Adrenomedullin has mitogenic effects on human oral keratinocytes: involvement of cyclic AMP

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Abstract The effects of the novel vasoactive regulatory peptide, adrenomedullin, on human oral keratinocytes was investigated. Adrenomedullin, acting via its specific receptor, stimulated a dose-dependent increase in DNA synthesis, and, in addition, stimulated further changes in the cell cycle resulting in the proliferation of keratinocytes. When cells were incubated in the presence of increasing concentrations of adrenomedullin, there was a rapid and dose-dependent rise in intracellular cyclic AMP levels. Stimulation of mitogenesis and cell proliferation in these cells were mimicked by the cell permeable cAMP analogue, di-butyryl cAMP. Adrenomedullin-stimulated mitogenesis was attenuated by the adenylyl cyclase inhibitor SQ22,536, but was unaffected by inhibitors of PKC, tyrosine kinase or the CGRP receptor antagonist, CGRP(8–37). These data identify adrenomedullin as a new mitogenic regulatory peptide of keratinocytes acting via the cAMP cascade.

Key words: Adrenomedullin; Oral keratinocyte; Cyclic AMP; Mitogenesis

1. Introduction

Adrenomedullin (AM) is a newly discovered member of the calcitonin peptide family and is a vasoactive 52 amino acid peptide [1]. AM mRNA and protein are widely distributed in human and rat tissues, including the heart, aorta, lung and adrenal gland, and it is detectable in the plasma [2–5]. Binding sites for AM have been identified in many human tissues such as the adrenal glands, lung, heart, kidney and in primary cultures of aortic endothelial cells and vascular smooth muscle cells (VSMC) [3–6]. These findings have identified AM as a novel component of the homeostatic regulatory peptide system, but the cellular responses and the signalling pathways that are mediated by this peptide are not fully elucidated.

Previous studies have shown that AM stimulates increases in intracellular cAMP levels in VSMC and rat adrenocortical cells [6]. It has also been shown to elevate intracellular Ca^{2+} in bovine aortic endothelial cells via a G-protein which is cholera-toxin sensitive [6]. Studies with AM, however, have been complicated by the knowledge that some of the effects can be blocked by the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP(8–37) [1]. It has been shown that the AM can bind to the CGRP receptor and CGRP(8–37) can bind to AM receptor, albeit weakly [8], suggesting that the effects induced by AM may be mediated via CGRP receptors. We have demonstrated, previously, that the cloned rat AM receptor binds AM specifically and this binding is not displaced by CGRP [7]. In order to study and characterise further the cellular effects of AM, a cell line is required which has specific AM and CGRP receptors.

This study reports that H357 oral keratinocytes express receptors of specific high affinity for AM, but not CGRP, and that AM causes increases in cAMP accumulation, induces mitogenesis and stimulates cell proliferation. Furthermore, these effects are mimicked by the cell permeable cAMP analogue, di-butyryl cAMP. AM-stimulated mitogenesis of H357 cells is abolished in the presence of SQ22,536, a specific antagonist of adenylyl cyclase. Inhibitors of PKC (Ro31-8220), tyrosine kinase (lavendustin A) or the CGRP receptor antagonist, CGRP(8–37) have no effect on AM-stimulated mitogenic events. Our results identify AM as a new peptide growth factor acting via its own specific receptor and the cAMP cascade.

2. Materials and methods

2.1. Materials

Human AM, CGRP(8–37), CGRP I, II, amylin and [^{125}I]AM were obtained from Phoenix Pharmaceuticals Inc., San Francisco, CA, USA. Tissue culture materials were from Life Technologies Ltd., Paisley, UK. [^{3}H]cAMP was from Amersham International Ltd., Amersham, UK. SQ22,536 and lavendustin A were obtained from Semat Technical (UK) Ltd., Herts, UK. All other reagents were the best grade available.

2.2. Cell culture

H357 cells were maintained in keratinocyte growth medium (KGM) supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C [9]. Thymidine incorporation assays were carried out using confluent, quiescent cultures of H357 cells, in 6-well plates, that were incubated in KGM containing 1 μCi/ml [^{3}H]thymidine and increasing concentrations of AM in the presence of 50 μM of IBMX. In other experiments cells were incubated in the presence of 10 nmol/l AM and SQ22,536 (10 mol/l), Ro31-8220 (150 nmol/l), lavendustin A (500 nmol/l) or CGRP(8–37) (1 μmol/l). After 24 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material [10].

In other experiments, semi-confluent quiescent cultures of H357, in 96-well plates, were incubated in KGM in the absence or presence of various concentrations of AM ± inhibitors and 50 μM IBMX. After 24 h, cell proliferation was measured by adding 20 μl of MTS tetrazolium [11,12] and incubating the plates for up to 4 h in a humidified atmosphere. MTS tetrazolium is reduced by proliferating cells into a coloured formazan product that is soluble in tissue culture medium. Absorbance was recorded at 490 nm with a 96-well plate reader.

2.3. Receptor-binding assays

The binding assay used was a modified form of a previously de-

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Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; cAMP, cyclic adenosine monophosphate; dbcAMP, di-butyryl cAMP; HUVEC, human umbilical vein endothelial cells; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate buffered saline; PKC, protein kinase C

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scribed method [8]. Confluent H357 cells were incubated at room temperature for 60 min with 0.1 nmol/l [^{125}I]AM and increasing concentrations of unlabelled AM, CGRP I, CGRP II, CGRP(8–37) or amylin, in binding buffer (20 mmol/l HEPES, pH 7.4, 5 mmol/l $MgCl_2$, 10 mmol/l NaCl, 4 mmol/l KCl, 1 mmol/l EDTA). Cells were solubilised in 0.1 mol/l NaOH and tracer bound to the cells was determined by γ spectroscopy.

2.4. cAMP measurements

H357 cells were washed twice with PBS and incubated in KGM with various factors as indicated for 20 min in the presence of 50 μM IBMX. Medium was then removed and cAMP was measured in an aliquot using an in-house specific binding protein assay [13]. Results shown are derived from four separate experiments carried out in triplicate.

2.5. Statistical analysis

Arithmetic means and standard deviations were calculated. One-way analysis of variance was used to test whether factors had an effect on basal (control) levels of cAMP, cell proliferation and mitogenesis, and Student's *t*-test was used to determine the effect of inhibitors on AM-stimulated events using the Minitab statistics software package. Binding parameters were determined using the LIGAND program [14].

3. Results and discussion

The binding characteristics of AM receptors were studied in the H357 human oral cell line. Binding of [^{125}I]AM was shown to be saturable for the receptor (Fig. 1A) and analysis of the data, by the curve fitting program LIGAND, and Hill analysis predicted the probability of a single site model vs a two-site model ($P < 0.05$; Hill coefficient 0.891). Scatchard analysis revealed the presence of a single population of binding sites (Fig. 1B) and the calculated K_D of the receptor was 8.25 nmol/l with a B_{max} of 466 fmol/mg protein. Fig. 1C shows the displacement of [^{125}I]AM by unlabelled AM, CGRP I, CGRP II, CGRP(8–37) or amylin on intact H357 cells. Displacement of [^{125}I]AM binding by unlabelled peptide showed that AM tracer was displaced in a dose-dependent manner and up to 90% was displaced by 50 nmol/l AM. Fig. 1C shows that CGRP I, CGRP II, CGRP(8–37) or amylin did not significantly displace [^{125}I]AM binding in parallel cultures of H357 cells, even at concentrations up to 10^{-6} mol/l. These data demonstrate that H357 cells express specific receptors for AM.

There was a significant increase of intracellular cAMP production by H357 cells in response to AM (Fig. 2) with threshold stimulation occurring around 10 pmol/l, peak response at 10 nmol/l and an EC_{50} of 1 nmol/l. Although it has been reported that AM can stimulate increases in both cAMP levels and intracellular calcium mobilisation [7], studies have shown that AM-stimulated cAMP actions can be attenuated by cholera toxin pretreatment, suggesting its effects can partly be mediated via cytosolic calcium [7]. No mobilisation of calcium was seen in the H357 cells in response to AM (data not shown). Coupling of the receptor to adenylyl cyclase was further assessed by exposing cells to 10 nmol/l SQ22,536, a potent and specific inhibitor of adenylyl cyclase, in the absence or presence of 10 nmol/l AM. Fig. 2 (inset) shows that the cAMP response to AM was significantly ($P < 0.001$) attenuated in the presence of SQ22,536. Inhibitors of PKC or tyrosine kinase activity (10 nmol/l of either Ro31-8220 or lavendustin A, respectively) had no effect on cAMP levels in response to AM (Fig. 2, inset). The CGRP receptor antagonist, CGRP(8–37), was without effect on AM-stimulated cAMP levels. These data suggest that AM does not activate either PKC or tyrosine kinase and the stimulatory effect of AM on cAMP levels appears to be mediated via specific AM receptors coupled to adenylyl cyclase.

Previous studies using AM and CGRP(8–37) have demonstrated that some of the effects of AM are mediated via CGRP receptors. These include AM-stimulated rises in cAMP levels in neuroblastoma cells [15] and AM-mediated vasodilation in the microvasculature of the hamster cheek pouch and rat skin [16]. However, it has also been reported that some effects of AM are mediated by AM receptors and not via CGRP receptors. These include AM-stimulated cAMP production in HUVECs [17] and vasodepressor responses to AM in the hindquarter vascular bed of the rat [18]. The data presented in this study (Fig. 2) indicate that in human oral keratinocytes AM acts via cAMP by binding to its own specific receptor.

When H357 cells, labelled with [^{3}H]thymidine, were incu-

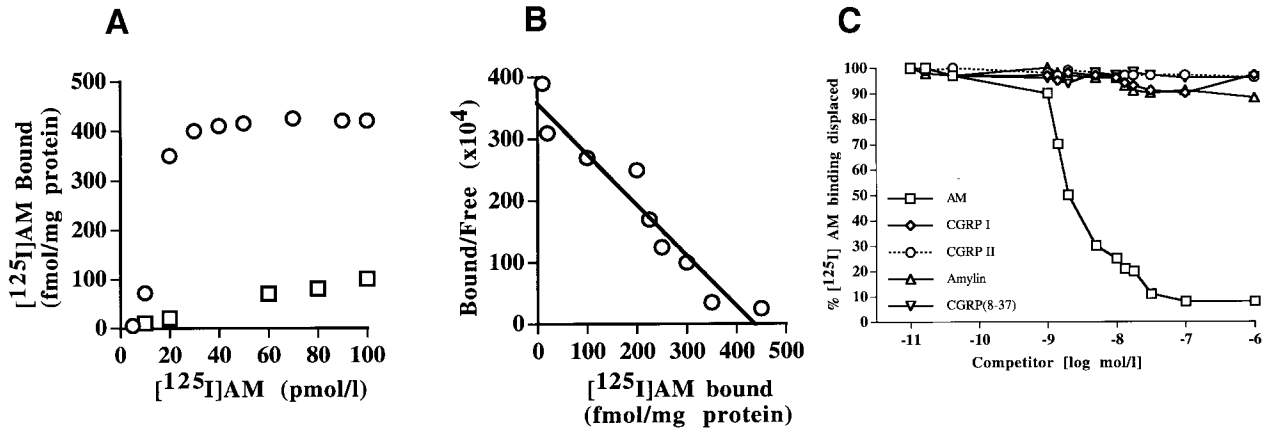

Fig. 1. Saturation curve, Scatchard analysis and displacement curves of human [^{125}I]AM binding. A: Concentration dependence of [^{125}I]AM binding to intact H357 cells showing specific (circles) and non-specific (squares) binding. B: Scatchard analysis of specific [^{125}I]AM binding. C: Displacement of [^{125}I]AM binding by increasing concentrations of AM, CGRP I, CGRP II, CGRP(8-37) or amylin. Each point represents the mean of triplicate determinations.

bated in the presence of AM there was a concentration-dependent increase in [^{3}H]thymidine incorporation as shown in Fig. 3. Maximum incorporation was observed at 10 nmol/l AM. Since it was clear that AM mediated its effects via cAMP it was decided to investigate whether the mitogenic effects of AM were also cAMP related. AM-stimulated H357 cells, labelled with [^{3}H]thymidine, were incubated in the presence of various enzyme inhibitors. Fig. 3 (inset) shows that SQ22,536 had profound inhibitory effects on [^{3}H]thymidine uptake whereas Ro31-8220 or lavendustin A had no effect. These results strongly suggest that AM-induced DNA synthesis in H357 cells is not mediated via PKC or tyrosine kinase. Furthermore, addition of the CGRP receptor antagonist CGRP(8–37) to the [^{3}H]thymidine-labelled cells did not affect DNA synthesis in response to 10 nmol/l AM, lending additional support that the effects of AM are mediated by specific AM receptors and not by CGRP receptors.

Cell proliferation studies, using an MTS tetrazolium-based colorimetric assay, demonstrated that AM caused significant increases in cell proliferation with maximum incorporation occurring at 1 nmol/l AM ($2.59\times10^5\pm0.18$ cells vs $1.02\times10^5\pm1.09$ untreated cells; $P<0.001$ compared to control). When cells were incubated with SQ22,536, cellular proliferation was significantly attenuated in response to 1 nmol/l AM ($1.15\times10^5\pm0.08$ cells vs $2.59\times10^5\pm0.18$ untreated cells; $P<0.001$ compared to AM alone). Furthermore, the cell-permeable cAMP analogue, dbcAMP, also caused a significant increase in cell growth ($2.22\times10^5\pm0.19$ cells vs $1.02\times10^5\pm0.09$ untreated cells; $P<0.001$ compared to control), suggesting that this effect on proliferation is mediated via cAMP or a cAMP-mediated mechanism. However, the PKC inhibitor, Ro31-8220, the tyrosine kinase inhibitor, lavendustin A, and CGRP(8–37) had no effect on cell proliferation in response to 1 nmol/l AM at any concentration used (data not shown).

Increases in levels of cAMP have been identified as a mitogenic signal for many cells and AM has been shown to stimulate cAMP production in a wide variety of cells. These include Schwann cells [19,20], breast tumour cells [21], thyroid epithelial cells [22] and AML193, a leukaemic cell line [23]. It

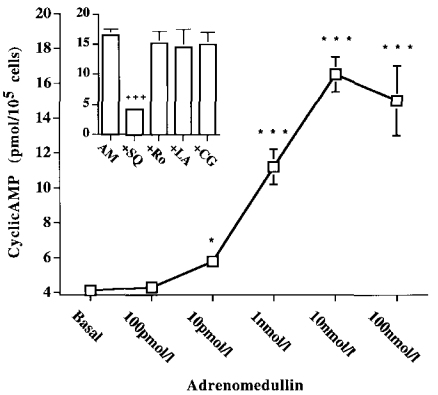

Fig. 2. Dose-dependent increases in cAMP production in H357 cells in response to AM. Inset: Bar graph showing the effects of various inhibitors on 10 nmol/l AM-stimulated cAMP levels. SQ, SQ22,536 (10 nmol/l); Ro, Ro31-8220 (150 nmol/l); LA, lavendustin A (500 nmol/l); CG, CGRP(8-37) (1 μmol/l). Values are means ± S.E.M., $n=4$. $^*P<0.05$, $^{***}P<0.001$ compared with basal levels of cAMP (analysis of variance). $^{+++}P<0.001$ compared with AM alone (Student's t-test).

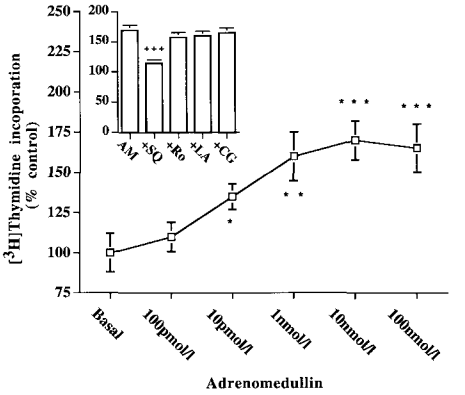

Fig. 3. Dose-dependent increases in [^{3}H]thymidine uptake in intact H357 cells in response to AM. Inset: Bar graph showing the effects of various agents on 10 nmol/l AM-stimulated cAMP levels. SQ, SQ22,537 (10 nmol/l); Ro, Ro31-8220 (150 nmol/l); LA, lavendustin A (500 nmol/l); CG, CGRP(8-37) (1 μmol/l). Values are means ± S.E.M., $n=4$. $^*P<0.05$, $^{***}P<0.001$ compared with basal levels of DNA synthesis (analysis of variance). $^{+++}P<0.001$ compared with AM alone (Student's t-test).

is clear that AM acts in a similar manner on cultured oral keratinocytes. Previous studies have shown that regulatory peptides have a role in controlling cell proliferation, in addition to the growing list of neuropeptides and growth factors that are known to promote cell proliferation [24–27].

Many peptides with mitogenic properties, such as endothelin, angiotensin II and vasopressin, mediate their cellular effects via activation of phospholipase C and, thus, production of inositol phosphates and diacylglycerol, as well as PKC activation [25–27]. Experiments conducted in this study suggest that PKC is not activated by AM in H357 cells. Additionally, in experiments where cells were labelled with [^{3}H]inositol, AM did not induce the production of inositol trisphosphate, suggesting AM does not activate phospholipase C (data not shown). In contrast, our findings indicate that AM is a novel mitogenic peptide for human oral keratinocytes and that these mitogenic effects are mediated via cAMP, a signal that is known to cause increased/enhanced cell proliferation in a number of cell types. These results lend further support for the role of peptides, including AM, in the control and regulation of cell proliferation.

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