in Swiss 3T3 cells, and this may account for the differences seen.

Thus we have identified a cell line, the Rat-2 fibroblast, that secretes adrenomedullin and expresses specific adrenomedullin receptors in the absence of CGRP receptors. The adrenomedullin receptors are functionally coupled to adenylate cyclase, which produces a dose-dependent increase in cAMP and decreases mitogen-stimulated MAPK activity. Therefore adrenomedullin may be a candidate for an autocrine regulator of cellular proliferation.

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