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Adrenomedullin inhibits prostate cancer cell proliferation through a cAMP-independent autocrine mechanism☆

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Abstract

Adrenomedullin (AM) is a multifunctional peptide expressed in the normal and malignant prostate, and in prostate cancer cells. To elucidate the potential role of AM in prostate cancer, we have transfected the human AM gene into PC-3, DU 145, and LNCaP prostate cancer cells. Northern blot, Western blot, and radioimmunoassay techniques confirmed an increase in the synthesis and secretion of the 6 kDa mature peptide, in the AM-transfected clones. Proliferation and cell cycle assays demonstrated that AM overexpression inhibited cell proliferation in PC-3 and LNCaP cells through a G0/G1 cell cycle arrest, but not in DU 145 cells. In vivo growth assays also confirmed that, at least in PC-3, AM produced a very significant reduction of tumor volume. In addition, the three cell lines expressed the CL/RCP/RAMP-2 receptor complex by RT-PCR, which suggests that AM peptide acts through an autocrine loop in prostate cancer cells. Although cAMP elevation is the most common pathway involved in AM signalling, stimulation of PC-3, DU 145, and LNCaP with synthetic AM did not increase intracellular cAMP. However, short-term stimulation of PC-3 cells with synthetic AM increased ERK1/2 activation. On the contrary, long-term stimulation, or AM overexpression, caused a reduction in the basal activation of ERK1/2. In summary, our results demonstrate that AM (either overexpressed or exogenously added) causes an inhibition of prostate cancer cell growth. This inhibition does not depend on changes in intracellular cAMP levels, but may be related to ERK1/2 activation.

Keywords: Adrenomedullin; Prostate cancer; cAMP; MAPK; Growth inhibition

Prostate carcinoma is the second leading cause of cancer mortality among men in the US [1]. Although some of the molecular mechanisms involved in prostate cancer progression have been elucidated, the development of the disease is largely unknown. In addition, the limited success of prostate cancer therapies requires that additional targets for therapy be identified and tested. We and others have previously described the expression of adrenomedullin (AM), a 52-aminoacid peptide isolated from a pheochromocytoma [2], in the normal and malignant prostate [3–5]. Although numerous functions have been attributed to AM in different organs, such as vasodilation, bronchodilation, hormone secretion control, and regulation of cell growth [6], the role of AM in the prostate and in prostate carcinoma is not well understood. The effect of AM on cell growth has been studied in several cell types. AM stimulates proliferation of *zona glomerulosa* cells, skin fibroblasts, glioblastoma, and lung cancer cells [6,7]. However, AM causes growth inhibition of myocytes, cardiac fibroblasts, vascular

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smooth muscle cells (VSMC), chondrocytes, and mesangial cells [6,7]. The reason for this opposite effect on cell growth depending upon the cell type is still to be determined.

Three different AM receptors have been described: L1, RDC1, and the calcitonin receptor-like receptor (CL), which belong to the seven transmembrane G-coupled receptors [6]. Among these, recent publications show that CL is the most important and widespread receptor involved in AM function. CL requires the presence of certain regulatory proteins with a single transmembrane domain known as receptor activity-modifying proteins (RAMPs) [8]. CL acts as a calcitonin gene related protein (CGRP) or AM receptor, when coupled with RAMP-1. However, the complex binds CGRP with much more affinity than AM [8]. RAMP-2 or RAMP-3 coupled with CL binds specifically to AM [8]. The receptor component protein (RCP) is responsible for the initiation of the intracellular transduction of the signal [9,10].

The activation of the receptor by AM has commonly been linked to elevation of cAMP [6]. However, recent studies have shown that alternative intracellular pathways may act in AM signalling. These include, among others, elevation of Ca^{+2} levels [11], activation of the cGMP-NO pathway [6,12,13] or the involvement of the mitogen-activated protein kinases (MAPKs) [14,15].

In order to clarify the role of AM in prostate cancer, we previously generated PC-3 cell clones which overexpressed the rat AM gene, and found, as a result, a striking cell growth inhibition both in vitro and in vivo in these clones [16]. In this study we have expanded the study to other prostate cancer cells (LNCaP and DU 145) and analyzed the effect of human AM overexpression. We show here that, indeed, increased expression of AM or exogenous treatment with the AM peptide produce cell growth inhibition in prostate cancer cells. We also demonstrate that prostate cancer cells express AM receptors, which suggests an autocrine loop for AM activity. Intracellular signalling seems to involve ERK1/2 activation rather than an increase in cAMP levels in prostate cells.

Materials and methods

Vector design. The pSecTagB plasmid (Invitrogen, Carlsbad, CA) containing the complete sequence of the human AM (hAM) was kindly donated by Dr. Alfredo Martínez (NCI, NIH, Bethesda, MD). hAM was then subcloned into the pcDNA3.1/Hygro plasmid (Invitrogen) by PCR, using the following primers: CATGCTAGCGCCACCATGA AGCTGGTTTCCGTC (forward), and CTAGGATCCCTAAGGAA AGTGGGGAGCACT (reverse), under the following conditions: 94 °C, 45 s; 55 °C, 45 s; and 72 °C, 75 s (35 cycles). The PCR product included sequences for the restriction enzymes *Nhe*I (at the 5′ position) and *Bam*HI (at the 3′ end), and for the Kozak sequence (GCCACC) (at the 5′ end).

Cell culture, proliferation, and cell cycle analysis. Prostate cancer cell lines (PC-3, DU 145, and LNCaP) were cultured in 5% CO_2 at 37 °C in RPMI 1640 medium with Glutamax-I (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were stably transfected with the pcDNA3.1/Hygro plasmid containing hAM or the plasmid alone (as a control). Transfections were done with Fugene 6 (Roche, Penzberg, Germany) following the manufacturer's protocol. Selection of cell clones was performed with 150 μg/ml hygromycin B (Invitrogen). Up to 20 individual clones per cell line were randomly chosen to study AM expression. In the case of LNCaP, we could not establish cell clones with stable expression of AM. Cell clones lost expression of AM after several days in culture, and therefore the experiments were conducted with transiently transfected cells.

Proliferation assays of the stably transfected clones were conducted in 96-well plates with 1000 or 1500 cells/well in serum free medium. After 24 h, 10% FCS or conditioned medium from PC-3 cells was added to the cultures and cell viability was assessed for the following 5 days using the MTT assay (Roche). Proliferation assays were also performed in parental cell lines by daily addition of the human AM (hAM) peptide (Peninsula–Bachem, San Carlos, CA) at concentrations ranging from 0.1 to 1000 nM. For these experiments, cells were cultured with low amount of FCS (1%). We have previously tested that cells cultured in 1% FCS maintain considerable good growth capabilities, while reduction of the serum below 0.5–1% leads to significant cell growth decrease (stress conditions).

For cell cytometry analysis, synchronized cells were grown in complete medium for 24 h, collected, and processed with DNA Prep Coulter (Beckman–Coulter, Fullerton, CA). Cells were analyzed in a FacsCalibur flow cytometer with an excitation wavelength of 488 nm. Anchorage independent growth in soft agar was also evaluated as previously described [16]. Representative photographs were taken with a Leica phase contrast microscope and are occupied by the colonies was calculated by image analysis (analySYS 3.0, Soft Imaging System, GmbH, Munster, Germany).

In vivo tumor growth. PC-3 cell clones were subcutaneously injected (1×10^6 cells per mouse) in BALB/c athymic nude mice (4-week-old males, 5 mice per group). Tumor size was measured weekly and volumes were calculated using the previously described formula $V = (w^2 \times l)/2$ [17]. Mice were sacrificed when tumors reached 1 cm^3, or 5 weeks after the initiation of the experiment. All mice were treated in accordance with the guidelines for the Animal Care Ethics Comission of our institution (Universidad de Navarra) under an approved animal protocol.

Northern blot and RT-PCR. Total RNA was obtained with the Ultraspec RNA Kit (Biotecx, S. Loop E. Houston, TX) following the manufacturer's instructions. Northern blot analysis for AM and S9 mRNA was carried out as previously described [18]. One microgram of DNAse-treated RNA was reversed transcribed into cDNA using the First Strand SuperScript II RT kit (Invitrogen). For PCR amplifications, a Perkin–Elmer 2400 thermocycler was used, and PCR products were run through 1% agarose gels and scanned with Gelprinter SuperII (*Tecnología para Diagnóstico e Investigación*, Madrid, Spain). The sequence of the primers and melting temperatures used for the PCR are shown in Table 1. Thirty-five cycles of PCR were used for the amplification.

Western blot and RIA. Soluble proteins were extracted with RIPA buffer for AM detection or with a specific lysis buffer (25 mM sodium β-glycerolphosphate, 1 mM sodium orthovanadate, 25 mM NaF, 1 mM $Na_2P_3O_4$, 1% Triton X-100, 0.05% sodium deoxycholate, 1 mM benzamidine, 10% glycerol, 0.1% β-mercaptoethanol, 0.01% SDS, 5 mM EDTA, and 5 mM EGTA, all from Sigma) for phosphorylated protein detection. Both extraction buffers contained a cocktail of protease inhibitors (Complete; Roche Diagnostics, Penzberg, Germany).

Table 1
Sequence of the primers and melting temperatures for the amplification by RT-PCR of AM, CL, RCP, RAMPs(1–3), and β-actin, in PC-3, DU 145, LNCaP, and their cell transfectants

Gene name	GenBank Accession No.	Primer sense	Sequence (5′ → 3′)	Position	Melting *T*
AM	D14874	S	AAGAAGTGGAATAAGTGGGCT	250	55
		AS	TGTGAACTGGTAGATCTGGT	540	
CL	U17473	S	TGCTCTGTGAAGGCATTTAC	1159	63
		AS	CAGAATTGCTTGAACTCTC	1655	
RCP	AF073792	S	TGGGCAAGAGAACTTGAACA	111	60
		AS	TTCCACGATCAGCTGGATCT	306	
RAMP1	NM005855	S	GAGACGCTGTGGTGTGACTG	189	61
		AS	TCGGCTACTCTGGACTCCTG	614	
RAMP2	AJ001015	S	GGACGGTGAAGAACTATGAG	235	60
		AS	ATCATGGCCAGGAGTACATC	517	
RAMP3	AJ001016	S	TGGAAGTGGTGCAACCTGTC	189	63
		AS	CACGGTGCAGTTGGAGAAGA	347	
β-Actin	BC001301	S	TCCATCGTCCACCGCAATG	1169	55
		AS	TTGTGAACTTTGGGGGATGCTC	1398	

Protein concentration was spectrophotometrically measured (BCA protein assay kit; Pierce, Rockford, IL, USA) and 60 μg of total soluble proteins was separated by SDS–PAGE in 4–12% Bis–Tris polyacrylamide pre-cast gels (NuPage, Invitrogen). Electrophoresis was performed in Mes buffer in the presence of 5% of 2-β-mercaptoethanol for 45 min at 200 V. Proteins were then transferred to a 0.22 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 30 V for 1 h, and membranes were stained with Ponçeau solution. Blots were then incubated for 2 h at RT in blocking solution (Tropix, Bedford, MA, USA). AM detection was conducted as previously described [5] using a polyclonal antiserum raised in rabbit that recognizes the amidated $AM_{22–52}$ synthetic human sequence, at 1:1000 dilution. The amidated-$AM_{22–52}$ peptide was used for the absorption controls, at a concentration of 10 μM. Phosphorylated and total ERK1/2 antisera (Cell Signalling, Beverly, MA, USA) were used at a 1:1000 dilution. After incubation with the AP-conjugated anti-rabbit secondary antibody (1:20,000 dilution), a chemoluminescence kit (Tropix) was used for visualization.

Cell supernatants were collected after centrifugation (12,000*g*, at 4 °C, 30 min) and concentration of AM was measured by RIA analysis, as previously described [16]. A volume of 0.1 ml of medium, or standard AM (Phoenix Pharmaceuticals, Mountain View, CA) was incubated for 18 h at 4 °C with 0.1 ml of primary antibody. After incubation, 0.1 ml of ^{125}I-AM was added (10,000 cpm) and the mixture was incubated at 4 °C for 18 h. Bound tracer was separated by polyethylene glycol-facilitated precipitation with goat anti-rabbit serum and normal rabbit serum. The supernatant was aspirated and radioactivity of the pellets was determined with a DPC Gambyt CR γ counter.

Measurement of total cAMP. The Biotrak cAMP enzyme immunoassay kit (Amersham, UK) was used following the manufacturer's instructions. Cells (50,000–75,000) were seeded in 24-well plates. After overnight incubation, wells were washed three times with serum- and antibiotic-free medium, and blocked for 15 min in 1% BSA and 10^{-4} M isobutylmethylxanthine (both from Sigma). Cells were then supplemented with synthetic AM in doses ranging from 1 nM to 1 μM and incubated at 37 °C for 10 min. One micromolar of forskolin (Sigma, St. Louis, MO) was used as a control. Treatments were stopped with a lysis buffer provided by the kit and plates were finally read at 450 nm in a Multiskan Ascent ELISA plate reader (Labsystems).

Results

Transfected prostate cancer cells produce and secrete mature AM peptide

PC-3 and DU 145 prostate cancer cells were stably transfected with an expression vector containing the complete coding sequence for the hAM gene. Up to 20 clones of each cell line were isolated and tested for hAM mRNA and protein expression, and four PC-3 clones (hAM12, hAM22, hAM31, and hAM32), and DU 145 clones (hAM5, hAM6, hAM9, and hAM11) were selected for further studies. In the case of LNCaP, since it was not possible to obtain stable AM-expressing clones (as explained in Materials and methods), all the experiments were conducted with transient transfections. Fig. 1A shows AM mRNA expression of the parental or transfected cells. An intense band of 0.8 kb corresponding to the expression of exogenous AM mRNA was found in the transfected clones. Both parental and transfected cells showed a faint band of 1.4 kb, corresponding to the endogenous AM mRNA, which was also observed in the control H157 lung cell line [19].

AM protein production was studied by Western blot (Fig. 1B). Only cell lysates from the AM-transfected clones showed the 6 kDa band corresponding to the AM mature peptide, which was also observed in the bovine adrenal medulla (M.Ad.) used as a control [20]. Remarkably, PC-3 and DU 145 cell clones with high

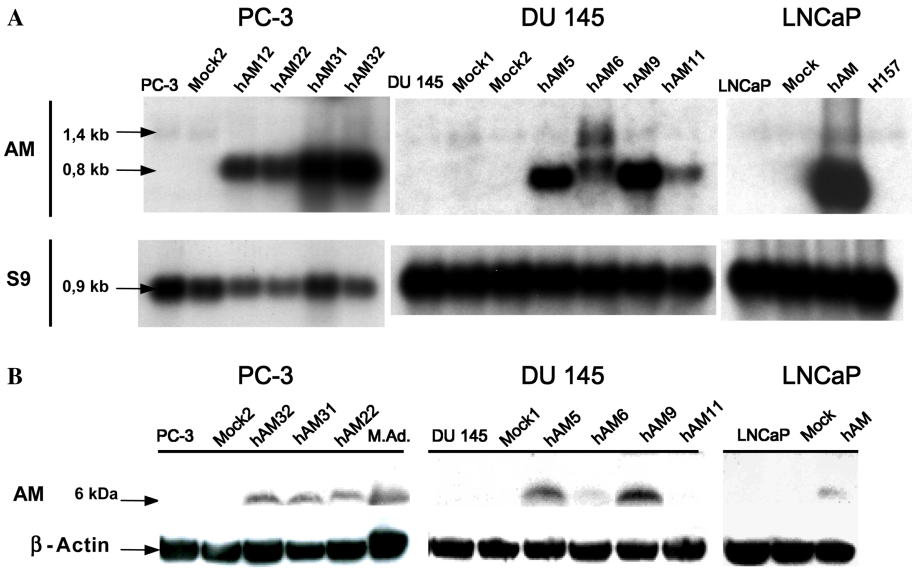

Fig. 1. Characterization of AM expression. (A) Northern blots in the upper panels show a faint band for the endogenous AM mRNA (1.4 kb) and a strong band corresponding to the ectopic hAM mRNA expression (0.8 kb), which is exclusively found in AM-transfected cells. RNA from H157 cells (a lung cancer cell line) was used as a positive control. In the lower panels, hybridization for S9 mRNA was used as an internal control. (B) Western blot for AM only shows the 6 kDa band corresponding to the mature AM peptide, in the cells transfected with AM. Bovine adrenal medulla (M.Ad.) was used as positive control for AM and β-actin as a loading control. Both experiments were performed three times with different RNA and protein extracts.

expression of AM mRNA (i.e., hAM32 and hAM9) also showed high expression of AM peptide (Fig. 1B). To determine whether AM was secreted into the cell culture media we performed RIA analysis (Table 2). Overexpression of AM caused a very significant increase in the levels of secreted AM peptide, in the three cell lines analyzed. This increase, however, was not homogeneous in all the cell clones. The PC-3 clone hAM32 showed the highest amount of secreted AM (162.6 ± 52.1 pg/μg of total protein), and LNCaP-transfected cells had also high levels of secreted AM (93.09 ± 9.08 pg/μg protein).

Table 2
RIA analysis for hAM of the culture media of PC-3, DU 145, LNCaP, and their cell transfectants

Cell line	Clone	hAM (pg/μg protein)
PC-3	PC-3	0.047 ± 0.005
	Mock2	0.030 ± 0.001
	hAM22	1.030 ± 0.032
	hAM31	2.529 ± 1.16
	hAM32	162.6 ± 52.1
DU 145	DU 145	0.547 ± 0.172
	Mock2	0.780 ± 0.273
	hAM5	1.166 ± 0.320
	hAM6	7.440 ± 0.093
	hAM9	7.446 ± 0.521
LNCaP	LNCaP	1.41 ± 0.80
	Mock	3.24 ± 0.14
	hAM	93.09 ± 9.08

AM inhibits PC-3 and LNCaP prostate cancer cell proliferation

To study the effect of AM overexpression in prostate cancer cells, MTT proliferation assays were conducted. As seen in Fig. 2, PC-3 (Fig. 2A) and LNCaP (Fig. 2B) cells transfected with AM significantly reduced ($p < 0.01$) their in vitro proliferation rates compared to controls. In contrast, cell proliferation in DU 145 clones overexpressing hAM was not significantly altered ($p = 0.652$) with respect to controls (Fig. 2C). Proliferation was also studied in PC-3 parental cells cultured in the presence of conditioned media from PC-3-transfected clones (Fig. 2D). Culture of PC-3 with supernatants from hAM22 and hAM31 for 4 days did not result in a significant cell growth inhibition compared to controls ($p = 0.05$). However, culture of PC-3 cells with supernatants from hAM32 (the clone with the highest expression and secretion of AM) for 4 days caused a dramatic cell growth inhibition ($p < 0.001$) (Fig. 2D).

The inhibitory effect of AM was further confirmed by addition of synthetic AM peptide to the parental prostate cancer cells (Fig. 3A). Addition of hAM at concentrations ranging from 10 to 1000 nM produced a very significant reduction ($p < 0.01$) of cell proliferation in a dose-dependent manner (up to 20% decrease for PC-3 and 50% for LNCaP). Lower concentrations (0.1 and 1 nM) did not cause such an effect. In the case of DU 145, only the highest concentration of AM (1000 nM) produced significant ($p < 0.05$) cell growth inhibition (Fig. 3A).

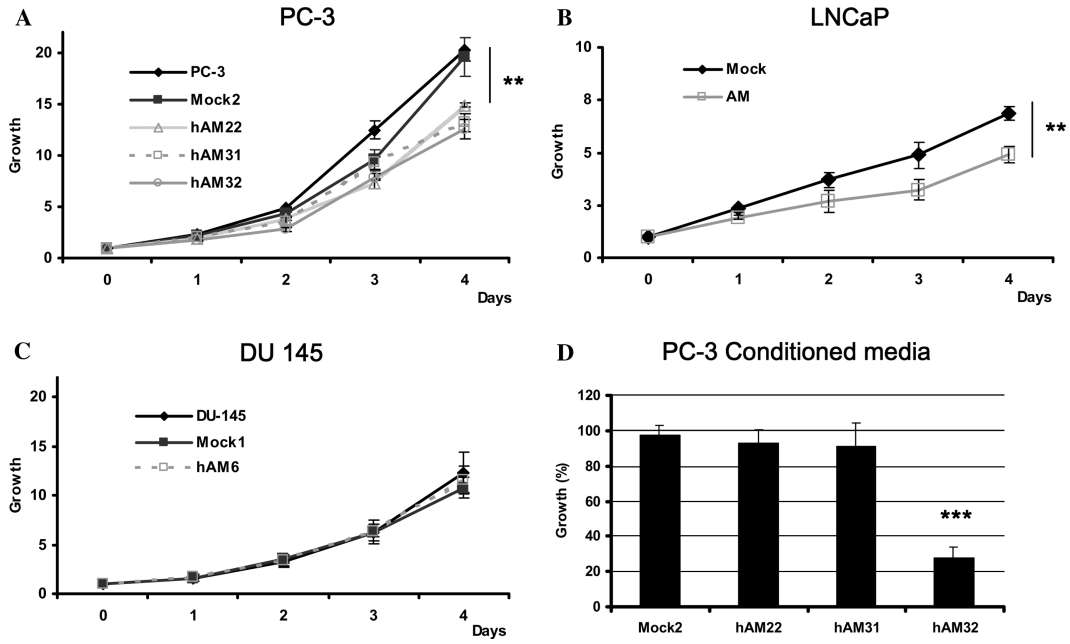

Fig. 2. Proliferation assays (MTTs) for the prostate cancer cell lines studies. (A) AM-overexpressing clones (hAM22, hAM31, and hAM32) show a significant decrease (**$p < 0.01$) in cell proliferation compared to controls (PC-3 and Mock2). (B) LNCaP cells overexpressing AM (transient transfection) also show significant reduction (**$p < 0.01$) in cell proliferation compared to mock-transfected cells. (C) Proliferation rates of DU 145 cells overexpressing AM (hAM6) are similar to those of controls (DU 145 and Mock1). (D) Parental PC-3 cells cultured for 4 days in the presence of supernatants from hAM31, and hAM22 had slightly lower proliferation rates (non-significant) than PC-3 cells cultured in the presence of supernatants from controls (parental PC-3 and Mock2). However, PC-3 cells cultured in the presence of supernatants from hAM32 (the clone with the highest secretion of AM) resulted in a dramatic cell growth inhibition (***$p < 0.001$). Data were analyzed by ANOVA for (A,C,D), and by Student's t test for (C). Each experiment was repeated at least three times.

We next evaluated whether overexpression of AM leads to cell growth retardation in anchorage independent conditions (Fig. 3B). These experiments were conducted for PC-3 and DU 145 cells. Quantification by image analysis revealed that PC-3 clones overexpressing AM (hAM22, hAM31, and hAM32) suffered a significant decrease ($p < 0.01$, 60% reduction, approximately, for hAM32) in the average area occupied by the colonies compared to controls (parental PC-3 and Mock2) (Fig. 3B). In contrast, and in keeping with our MTT results, the area occupied by the DU 145 cell clones hAM5, hAM6, and hAM9 (which overexpress AM) did not differ from controls (result not shown).

Cell cycle analysis was performed in parental and transfected PC-3 cells to determine whether overexpression of AM caused cell cycle arrest. In PC-3 and Mock2-transfected cells, the percentage of cells in the different phases of the cell cycle was similar (Fig. 3C). However, in the clones transfected with AM (hAM31 and hAM32), there was a significant increase in the percentage of cells in the G0/G1 phase, and a significant decrease in the S and G2/M phases ($p = 0.02$) (Fig. 3C).

AM inhibits PC-3 tumorigenesis in nude mice

To evaluate the effect of AM overexpression in vivo we inoculated clones Mock2, hAM31, and hAM32 into nude mice (Fig. 3D). Five weeks after subcutaneous injection, tumors reached an average volume of 800 mm^3 for controls. Tumor growth was significantly reduced (by 50%, approximately, $p = 0.013$) when clones hAM31 and hAM32 were injected, compared to controls. These results confirm our in vitro data and demonstrate that overexpression of AM leads to cell growth inhibition and decrease in tumorigenicity.

Postate cancer cells express AM receptors

Expression of the AM receptor complex CL/RCP/RAMP was analyzed by RT-PCR (Fig. 4). All of the prostate cell lines tested expressed the 497 bp band corresponding to CL, and the 196 bp band for RCP. RAMP-1 was only detected in LNCaP cells (in both controls and AM-transfected cells). The normal human bronchiolar epithelium (NHBE) derived cDNA was used as a control for RAMP-1. All of the prostate cell lines showed the band with the expected size for RAMP-2 (283 bp).

AM does not increase cAMP levels in PC-3, LNCaP, or DU 145 cells, but affects the MAPK pathway

AM signalling pathway in many cellular systems has been linked to intracellular elevation of cAMP. For this

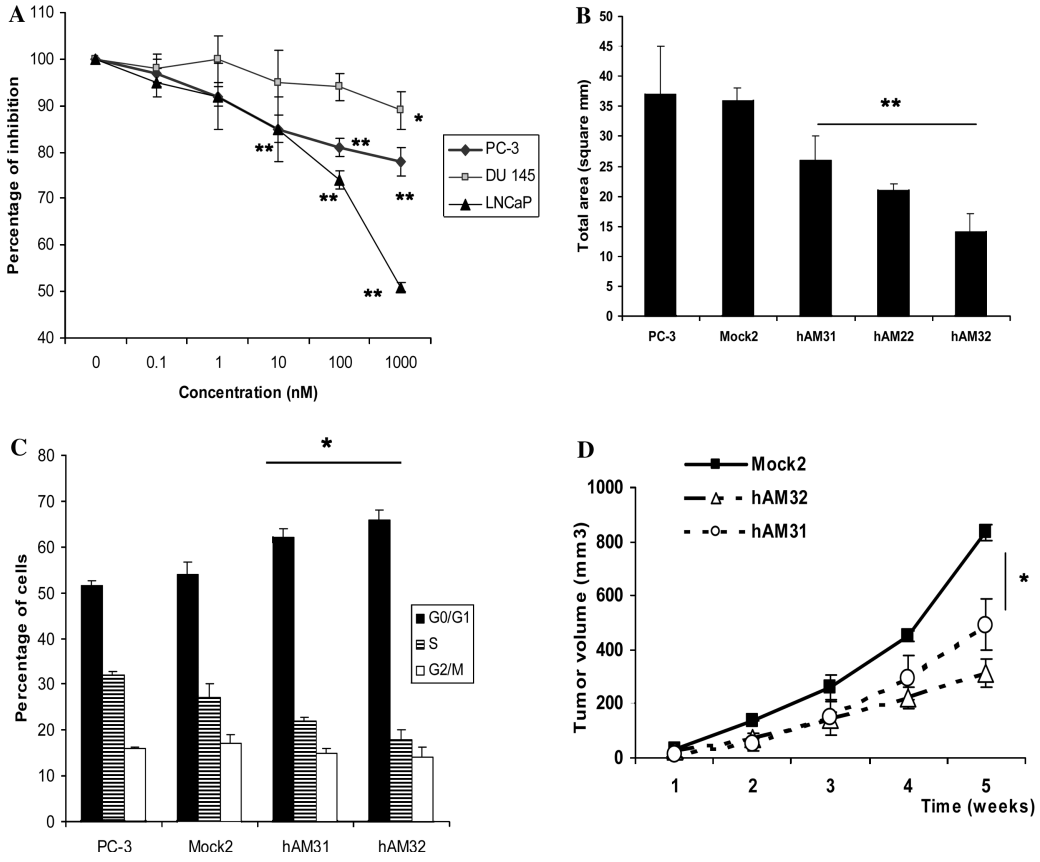

Fig. 3. (A) MTT proliferation assays in parental cell lines with different concentrations of hAM in the culture medium. Cell proliferation is significantly decreased (**$p < 0.01$) in LNCaP and PC-3 cells at hAM concentrations ranging from 10 to 1000 nM, with respect to controls. On the contrary, cell proliferation is only significantly reduced (*$p < 0.05$) in DU 145 with concentrations of 1000 nM of AM. (B) Cell growth in anchorage-independent conditions. PC-3 cell clones overexpressing AM have a significantly reduced (*$p < 0.01$) anchorage-independent growth (as revealed by the total area occupied by the colonies) than controls. (C) Cell cycle analysis shows that in the clones transfected with AM (hAM31 and hAM32) there is a significant increase in the percentage of cells in the G0/G1 phase, and a decrease in the percentage of cells in the S and G2/M phases (*$p = 0.02$). (D) Tumor volume in nude mice is significantly lower (*$p = 0.013$) when the injected cells overexpress AM (clones hAM31 and hAM32) instead of controls (Mock2). Data were analyzed by ANOVA. The in vitro experiments (A,B,C) were repeated at least three times. The in vivo experiment (D) was performed with 5 mice per group.

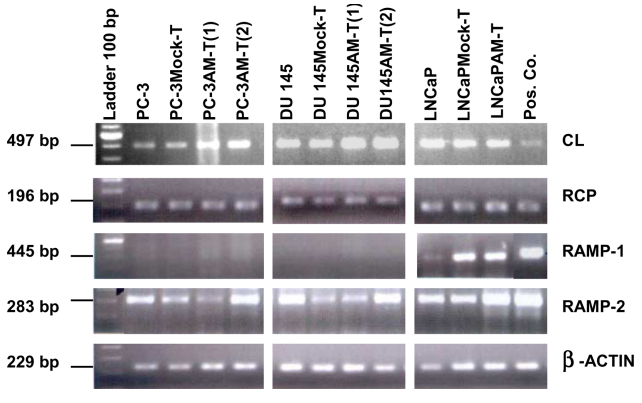

Fig. 4. Expression of AM receptors by RT-PCR. CL and RCP is expressed in PC-3, DU 145, and LNCaP cells and their transfected (T) clones. RAMP-1 is only expressed in LNCaP parental and transfected cells. RAMP-2 is detected in PC-3, DU 145, and LNCaP cells and their transfected clones. β-Actin served as an internal control. PCRs were performed at least three times.

reason, we first evaluated the effect of AM stimulation on cAMP levels in prostate cancer cells. Total cAMP levels were not altered in PC-3, DU 145, and LNCaP parental cells after AM stimulation, regardless of the AM dose or exposure time (results for PC-3 cells are shown in Figs. 5A and B).

Since AM stimulation has also been related to changes in the MAPK signalling cascade in some cell types, ERK1/2 levels were analyzed in PC-3 cells. Fig. 5C shows the increase in phosphorylated ERK1/2 levels, 5 min after AM stimulation (which was maintained up to 20 min after the treatment). However, long-term AM stimulation (longer than 40 min) resulted in a decrease in phosphorylated ERK1/2. Treatments for 1 h reduced phosphorylated ERK1/2 below control levels. In AM-overexpressing cells, decrease in phosphorylated ERK1/2 levels was dramatic compared to controls (Fig. 5D). These results show that long-term

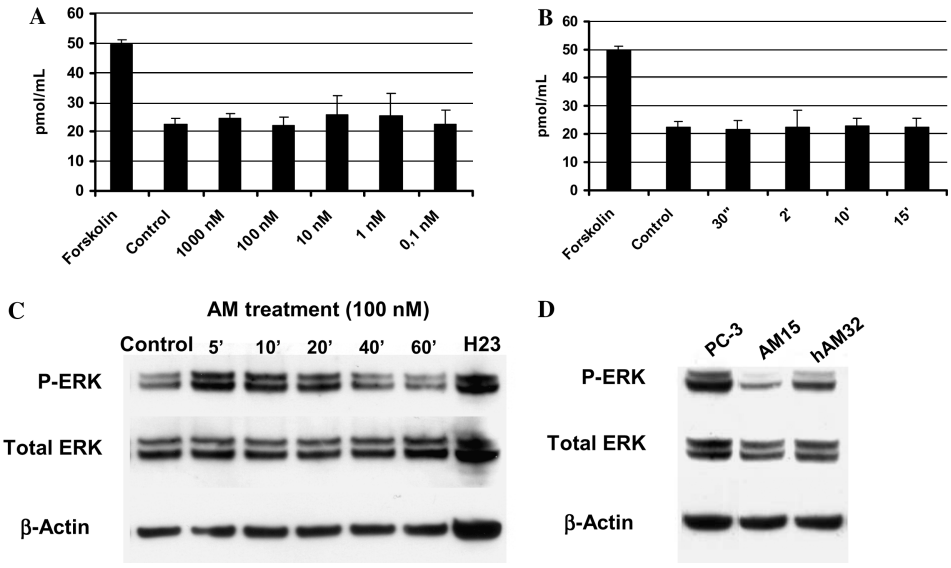

Fig. 5. Intracellular signalling of AM action. (A) PC-3 cells treated with different concentrations of hAM (10 min after incubation) show no change in cAMP levels. (B) PC-3 cells in a time-course experiment after hAM stimulation (1 nM) show no change in cAMP levels either. (C) Treatment of PC-3 cells for 5–20 min causes an increase in phosphorylated ERK1/2, whereas longer treatments (40–60 min) produce a decrease of such levels compared to untreated cells. Total ERK1/2 levels are not affected. (D) PC-3 clones overexpressing AM (hAM32, transfected with hAM; or AM15 transfected with rAM) show reduced phosphorylated ERK1/2 levels, as compared to controls (parental PC-3 cells). Total ERK remains similar to controls in the transfected cells. β-Actin served as loading control. Data for (A,B) were analyzed by ANOVA. All experiments were repeated three times.

AM stimulation reduces the activation of phosphorylated ERK1/2.

Discussion

Adrenomedullin is expressed in a variety of tissues and cancer cells, including prostate [3,6,7]. However, its role in prostate carcinogenesis is still poorly understood. Previous studies have shown that AM can either stimulate or inhibit cell growth, depending upon the cell type [6,7,15,21]. We have analyzed the effect of AM stimulation and AM overexpression in PC-3, LNCaP, and DU 145 cell lines. For PC-3 and DU 145 cells, we were able to obtain stable cell clones overexpressing AM, whereas for LNCaP, AM expression was shut down approximately 1 week after transfections, probably due to silencing of the expression vector. Therefore, transient transfections were used for experiments with LNCaP. This approach has allowed us to have prostate cells that produce high and constant levels of AM mRNA and protein, which are secreted into the culture medium.

MTT experiments in cells overexpressing AM have shown a significant cell growth inhibition in PC-3 and LNCaP cells. These results were corroborated by the addition of the hAM peptide to the culture medium. In contrast, proliferation is not decreased in DU 145 clones overexpressing AM, and only addition of high concentrations of hAM in the culture medium reduced proliferation of parental DU 145 cells. This result shows that DU 145 is refractory to the AM inhibitory effect observed in PC-3 and LNCaP cells. We have analyzed with further experiments the inhibitory effect of AM in PC-3 cells overexpressing hAM. Cell cycle analysis, anchorage independent growth assays, and culture of parental PC-3 cells with supernatants from AM-transfected clones confirmed cell growth inhibition and cell cycle arrest at the G0/G1 phase in vitro. Moreover, we have shown in in vivo assays on nude mice that AM reduces significantly tumor growth. Our previous results overexpressing rat AM in PC-3 cells are in keeping with our present results [16].

Rocchi et al. [4], studied proliferation of PC-3, LNCaP, and DU 145 cells after AM stimulation. These authors describe no effect in PC-3 and LNCaP cells, and a slight increase in cell growth in DU 145 cells after treatment with 2×10^{-7} M AM for 8 days. Although these results are in disagreement with ours, the experimental conditions were different. Those authors used serum-free TIS (transferrin, insulin, selenite) medium. In our experience, these conditions make prostate cells grow under stress and very slowly. This is probably the reason why they needed the proliferation assays to last for so long (8 days). Instead, we have used 1% FCS, which is the lowest amount of serum needed for the prostate cells to grow without stressful conditions (unpublished observations). Interestingly, Martínez et al. [21] have shown that the effect of AM on mammary cancer cell growth is critically dependent upon

stress culture conditions. Thus, AM-overexpessing T47D cells cultured in the presence of serum grew at lower rates than control cells, whereas under stress conditions (without serum), proliferation rates were increased [21]. AM also demonstrated to have an anti-apoptotic role in T47D cells cultured in serum deprived medium or TNF-α treatment. This result correlated with a decreased expression of pro-apoptotic molecules, such as Bax, Bid, and caspase-8, in T47D cell clones overexpressing AM [21]. Similarly, treatment of VSMC cells with AM results in cell growth inhibition or proliferation, depending on the presence or absence of serum [15]. Thus, AM has a mitogenic effect in VSMC cells grown under stress conditions (serum deprivation), whereas it exerts an anti-mitogenic effect when cells are grown with serum [15]. In our unpublished experiments using prostate cancer cells in culture medium containing TIS instead of serum, we did not find a cell growth inhibitory effect after AM stimulation (in agreement with Rocchi et al. [4]). All these considerations point out that stressful culture conditions can alter cell response to AM. This issue, which was suggested by Martínez et al. [21], should be taken into consideration for further proliferation assays and to re-evaluate previous controversial cell growth results after AM stimulation.

PC-3, LNCaP, DU 145, and their AM-overexpressing clones express the receptor complex CL/RCP/RAMP-2. In addition, LNCaP cells express RAMP-1. This result suggests that prostate cancer cells can produce and secrete AM into the culture media and be responsive to the peptide through an autocrine/paracrine mechanism. The presence of both AM and AM-receptors has been found in many other cell lines, such as glioblastoma [22], *zona glomerulosa* cells [23], hepatic stellate cells [24], and VSMC [15]. Therefore, an AM-mediated autocrine or paracrine activation loop is probably a very common way of action [25].

The elevation of cAMP has been described as the most common intracellular signalling process that occurs after activation of AM receptors [6]. However, other alternative intracellular pathways have been described. These include increase in Ca^{+2} levels, elevation of NO and cGMP, or modification of MAPK levels, especially ERK [6,14]. Our experiments show that the elevation of cAMP is not responsible for the effect observed in prostate cells due to AM stimulation. Treatment of cells with different doses of AM or exposure times showed no change in cAMP levels. Since MAPK pathways are related to cell proliferation and AM signalling led to significant cell growth inhibition in our study, we decided to analyze ERK1/2 levels. We have found that, indeed, AM treatment alters phosphorylated ERK1/2 levels. However, the response is different depending on the time after stimulation. Short-term stimulation (within 20 min) produces an increase in phospho-ERK, whereas long-term treatment leads to decreased levels of phosphorylated ERK1/2. Moreover, cell clones overexpressing AM, which have a sustained high expression of the peptide, show very low levels of phosphorylated ERK1/2 compared to control cells. Experiments by other groups in different cell lines have shown variations in ERK levels after AM stimulation [14,15,26,27]. It is a common finding that when AM stimulates proliferation, levels of activated ERK increase, and when AM inhibits proliferation, levels of activated ERK decrease [14,15,26,27], which is coincident with our results.

The mechanism by which AM alters ERK levels is still unknown. Both pathways involving G-protein coupled receptors and tyrosine kinase receptors could be responsible. In this regard, the existence of a still uncharacterized tyrosine kinase receptor for AM has been suggested [14]. In VSMC cells, growth effects elicited by AM are independent of cAMP levels and lead to the activation of ERK [15]. This scenario could be similar to what happens in prostate cancer cells, since cAMP levels are not modified but active ERK levels are decreased, under the effect of AM. Further studies are needed to elucidate how AM exerts its effects in different cell types, including prostate cancer cells.

In conclusion, we demonstrate that hAM decreases cell proliferation in PC-3 and LNCaP cells both in vitro and in vivo. This effect is likely due to an autocrine mechanism which is independent of cAMP increase. Signalling pathways involve the alteration of MAPKs by decreasing phosphorylated levels of ERK1/2 as a consequence of AM stimulation.

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