Adrenomedullin stimulates DNA synthesis and cell proliferation via elevation of cAMP in Swiss 3T3 cells

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Abstract Our results demonstrate that the novel vasoactive regulatory peptide adrenomedullin is a potent mitogen for Swiss 3T3 cells. Acting via a specific adrenomedullin receptor, it stimulates a dose-dependent increase in DNA synthesis in synergy with insulin. Additionally, adrenomedullin stimulates further progression through the cell cycle resulting in cell proliferation, an effect that was further enhanced by the presence of insulin. Adrenomedullin rapidly induces accumulation of intracellular cAMP but does not stimulate an increase in intracellular Ca^{2+}, activation of protein kinase C, or tyrosine phosphorylation of intracellular substrates. Adrenomedullin-stimulated mitogenesis is markedly enhanced in Swiss 3T3 cells stably transfected with a constitutively activated Gsα, which are highly sensitive to agents that elevate cAMP, and is inhibited by the PKA inhibitor H-89. Adrenomedullin is, thus, identified as a novel mitogenic regulatory peptide acting via cAMP.

Key words: Neuropeptide; Signal transduction; Growth control

1. Introduction

Adrenomedullin is a novel 50 amino acid peptide which has a six-residue intramolecular disulfide-link ring structure and is a member of the calcitonin peptide family [1,2]. Adrenomedullin immunoreactivity and mRNA are widely distributed in rat and human tissues, including heart, aorta and lung, and the peptide is present in plasma [3]. Specific adrenomedullin-binding sites have been identified in a range of rat tissues, including lung and vascular tissue [4], and in cultured rat vascular smooth muscle (VSM) and bovine aortic endothelial (BAE) cells [5]. Although these findings suggest that adrenomedullin is a novel element in the homeostatic regulatory peptide network, the cellular responses and signal transduction pathways stimulated by this peptide through specific adrenomedullin receptors have not, as yet, been elucidated.

Previous studies have shown that adrenomedullin stimulates the accumulation of intracellular cAMP in VSM and BAE cells and induces a rise in intracellular Ca^{2+} via a cholera toxin-sensitive G-protein-dependent mechanism in BAE cells [5]. However, adrenomedullin mediated increases in intracellular cAMP can be blocked by the calcitonin-gene related peptide (CGRP) receptor antagonist CGRP (8–37) in some cell types [6]. Since adrenomedullin has been shown to interact with CGRP receptors and CGRP (8–37) binds very weakly to specific adrenomedullin receptors [7], the effects of adrenomedullin observed in some cell lines and tissues may be mediated via CGRP receptors. Indeed, the best characterized pharmacological action of adrenomedullin, namely the induction of a potent hypotensive response in rats, might be mediated by CGRP receptors [8]. However, the rat adrenomedullin receptor, which belongs to the G-protein-coupled receptor superfamily characterized by seven transmembrane domains [9], binds adrenomedullin but not CGRP [7]. Clearly, the examination of the signal transduction pathways activated by specific adrenomedullin receptors and the elucidation of the biological responses induced by this novel peptide at the cellular level requires a cell line which expresses adrenomedullin but not CGRP receptors.

Here, we report that Swiss 3T3 cells, a useful model to identify the extracellular and intracellular signals that elicit mitogenesis [10], display specific high-affinity adrenomedullin receptors but not CGRP receptors. We show, for the first time, that adrenomedullin acts as a potent synergistic mitogen for these cells. Adrenomedullin elicits accumulation of intracellular cAMP but does not induce Ca^{2+} mobilization, protein kinase C (PKC) activation, or tyrosine phosphorylation of intracellular substrates. Thus, our results identify adrenomedullin as a novel neuropeptide growth factor acting through specific adrenomedullin receptors to elevate intracellular cAMP.

2. Materials and methods

2.1. Materials

Rat adrenomedullin was obtained from Peptide Institute (Osaka, Japan). Iodogen reagent was obtained from Pierce (Rockford, IL). Bombesin, insulin, bacitracin, antimouse IgG and phosphoramidon were obtained from Sigma. The bromodeoxyuridine (BrDu) labelling and detection kit was obtained from Boehringer Mannheim Biochemica. The Biotrak cAMP RIA kit, [^{125}I]protein A, ^{125}I-labelled sheep antimouse IgG and Na^{125}I were obtained from Amersham (UK). PY20 and 4G10 anti-Tyr (P) mAbs were obtained from TCS Biologicals (UK). Tyr^{0}αCGRP and CGRP (8–37) were obtained from Peninsula Laboratory (Belmont, CA) and αCGRP from ASG University (Szedegl, Hungary). The Gsα (Q227Lαs) 3T3 cell line was a kind gift from I. Zachary (Department of Medicine, King's College School of Medicine and Dentistry, London, UK). All other reagents were of the purest grade available.

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Abbreviations: CGRP, calcitonin-gene related peptide; PKC, protein kinase C; FAK, focal adhesion kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PDB, phorbol 12,13-dibutyrate; IBMX, 3-isobutyl-1-methylxanthine; EGF, epidermal growth factor; MARCKS, myristoylated, alanine-rich C kinase substrate; PKA, protein kinase A; VSM, vascular smooth muscle; BAE, bovine aortic endothelial; Tyr (P) tyrosine phosphate; BrDu, bromodeoxyuridine; mAb, monoclonal antibody; RIA, radioimmunoassay.

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2.2. Cell culture, DNA synthesis, cell proliferation and measurement of bromodeoxyuridine labelling of cell nuclei

Stock cultures of Swiss 3T3 fibroblasts were maintained as previously described in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 10% CO_2 and 90% air at 37°C [11]. Thymidine incorporation assays and the measurement of cell proliferation were performed as previously described [11]. The immunofluorescence assay for the detection of BrDu incorporated into cellular DNA was performed using the BrDu labelling and detection kit from Boehringer Mannheim Biochemica.

2.3. Peptide iodination

Rat adrenomedullin was iodinated by the Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) method [12]. Briefly, 12.5 μg (2 nmol) of adrenomedullin in 10 μl of 0.2 M phosphate buffer (pH 7.2) were reacted with 37 MBq of $Na^{125}I$ and 10 μg of Iodogen reagent for 4 min at 22°C. The [^{125}I]peptide was purified by reversed-phase HPLC (Waters C_{18} Novapak, Millipore) using a 15–40% acetonitrile/water/0.05% trifluoroacetic acid gradient. Fractions showing binding were aliquoted, freeze-dried and stored at −80°C. The specific activity of the adrenomedullin label was 10.4 Bq/fmol. Tyr^0αCGRP was iodinated by the Iodogen method [13]. The specific activity of the CGRP label was 35.9 Bq/fmol.

2.4. Receptor-binding assays

Confluent Swiss 3T3 cells were incubated for 120 min at room temperature in 1 ml of binding buffer (20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl, 1 mM EDTA, 1 μM phosphoramidon, 0.25 mg/ml bacitracin and 0.1% BSA) containing 1000 Bq (100 pM) [^{125}I]adrenomedullin. Bound and free label were separated by aspiration of binding buffer and washing 3 × with binding buffer without label at 4°C. Cells were solubilized in 0.1 M NaOH and bound label counted. Non-specific binding was determined in the presence of 500 nM unlabelled rat adrenomedullin. Specific binding was defined as total binding minus non-specific binding. CGRP-binding assays were performed using a similar protocol with the same assay buffer and incubation conditions except with 1000 Bq (27.8 pM) of Tyr^0αCGRP. Non-specific binding was determined in the presence of 200 nM rat αCGRP.

2.5. Measurement of cAMP accumulation

Quiescent cultures of cells were washed 3 × with DMEM and incubated with DMEM/Waymouth's medium (1:1 v/v) with factors as indicated for the times shown. Medium was then removed and cAMP extracted by incubating with 0.1 M HCl for 20 min at 4°C. The HCl extract was then neutralized and the cAMP concentration determined using the acetylation protocol of the Biotrak cAMP RIA system (Amersham). Protein determinations were performed by solubilizing the cell layer in 1 ml of 2% Na_2CO_3, 0.1 M NaOH, 1% SDS and using the BCA protein assay (Pierce).

2.6. Measurement of intracellular calcium, antiphosphotyrosine immunoprecipitations and 80K/MARCKS phosphorylation

These procedures were performed as previously described [14,15].

3. Results and discussion

3.1. [^{125}I]adrenomedullin binds to intact Swiss 3T3 cells

To examine whether Swiss 3T3 cells express specific adrenomedullin receptors, quiescent cultures of these cells were incubated with ^{125}I-labelled peptide in the presence of increasing concentrations of unlabelled adrenomedullin. As shown in Fig. 1, upper panel, unlabelled peptide competed with [^{125}I]adrenomedullin for binding to Swiss 3T3 cells in a concentration dependent manner, with an IC_{50} of 3.5 ± 0.6 nM and a B_{max} of 26.3 ± 3.9 fmol/10^5 cells. In contrast, no binding of labelled CGRP could be demonstrated in parallel cultures of Swiss 3T3 cells using the same assay conditions (Fig. 1, upper panel, inset). As a control we verified that the [^{125}I]CGRP used in these experiments bound specifically to SK-N-MC neuroblastoma cells, a cell line known to express specific CGRP receptors [4] (Fig. 1, upper panel, inset). Furthermore, neither CGRP nor CGRP (8–37) inhibited [^{125}I]adrenomedullin-binding to Swiss 3T3 cells at concentrations up to 1 μM (results not shown). These experiments demonstrate the expression of specific adrenomedullin but not CGRP receptors in quiescent Swiss 3T3 cells.

3.2. Adrenomedullin stimulates DNA synthesis and cell proliferation

Regulatory peptides are increasingly implicated in the control of cell proliferation [16] and, therefore, we next determined whether adrenomedullin can act as a growth factor for Swiss 3T3 cells. Treatment of confluent, quiescent cultures of these cells with adrenomedullin, in combination with insulin, stimulated a dose-dependent increase in [^{3}H]thymidine incorporation as shown in Fig. 1, lower panel. The maximum effect was seen at 100 nM adrenomedullin and was similar to that achieved with the potent neuropeptide mitogen bombesin at fully mitogenic concentrations [17]. These results were substantiated by the finding that treatment of cells with adrenomedullin stimulated the incorporation of BrDu into cell nuclei. The maximum effect was again seen at 100 nM adrenomedullin, in synergy with insulin, with 55 ± 0.5% of nuclei being labelled (Fig. 1, lower panel, inset). In agreement with the binding data presented above, the stimulation of DNA synthesis induced by adrenomedullin was not attenuated by the CGRP-antagonist CGRP (8–37) at 1 μM (results not shown). Adrenomedullin stimulated further progression through the cell cycle resulting in cell proliferation. As shown in Table 1, adrenomedullin added to cells maintained in conditioned medium elicited a statistically significant increase in cell number an effect that was further enhanced by the presence of insulin. The results presented in Fig. 1 and Table 1 demonstrate for the first time that adrenomedullin can synergistically induce long-term stimulation of DNA synthesis and cell division in cultured cells through specific adrenomedullin receptors.

3.3. Adrenomedullin stimulation of DNA synthesis is mediated via cAMP

The potent mitogenic activity of adrenomedullin in Swiss 3T3 cells led us to characterize the signal transduction pathways activated by this neuropeptide in these cells. Adrenomedullin has recently been reported to stimulate an increase in intracellular [Ca^{2+}] [5] and many other mitogenic neuropep-

Table 1
Effect of adrenomedullin or bombesin on the proliferation of Swiss 3T3 cells

Addition	Cell number (×10^{-5})	
	–	+Ins
–	3.39 ± 0.14	4.07 ± 0.08
Adrenomedullin	4.52 ± 0.06*	7.14 ± 0.13*
Bombesin	4.45 ± 0.14*	9.15 ± 0.08*

6-day-old cultures of Swiss 3T3 cells in conditioned medium were treated with various factors: 100 nM adrenomedullin with 50 μM IBMX, or 10 nM bombesin (Bom) or control (−) in the absence or presence of 1 μg/ml of insulin (± Ins). Cell number was then determined after 72 h as described in section 2. Results shown are the mean ± S.E. values of two experiments performed in qudruplicate.
*$P < 0.001$ in Student's t test compared with control without insulin for factors in the absence of insulin and with control with insulin for factors in the presence of insulin.

tides, including bombesin, stimulate calcium mobilization from intracellular stores in Swiss 3T3 cells [18]. Therefore, we assessed the ability of adrenomedullin to mobilize calcium in these cells. As shown in Fig. 2, panel A, adrenomedullin did not increase the intracellular [Ca^{2+}] over a concentration range from 10 to 100 nM (at which levels it has been demonstrated to elevate intracellular [Ca^{2+}] in BAE cells) whereas subsequent addition of bombesin caused a rapid increase in intracellular [Ca^{2+}]. No mobilization of Ca^{2+} was seen with adrenomedullin in combination with insulin (data not shown).

In intact cells, the activation of phorbol ester-sensitive isoforms of PKC results in the phosphorylation of an acidic 80 kDa protein 80K/MARCKS [13]. Fig. 2, panel B, shows that phorbol 12,13-dibutyrate (PDB) caused a marked increase in the phosphorylation of 80K/MARCKS whereas adrenomedullin alone or in combination with insulin had no effect. These results strongly suggest that adrenomedullin does not activate PKC. Additionally, we did not detect any increase in the production of inositol phosphates when [^{3}H]inositol-labelled Swiss 3T3 cells were treated with adrenomedullin suggesting that this peptide does not activate phospholipase C (data not shown).

Bombesin and other neuropeptides have been shown to stimulate tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells, including major bands migrating with apparent molecular masses of 60–70 kDa and 110–130 kDa [19]. The focal adhesion proteins paxillin, p125^{FAK} and p130 are prominent tyrosine phosphorylated substrates in neuropeptide-treated Swiss 3T3 cells [19]. As shown in Fig. 2, panel C, adrenomedullin did not stimulate tyrosine phosphorylation of any proteins at concentrations that promote DNA synthesis.

An increase in the intracellular concentration of cAMP has been identified as one of the mitogenic signals for Swiss 3T3 cells [10,11] and adrenomedullin has been shown to elevate cAMP in several cell types [1,5]. Consequently, we examined whether adrenomedullin also increases cAMP in Swiss 3T3 cells. As shown in Fig. 3, adrenomedullin stimulated a rapid rise in intracellular cAMP levels (to 6-fold above basal levels) which peaked within 5 min of addition and persisted for at least 3 h. Adrenomedullin increased intracellular cAMP in a dose-dependent manner with a maximal response seen at 10 nM (Fig. 3, inset). The results presented in Figs. 2 and 3 indicate that adrenomedullin acts via cAMP rather than through phospholipase C-dependent signalling pathways utilized by many other mitogenic regulatory peptides.

We utilized two additional experimental approaches to provide further evidence that the mitogenic effects of adrenomedullin are mediated via elevation of intracellular cAMP. Swiss 3T3 cells stably transfected with a constitutively activated Gsα subunit are highly sensitive to agents that elevate cAMP [20]. In these cells, adrenomedullin-stimulated mitogenesis was markedly enhanced with a shift in the dose response such that maximal DNA synthesis was seen at a concentration as low as 0.1 nM (Fig. 4). To further investigate the role of cAMP in adrenomedullin-stimulated DNA synthesis, we utilized the PKA inhibitor H-89 [21]. As shown in Fig. 5, inset, addition of 5 μM H-89 resulted in a 50% inhibition of adrenomedullin-

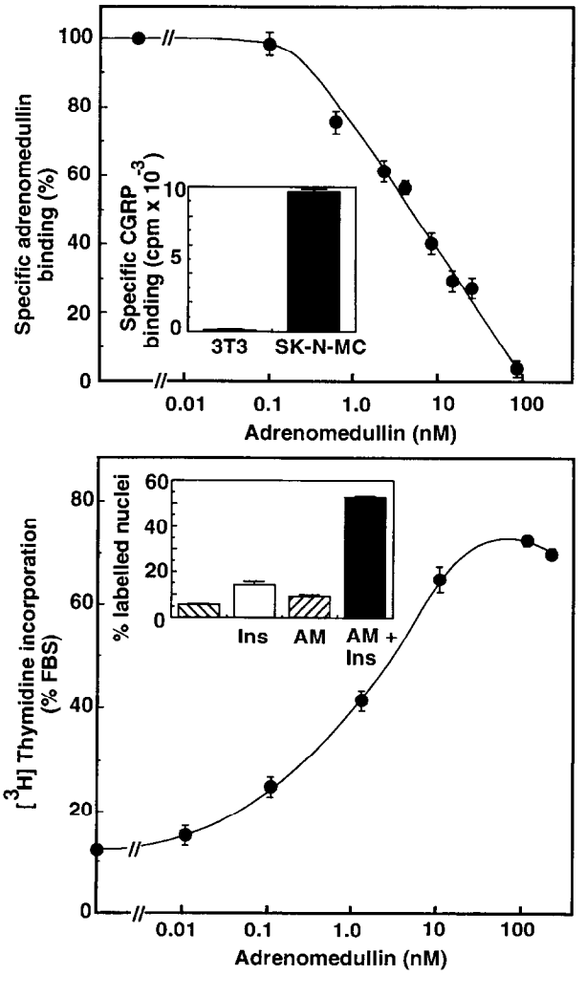

Fig. 1. Binding of [^{125}I]adrenomedullin to intact Swiss 3T3 cells and adrenomedullin stimulation of DNA synthesis. (Upper) Confluent, quiescent Swiss 3T3 cells were incubated for 2 h at room temperature with [^{125}I]adrenomedullin in the presence of increasing concentrations of cold adrenomedullin. Bound and free label were separated by washing, the cells solubilized and bound label counted. Specific binding was calculated as total binding minus non-specific binding as determined in the presence of 500 nM unlabelled adrenomedullin. Results presented are the mean ± S.E. values of three separate experiments each performed in sextuplet. (Upper, inset) Confluent, quiescent Swiss 3T3 and SK-N-MC cells were incubated for 2 h with [^{125}I]CGRP. Bound and free label were separated by washing, the cells solubilized and bound label counted. Specific binding was calculated as total binding minus non-specific binding as determined in the presence of 500 nM CGRP and is presented as cpm/10^6 cells. The results shown are the mean ± S.E. values of three separate experiments each performed in sextuplet. (Lower) Confluent, quiescent cultures of Swiss 3T3 cells were incubated in DMEM/Waymouth medium containing [^{3}H]thymidine and increasing concentrations of adrenomedullin in the presence of 50 μM IBMX and 0.5 μg/ml insulin. After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by 10% FBS, and data are shown as mean ± S.E. (n = 12). (Lower, inset) Confluent, quiescent cultures of Swiss 3T3 cells were incubated in DMEM/Waymouth medium containing bromodeoxyuridine (BrDu) labelling reagent and vehicle (−), 0.5 μg/ml insulin (Ins) and 100 nM adrenomedullin with 50 μM IBMX in the absence (AM) or presence (AM+Ins) 0.5 μg/ml insulin. After 40 h, BrDu incorporation into cell nuclei was determined by using a specific antibromodeoxyuridine monoclonal antibody detected by a fluoroscein-conjugated secondary antibody. Labelled nuclei were visualized by fluorescence microscopy. Results are presented as the% labelled nuclei ± S.E. in five distinct fields from two separate experiments.

stimulated DNA synthesis while no effect was seen on EGF-stimulated mitogenesis, which is not mediated via cAMP [10].

3.4. Conclusions

An increasing number of neuropeptides and vasoactive peptides have been shown to act as local hormones or transmitters exerting their actions in a paracrine or autocrine manner [22]. These regulatory peptides elicit a wide range of short-term physiological events such as neuronal excitation and are increasingly implicated in the long-term modulation of cell growth and differentiation. The results presented here demonstrate, for the first time, that the vasoactive regulatory peptide adrenomedullin is a potent growth factor for quiescent cultures of Swiss 3T3 cells incubated in the presence of insulin.

Many mitogenic neuropeptides, such as bombesin, vasopressin, bradykinin and endothelin, elicit their effects via polyphosphoinositide hydrolysis leading to elevation of intracellular Ca^{2+} and activation of PKC [22]. These peptides also promote cytoskeletal reorganization and tyrosine phosphorylation of focal adhesion proteins [19]. In contrast, our results indicate that the mitogenic effects of adrenomedullin are elicited via the elevation intracellular cAMP which is one of the mitogenic signals for Swiss 3T3 cells [10,11]. Acting via specific adrenomedullin, but not CGRP receptors, adrenomedullin stimulates a persistent elevation in intracellular cAMP but does not promote an increase in intracellular $[Ca^{2+}]$, activation of PKC or the tyrosine phosphorylation of distinct intracellular substrates. Further evidence that the mitogenic effects of adrenomedullin are mediated via its ability to elevate intracellular cAMP comes from the use of Swiss 3T3 cells stably transfected with a constitutively activated Gsα subunit. Thus, the findings

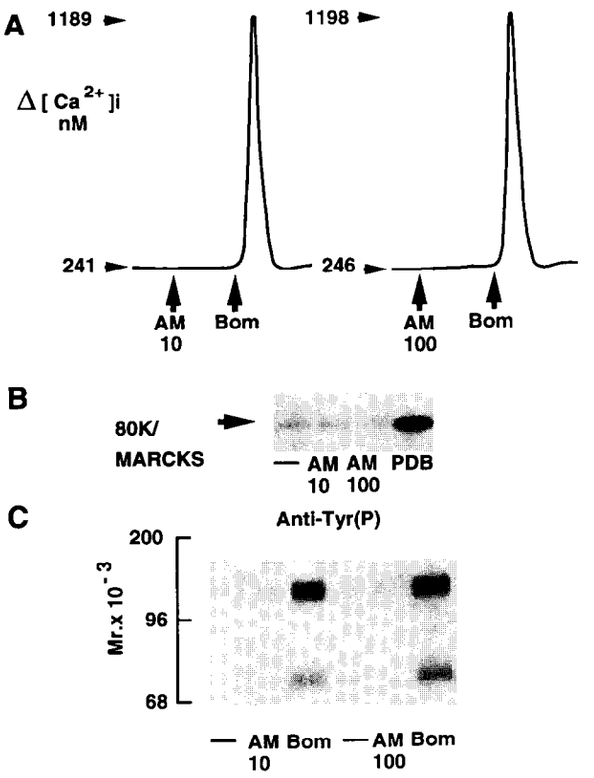

Fig. 2. Adrenomedullin does not induce Ca^{2+} mobilization, PKC activation, or tyrosine phosphorylation of intracellular substrates. (A) Confluent, quiescent cultures of Swiss 3T3 cells were washed and loaded for 15 min with Fura-2 and then scraped and transferred to a cuvette. The cells were stirred at 37°C in a Perkin-Elmer LS5 luminescence spectrophotometer and fluorescence was measured and recorded continuously. Adrenomedullin at doses of 10 nM (AM 10) and 100 nM (AM 100) were used in separate experiments. Bombesin at 10 nM (Bom) was added after the adrenomedullin as a positive control. Results are expressed as calculated increase in intracellular Ca^{2+} in nM ($[Ca^{2+}]_i$) and are representative of three independent experiments. (B) Confluent dishes of Swiss 3T3 cells were incubated overnight in phosphate-free DMEM with 50 μCi/ml $^{32}P_i$. Cells were treated for 10 min with vehicle (−), adrenomedullin (with 50 μM IBMX) at doses of 10 nM (AM 10) and 100 nM (AM 100) or 200 nM phorbol 12,13-dibutyrate (PDB). The cells were lysed, 80K/MARCKS immunoprecipitated with a specific antibody and the immunoprecipitates analysed by SDS-PAGE and subsequent autoradiography. (C) Quiescent cultures of Swiss 3T3 cells were treated for 5 min with vehicle (−), adrenomedullin (with 50 μM IBMX) at doses of 10 nM (AM 10) and 100 nM (AM 100) or bombesin at 10 nM (Bom), lysed and proteins were immunoprecipitated at 4°C overnight with a monoclonal antibody directed against phosphotyrosine. After separation by SDS-PAGE, proteins were transferred to Immobilon membranes which were incubated in 5% non-fat dried milk in phosphate-buffered saline with a mixture of PY20 and 4G10 antiphosphotyrosine monoclonal antibodies. Immunoreactive bands were visualized using ^{125}I-labelled sheep antimouse IgG followed by autoradiography.

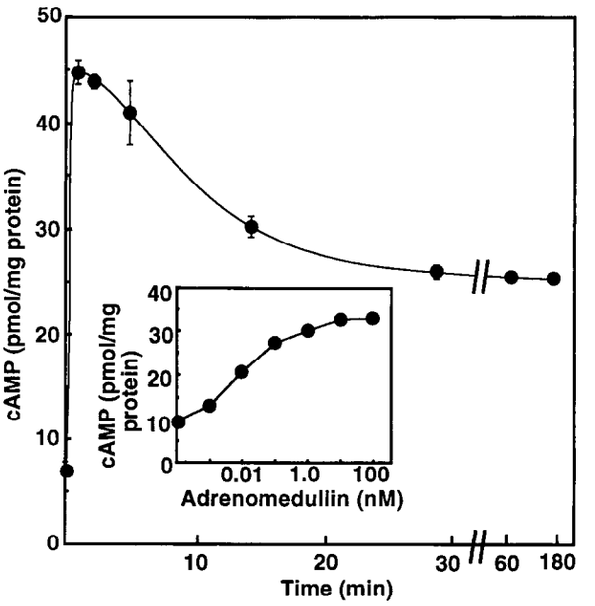

Fig. 3. Time- and dose-dependent stimulation of the intracellular accumulation of cAMP by adrenomedullin. Confluent, quiescent cultures of Swiss 3T3 cells were incubated for various times in the presence of 100 nM adrenomedullin with 50 μM IBMX. The medium was removed and cAMP was extracted by incubating with 0.1 M HCl and cAMP concentration determined using a specific RIA. Results are expressed as pmol/mg of cellular protein and are the mean ± S.E. values of three separate experiments each performed in duplicate. Where error bars are not visible, they lie within the borders of the symbol. (Inset) Confluent, quiescent cultures of Swiss 3T3 cells were incubated for 15 min in the presence of increasing concentrations of adrenomedullin with 50 μM IBMX. The medium was removed and cAMP was extracted by incubating with 0.1 M HCl and cAMP concentration determined using a specific RIA. Results are expressed as pmol/mg of cellular protein and are the mean ± S.E. values of three separate experiments each performed in duplicate. Where error bars are not visible, they lie within the borders of the symbol.

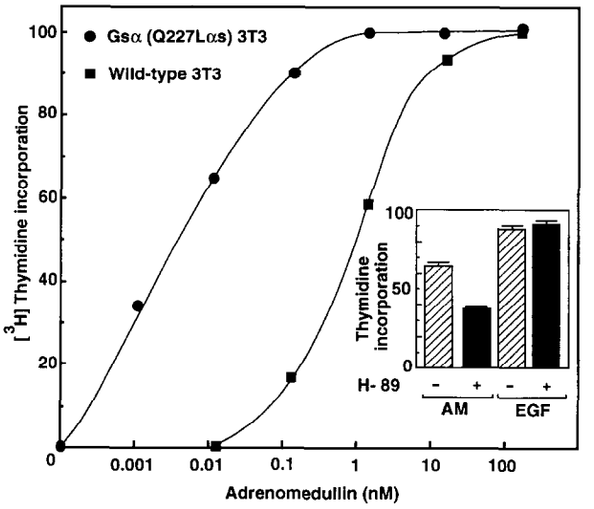

Fig. 4. Adrenomedullin stimulates mitogenesis via elevation of cAMP in Swiss 3T3 cells Confluent, quiescent cultures of either wild type Swiss 3T3 cells (■) or Swiss 3T3 cells stably transfected with an activated Gsα subunit [Gsα(Q227Lαs)3T3] (●) were incubated in DMEM/Waymouth medium containing [^{3}H]thymidine and increasing concentrations of adrenomedullin in the presence of 50 μM IBMX and 0.5 μg/ml insulin. After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are the mean values of duplicates, are expressed as a percentage of the incorporation induced by 10% FBS, and are representative of three independent experiments. (Inset) Confluent, quiescent cultures of Swiss 3T3 cells were incubated in DMEM/Waymouth medium containing [^{3}H]thymidine either 100 nM adrenomedullin with 50 μM IBMX and 0.5 μg/ml insulin (AM) or 5 ng/ml EGF with 0.5 μg/ml insulin (EGF) in the presence or absence of 5 μM H-89. After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by 10% FBS and are the mean ± S.E. values of two experiments performed in duplicate.

presented here support the existence of multiple signalling pathways in the mitogenic actions of regulatory peptides and imply that the role of these peptides in the control of cell proliferation is likely to be broader than previously anticipated.

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