Angiotensin II stimulation of Na^+/K^+ATPase activity and cell growth by calcium-independent pathway in MCF-7 breast cancer cells

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Abstract

Here we demonstrated, by RT-PCR analysis, the expression of both angiotensin II (Ang II) receptor subtypes, AT1 and AT2, in a breast cancer epithelial cell line, MCF-7. Ang II was not able to affect the intracellular Ca^{2+} concentration in Fura-2 loaded cells suggesting that AT1-mediated phospholipid hydrolysis is not involved in its intracellular transduction pathway. Ang II modulated the activity of the Na^+/K^+ATPase in a dose- and time-dependent manner and was mitogenic, with a dose-dependent (1–1000 nM) proliferative effect and a maximal response at 100 nM. Both Na^+/K^+ATPase activation and stimulation of proliferation were mediated by binding of Ang II to AT1, as the effects were completely blocked by DuP 753, a specific AT1 antagonist. CGP 42112, an AT2 antagonist, did not affect Ang II actions.

The main conclusion of this study is that Ang II exerts its effects on cell proliferation and Na^+/K^+ATPase in breast cancer epithelial cells, MCF-7, via AT1 activation independently of the Ca^{2+} signalling mechanism.

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Introduction

Angiotensin II (Ang II) may have a role in the maintenance of epithelial structure and function (Vinson *et al.* 1995). Such function may include cilia beat activity (Saridogan *et al.* 1996), mitosis and tissue differentiation (Lees *et al.* 1993, Goldfarb 1994, McEwan *et al.* 1996), and the regulation of water and electrolyte transport (Wong *et al.* 1990, Norris *et al.* 1991, Quan & Baum 1996). Ang II appeared also to be a potentially important candidate as a growth factor or growth promoter, since it activates phosphatidylinositol turnover causing a rise in cytosolic free Ca^{2+} in a variety of cell types, a process linked to mitogenesis (Lundergan *et al.* 1999, Bataller *et al.* 2000, Hou *et al.* 2000, Kuwahara *et al.* 2000, Shen *et al.* 2001). In the proliferation and differentiation of various cells, including keratinocytes (Shen *et al.* 2001), lymphocytes (Marakhova *et al.* 2000), smooth muscle cells (Henningsen *et al.* 1984), astrocytes (Matsuda *et al.* 1996), 3T3 cells (Russo & Sweadner 1993) and retinal pigment epithelium (Burke *et al.* 1991), the activity of the Na^+/K^+ATPase appeared greatly involved. Both in *in vivo* and in cultured cells the Na^+/K^+ATPase activity is modulated by Ang II (Muscella *et al.* 1997, 2000, Bharatula *et al.* 1998, Hussain *et al.* 1998, Buhagiar *et al.* 1999, Yingst *et al.* 2000, Zhang & Mayeux 2001).

Two main subtypes of Ang II receptors, designated AT1 and AT2, have been identified on the basis of their relative binding affinities for peptide and non-peptide antagonists (Chiu *et al.* 1989) and by cloning (Murphy *et al.* 1991, Kambayashi *et al.* 1993, Mukoyama *et al.* 1993). The majority of the well-known actions of Ang II occur via the AT1 subtype, while AT2 mediates the anti-growth and apoptotic actions of Ang II (Gallinat *et al.* 2000). AT1 receptor is present in a wide variety of human and animal tissues, including normal and cancerous breast epithelial cells (Inwang *et al.* 1997, Tahmasebi *et al.* 1998). The only information about the functions of Ang II in human breast tissue has been obtained recently in a continuous human breast cancer cell line, MCF-7, where Ang II affected the expression of integrin (Berry *et al.* 2000). In these cells, no information regarding the expression of Ang II receptor subtypes is so far available.

The aim of this study was to determine the Ang II subtype receptors expressed by the breast cancer epithelial cell line, MCF-7, and the effects of Ang II on the activity of the Na^+/K^+ATPase and on cellular proliferation.

Materials and Methods

Reagents

Glutamine, gentamicin, human Asp1-Ile5-Ang II, ouabain, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Sigma Chemical Co. (Milan, Italy). 2-n-Butyl-4-chloro-5-hydroxymethyl-1-[(21-(1H-tetrazol-5-yl)biphenyl-4-yl)

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methyl]imidazole, potassium salt (DuP 753 or losartan) was from Du Pont Merck Pharmaceutica (Wilmington, DE, USA). CGP 42112 and saralasin were obtained from Fluka (Sigma-Aldrich, Milan, Italy). Fura-2 acetoxymethyl ester (Fura-2 AM) and thapsigargin were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Cell culture

Cells from the MCF-7 cell-line were derived originally from human breast cancer pleural effusion (Soule *et al.* 1973). Besides the MCF-7 cells grown in our laboratory, another line of MCF-7 cells was purchased from the Interlab Cell Line Collection, CBA, Genoa, Italy, and was used for comparison of results. Cells were propagated in 75 cm^2 flasks in DMEM containing 10% FBS, 2 mM glutamine and penicillin/streptomycin (100 U/ 100 μg/ml). Cells were grown at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 and were used from passages 8–12.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from MCF-7 cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. For RT-PCR 1 μg total RNA was reverse transcribed in (mM): Tris/HCl (pH 8·3) 50, KCl 75, $MgCl_2$ 3, dithiothreitol (DTT) 10, dNTP 0·5, oligo(dT)15 primer 0·5 μg and 15 u Moloney murine leukemia virus (M-MLV) RT (Promega).

PCR was performed in (mM): Tris/HCl (pH 8·8) 50, KCl 50, $MgCl_2$ 1·5, DTT 2, 0·1% Triton X-100, dNTP 0·2, 0·7 μM specific primers and 2·5 u Taq polymerase (Promega). The amplification profile consisted of denaturation at 95 °C for 30 s, annealing at 54 °C for 60 s and extension at 72 °C for 90 s for 30 cycles. AT1 sense and anti-sense primers were as follows: 5′-GGAAAC AGCTTGGTGGTG-3′ and 5′-GCACAATCGCCATA ATTATCC-3′ corresponding to sense and anti-sense, respectively, of bases 133–150 and 719–739 in the human AT1 sequence. AT2 primers were 5′-ATAATGATTTG GGGATTCAG-3′ and 5′-TTTTAAGCCACCCAGAT ATT-3′ corresponding to bases 1613–1633 and anti-sense 2314–2334, respectively, of AT2 sequence. AT1 and AT2 primers were purchased from Celbio (Milan, Italy). Reaction products were resolved by electrophoresis through 1% agarose gels and stained with ethidium bromide. Contamination by genomic DNA in sample RNA was excluded by amplifying the sample RNA directly by PCR without reverse transcriptase.

Measurement of intracellular free calcium ($[Ca^{2+}]_i$)

Growth medium was changed to fresh FBS-free culture medium for 12–18 h and then the cells were harvested by gentle trypsinization. Cells were loaded with 5 μM Fura 2-AM for 45 min at 37 °C in HEPES-buffered Krebs-Ringer solution (KRH) (in mM: 140 NaCl, 5·0 KCl, 1·0 $MgCl_2$, 2·0 $CaCl_2$, 6·0 glucose and 10 HEPES, pH 7·4) containing 0·2% Pluronic F-127 and 0·1% BSA. Loaded cells were washed and resuspended in KRH to a density of 7×10^6 cells/ml and incubated for 20 min at room temperature to ensure complete deesterification of the dye.

In a cuvette with a magnetic stirrer, 10 μl of loaded cells were incubated in KRH solution containing 0·1% BSA for fluorescence measurements using the spectrofluorometer JASCO FP 750 (Jasco Corporation, Tokyo, Japan). Excitation monochromators were set at 340 and 380 nm, with a chopper interval of 0·5 s, and the emission monochromator was set at 510 nm. $[Ca^{2+}]_i$ was calculated according to the equation of Grynkiewicz *et al.* (1985), using the software Spectra Manager provided by JASCO. R_{max} and R_{min} values were determined by inclusion of 20 μl Triton X-100 (0·01% final concentration) and 20 μl EGTA (5 mM final concentration) respectively.

Measurement of Na^+/K^+ATPase activity in MCF-7 cells

The enzyme activity was measured by using a coupled enzyme assay method (Nørby 1988) with some modifications. Briefly, the cells were grown on 96-well plates for one day in DMEM plus 10% FBS and then the media were replaced with 100 μl serum-free media for 24 h to induce quiescence. After incubation with the various agents, cells were immediately permeabilized by freezing for 10 min at −20 °C. The reaction was started by the addition of 200 μl reaction mixture (in mM: 20 KCl, 8 $MgCl_2$, 100 NaCl, 0·5 EGTA, 40 Tris, 10 phosho-enol pyruvate, 0·25 NADH, 1·0 fructose-1,6-diphosphate, 5 ATP, 1·1 U/ml lactate dehydrogenase, 0·9 U/ml pyruvate kinase, with or without 1 mM ouabain), directly into 96-well plates. The plates were placed in a spectrophotometer (Bio-Rad Laboratories, Milan, Italy) and the absorbance readings at 340 nm at 37 °C were taken at 1-min intervals. The slope of the disappearance curve of NADH represents the ATP hydrolysis rate. To obtain the Na^+/K^+ATPase activity, the slope of the activity in the presence of ouabain (ouabain-resistant ATP activity) was subtracted from the slope obtained in the absence of ouabain (total ATPase activity). The activity was expressed as μmol ADP/mg protein/h.

Cell proliferation assay

The conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) by MCF-7 cells was used as an indicator of cell number as described by Mosmann (1983). This method measures the reduction of MTT by active mitochondria which results in a colorimetric change, optical density measured at 570 mm.

MCF-7 cells (1×10^4 cells/well) were grown in 96-well plates for one day in DMEM plus 10% FBS. Preliminary studies had demonstrated that this cell number did not result in confluent cultures at the end of the observation period of 60 h and produced the optimal absorbance value for the MTT assay. The media were replaced with 100 µl serum-free media for 24 h to induce quiescence. Increasing Ang II concentrations (1–1000 nM) were added daily to each well for different lengths of time (from 12 to 60 h). To test the specificity of Ang II binding to receptors, in other wells 1 µM DuP 753 and 1 µM CGP 42112 were added 10 min before Ang II treatment. Control cells were treated daily with DuP 753 and CGP 42112 alone for the same time. Next, MTT (250 µg/ml) was added to the cells for a 4-h incubation and cells were lysed in acidified isopropanol. The plates were subsequently read on a Bio-Rad microplate reader at 570 nm. The results were expressed as per cent viability of each well calculated from the following:

$$\text{per cent proliferation} = \frac{\text{absorbance of test} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100$$

The data presented are the means ± standard deviation (S.D.) from eight replicate wells per microtitre plate and were replicated four times.

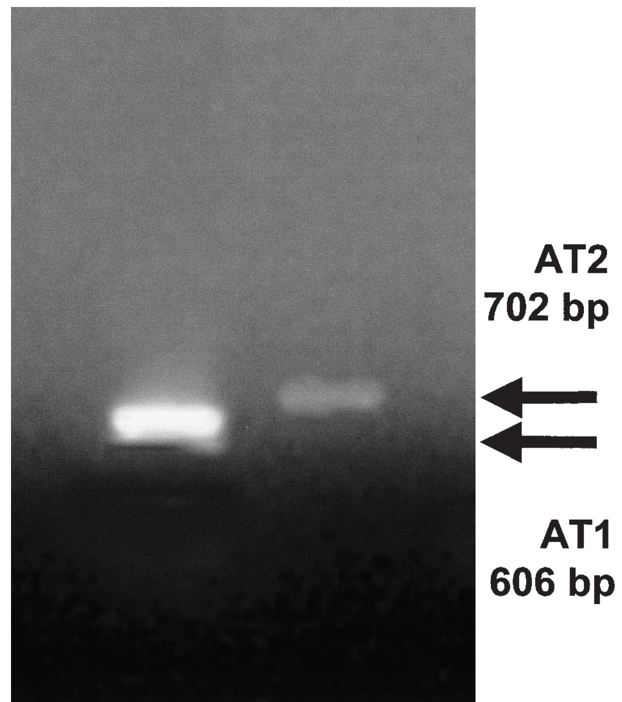

Figure 1 Agarose gel electrophoresis of RT-PCR products revealed the specific transcripts for AT1 and AT2 of Ang II receptors.

Total protein content

MCF-7 cells were plated in 96-well plates (1×10^4 cells/well), rested in serum-free medium for 24 h, and stimulated with a daily dose of Ang II. To test the effects of DuP 753, cells were pre-treated for 10 min and subsequently cultured with daily doses of Ang II and 1 µM DuP 753. Another group was treated with DuP 753 alone. Cells were still subconfluent at the end of the stimulation period of 60 h (~95% confluent). Total protein was determined by a modification of the method of Lowry *et al.* (1951).

Cell count

MCF-7 cells were seeded at 5×10^4 cells/well on 24-well plates, and cells were counted in a Thomas cell-chamber 12, 24, 48 and 60 h after treatment, as described above.

Statistical analysis

Experimental points represent the means ± standard deviation (S.D.) of 3–5 replicates. Statistical analysis was carried out using the Student's *t*-test for unpaired samples and the ANOVA. When indicated, post-hoc tests (Bonferroni/Dunn) were also performed. A P value less than 0·05 was considered to be significant.

Results

In order to make sure that the results obtained were not due to clonal variation of MCF-7 cells developed over time in our laboratory, all the experiments shown here were also performed on an MCF-7 cell line purchased from another institution; the results obtained were basically similar in both lines.

AT1 and AT2 gene expression in MCF-7 cells

The exponential phase of amplification for AT1 and AT2 mRNA was detected between 25 and 40 cycles, and further PCR experiments were performed using 30 cycles. As a control, total RNA was isolated from rat adrenal tissue which contains AT1 and AT2. PCR analysis of isolated DNA using the primers for the Ang II receptors yielded products of the expected sizes: 606 bp for AT1 and 702 bp for AT2 (Fig. 1). No bands were seen in the absence of RT, confirming the absence of genomic DNA contamination (data not shown).

Effects of Ang II and ATP on $[Ca^{2+}]_i$

The basal level of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was 128 ± 25 nM (mean ± S.D.; n=30). The effects of Ang II on the $[Ca^{2+}]_i$ were not significant in any

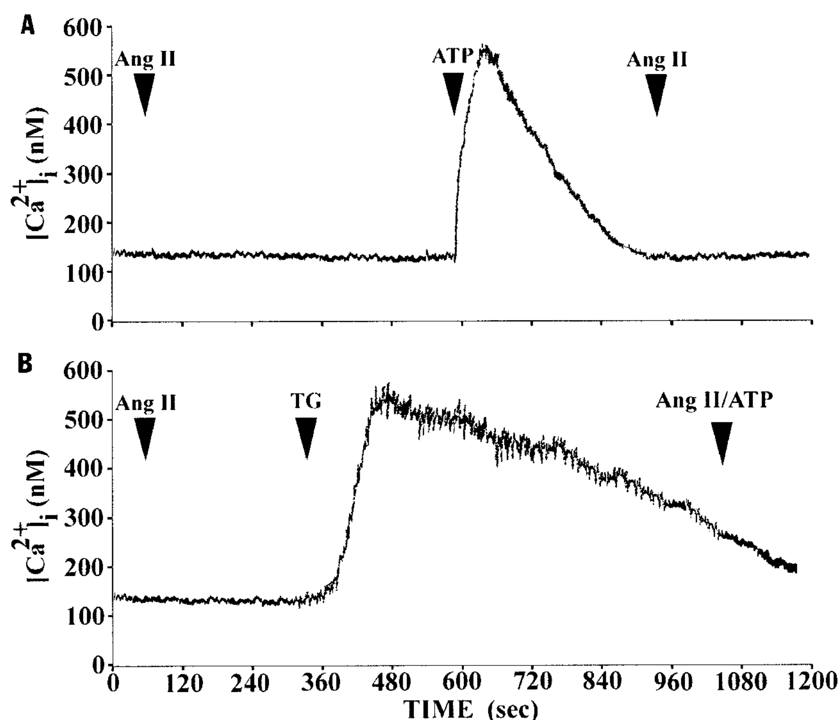

Figure 2 (A) Representative trace showing change in $[Ca^{2+}]_i$ in response to ATP (10 μM), but not to Ang II given before and after ATP. (B) After Ang II administration 1 μM thapsigargin (TG) was added at the indicated time to mobilize the endoplasmic reticulum Ca^{2+} stores; subsequent addition of Ang II and/or ATP did not have any effect. Results shown are representative of at least four experiments.

of the experimental conditions used (in the presence and in the absence of extracellular Ca^{2+}, in serum-starved cells or not, in cells incubated with an agonist, ATP, able to increase the $[Ca^{2+}]_i$). Since it is known that AT2 stimulation can inhibit the $[Ca^{2+}]_i$ transients evoked by AT1 (De Gasparo *et al.* 2000), MCF-7 cells were incubated for various times (1–30 min) with differing amounts of CGP 42112 (10 to 10 000 nM) and then Ang II was added (1 to 1000 nM); in all these experimental conditions, no variations in the $[Ca^{2+}]_i$ was obtained (data not shown).

As a positive control for the fluorimetric studies, extracellular ATP was used which is known to evoke $[Ca^{2+}]_i$ transients in MCF-7 cells following stimulation of phospholipase C through P2Y receptors (Flezar & Heisler 1993, Dixon *et al.* 1997, Wagstaff *et al.* 2000). Indeed, ATP (1–200 μM), produced a dose-dependent increase in $[Ca^{2+}]_i$ over basal (Fig. 2A). A threshold increase in $[Ca^{2+}]_i$ was observed at 1 μM with a maximum at 100 μM ATP (up to 512 ± 19 nM). No further increase was observed at 200 μM ATP. Various concentrations of Ang II added to MCF-7 cells prior to ATP exposure did not alter the ATP-evoked $[Ca^{2+}]_i$ transients (Fig. 2A). The microsomal Ca^{2+} ATPase inhibitor, thapsigargin (TG), was used to empty internal stores: in the presence of external Ca^{2+}, 0·1 μM TG caused a sustained elevation of $[Ca^{2+}]_i$ and subsequent addition of Ang II and/or ATP did not have any effect (Fig. 2B).

Effects of Ang II on Na^+/K^+ATPase activity

The method used to measure Na^+/K^+ATPase activity in MCF-7 cells was sensitive and reproducible. In standard culture conditions, the coefficient of variation of a single assay was 3·2% (n=8) and Na^+/K^+ATPase activity increased linearly with increasing numbers of cells. The baseline activity of Na^+/K^+ATPase in MCF-7 cells was 29·8 ± 0·9 μmol ADP/h/mg (mean ± S.D. of five independent experiments).

Ang II produced a significant increase in Na^+/K^+ATPase activity in a dose- and time-dependent manner (Fig. 3A and B). The maximal response, in subconfluent conditions, was obtained after 20-min incubation with 100 nM Ang II, and corresponded to a 2·6-fold increment in Na^+/K^+ATPase activity. Therefore, the protocol of 100 nM concentration for 20-min incubation was chosen in subsequent experiments. The ouabain-insensitive

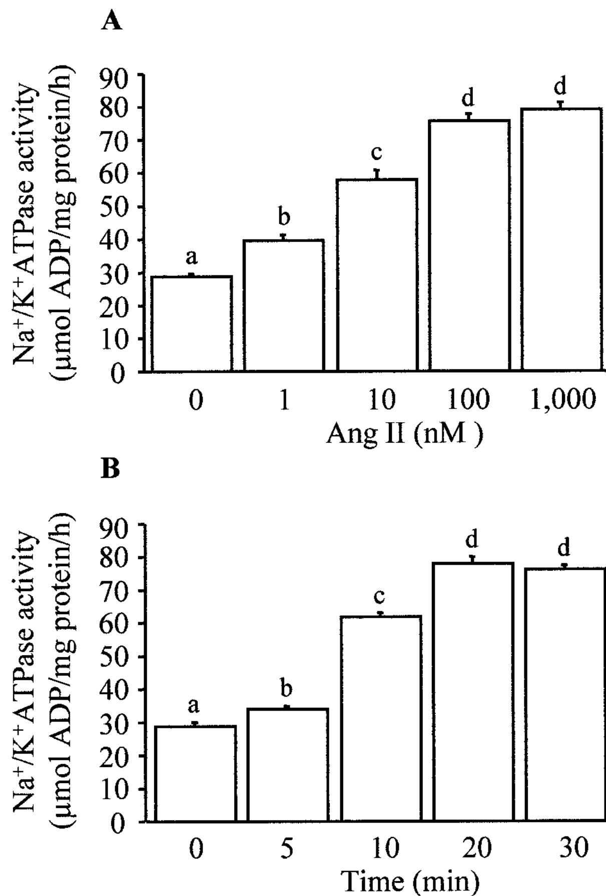

Figure 3 The effect of Ang II on Na^+/K^+ATPase activity is dose- and time-dependent. (A) MCF-7 cells were incubated without or with increasing concentrations of Asp1-Ile5-Ang II for 20 min at 37 °C. (B) MCF-7 cells were incubated without or with 100 nM Asp1-Ile5-Ang II for different time periods (5–30 min). The data are means ± S.D. of six different experiments run in triplicate and are presented as µmol ADP/h/mg protein. ANOVA for (A) and (B): $P<0{\cdot}0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

ATPase activity was not affected by any of Ang II concentrations used (6·5 ± 0·3 µmol ADP/h/mg protein). Saralasin (1 µM), a competitive Ang II antagonist, blocked Ang II increase of Na^+/K^+ATPase activity (data not shown).

To investigate the presence of Ang II receptors by which Ang II produces activation of the Na^+/K^+ATPase activity, the cells were incubated with the receptor antagonists, losartan and CGP 42112 (1 µM), prior to treatment with Ang II. CGP 42112, the AT2 antagonist, did not affect Ang II action, while the specific AT1 competitive antagonist, DuP 753, abolished the Ang II effect (Fig. 4). Losartan or CGP 42112 alone did not affect the basal Na^+/K^+ATPase activity.

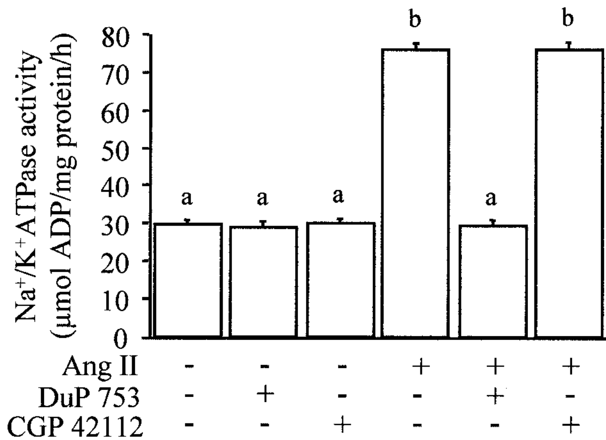

Figure 4 The AT1 receptor antagonist DuP 753 prevents Ang II induced Na^+/K^+ATPase activity but does not affect basal activity; CGP 42112, an AT2 receptor antagonist, did not affect Ang II actions. MCF-7 cells were treated with DuP 753 or CGP 42112 and then with either control medium or medium containing 100 nM Ang II for 20 min. Results are means ± S.D. of three different experiments run in triplicate and are presented as µmol ADP/h/mg protein. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests. $P<0{\cdot}0001$.

Effect of Ang II on cellular proliferation and total cellular protein

The relationship of the absorbance of MTT to cell number was verified in experiments in which 1200 to 80 000 cells were added to different wells of a microtitre plate. There was a significant ($P<0{\cdot}05$; $r=0{\cdot}98$) relationship between cell number and MTT absorbance.

MCF-7 cells were treated with Ang II (1 to 1000 nM) for 12, 24, 36, 48 and 60 h. Ang II had a replicating effect on MCF-7 cells in a dose- and time-dependent manner, with a maximal response obtained after 24-h administration of 100 nM Ang II (Fig. 5A). The proliferative effect of Ang II (100 nM) was abolished by pre-treatment with DuP 753 but not by CGP 42112 (Fig. 5B). In comparative experiments, Ang II also induced a dose- and time-dependent increase in total MCF-7 cell protein as measured by the method of Lowry *et al.* (1951) (Fig. 6A). Ang II-stimulated protein synthesis was abolished by DuP 753, and DuP 753 treatment alone did not affect total protein content of the MCF-7 cells. CGP 42112 did not affect Ang II-induced proliferation (data not shown). Comparable results were obtained when cell number was directly determined by cell counting. The actual number of untreated and Ang II-treated cells were counted in a Thomas cell-chamber: 100 nM Ang II significantly increased MCF-7 cell number after 12, 24, 48 and 60 h exposure (Fig. 6B).

Ang II (100 nM) did not increase the protein/cell number ratio (control cells = 168·9 ± 5·01 pg/cell; cells treated with 100 nM Ang II = 173 ± 5·72 pg/cell),

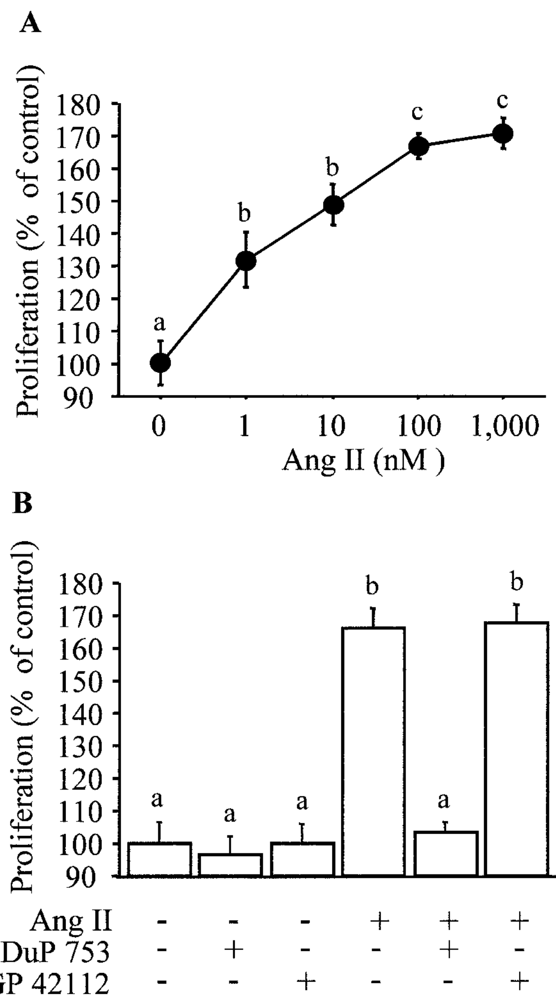

Figure 5 (A) The effect of Ang II on cell proliferation is dose-dependent. Serum-free media-rested MCF-7 cells were incubated without or with increasing concentrations of Ang II for 24 h. ANOVA: $P<0{\cdot}0001$. (B) The AT1 receptor antagonist, DuP 753, abolished the Ang II-induced increment of cell proliferation. CGP 42112, an AT2 receptor antagonist, did not affect Ang II action. MCF-7 cells were treated with DuP 753 or CGP 42112 and then with either control medium or medium containing 100 nM Ang II for 24 h. The data are means ± S.D. of four different experiments run in eight replicates and are presented as per cent proliferation of control. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests. $P<0{\cdot}0001$.

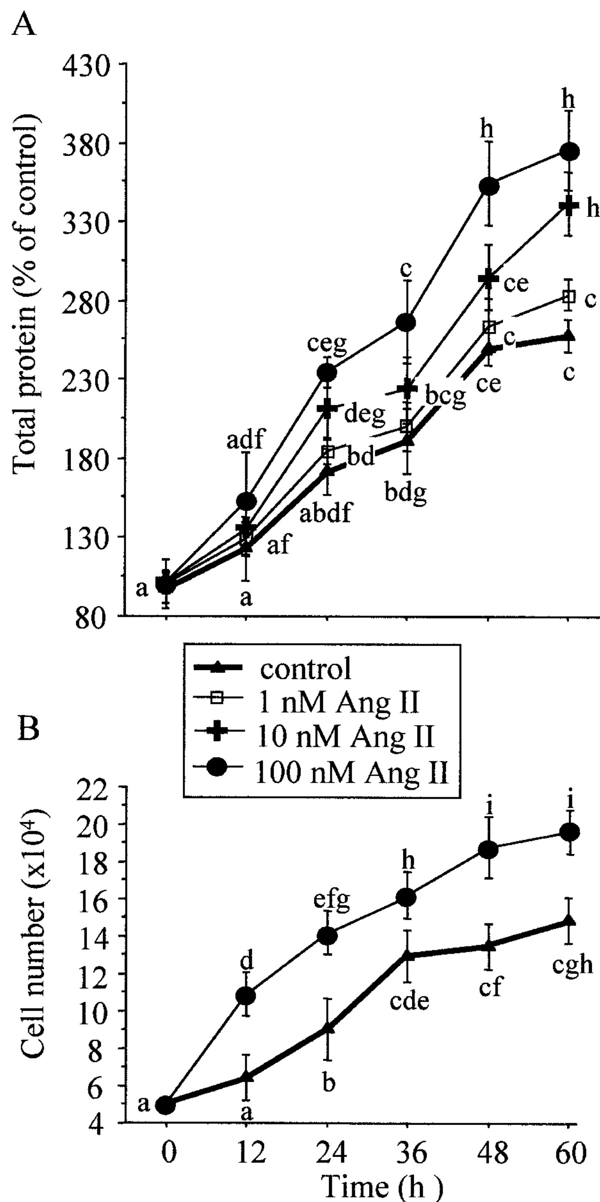

Figure 6 (A) The Ang II-induced increase in total cellular protein is dose- and time-dependent. Quiescent MCF-7 cells were incubated without or with increasing concentrations of Ang II for the indicated intervals. (B) Ang II increases the cell number. Serum-free media-rested MCF-7 cells were incubated without or with increasing concentrations of Ang II for the indicated intervals. The data are means ± S.D. of four different experiments run in eight replicates and are presented as per cent proliferation of control. ANOVA: $P<0{\cdot}0001$. Values with shared letters are not significantly different according to Bonferroni/Dunn post-hoc tests.

indicating that Ang II did not cause hypertrophy as in other cell types.

Discussion

Previous binding studies showed that normal and cancerous human breast express both the AT1 and the AT2 receptors (Inwang *et al.* 1997), therefore suggesting that Ang II could be involved in normal and abnormal tissue function (Tahmasebi *et al.* 1998). However, the precise role of Ang II in the breast is at the present time unclear, with the only functional information obtained in the

breast cancer epithelial cell line, MCF-7, where Ang II modulates integrin expression (Berry *et al.* 2000).

Our study is the first showing that MCF-7, a cancerous cell line derived from the pleural metastasis of a lobular human breast carcinoma, expresses mRNA for the AT1 and AT2 receptor subtypes. Resolution of RT-PCR products on agarose gels revealed that, under basal culture conditions, the specific transcripts for AT1 receptors exceed those for AT2 in accordance with the prevalence of AT1 receptors found in breast epithelial cells (Inwang *et al.* 1997).

Since AT1 receptor usually activates phosphatidylinositol turnover (Lundergan *et al.* 1999, Bataller *et al.* 2000, Hou *et al.* 2000, Kuwahara *et al.* 2000, Shen *et al.* 2001) or membrane-located Ca^{2+} channel (Gebke *et al.* 1998), we performed experiments aimed at understanding whether Ang II caused a rise in cytosolic free Ca^{2+} in MCF-7 cells. Results show that Ang II failed to evoke any increment in $[Ca^{2+}]_i$ while extracellular ATP (1–100 μM), used as a positive control, elevated $[Ca^{2+}]_i$ over basal in a dose-dependent manner. This result is consistent with other studies showing that in MCF-7 cells, ATP increases intracellular calcium levels through binding to a purinergic P2Y receptor (Flezar & Heisler 1993, Dixon *et al.* 1997, Wagstaff *et al.* 2000). The demonstration that Ang II is ineffective in eliciting a rise in $[Ca^{2+}]_i$ obviously suggests that its actions are mediated by other transduction mechanisms in MCF-7 cells. While this result is in contrast to those obtained in many cell types which exhibit Ca^{2+} mobilization leading to mitogenesis, it is consistent with other studies showing, for example, that Ang II activation of extra cellular-related kinase (ERK) is independent of Ca^{2+} mobilization (Li *et al.* 1998, Yang *et al.* 1999) or that the mechanisms by which Ang II inhibits the Na^+ pump in rat glomerulosa cells do not require Ca^{2+} transients (Yingst *et al.* 2000).

An AT1 receptor unable to mobilize calcium raises the question of whether it is functional or not, and to assess its functionality we looked for Ang II cellular targets. It is known that Ang II has important effects in controlling cellular electrolyte balance, and its role in the regulation of Na^+ transcellular movements through its actions on the activity of Na^+/K^+ATPase is well documented *in vivo* and in cultured cells (Aperia *et al.* 1994, Hussain *et al.* 1998, Buhagiar *et al.* 1999). We therefore assessed the ability of Ang II to modulate the Na^+/K^+ATPase activity of MCF-7 cells and showed that AT1 expressed by MCF-7 cells is functional, being able to regulate Na^+/K^+ATPase activity, as demonstrated in other cell types (Muscella *et al.* 1997, 2000, Marshall *et al.* 2000, Mondorf *et al.* 2000, Yingst *et al.* 2000, Zhang & Mayeux 2001), since the specific AT1 receptor subtype competitive antagonist DuP 753 completely blocked the Ang II effect. Based on this finding, the physiological role in ion homeostasis of Ang II can be extended to the control of the MCF-7 Na^+/K^+ATPase activity. This effect could be related to the process of secretion of epithelial cells or to the hyperpolarization observed in proliferating cells, including MCF-7 (Klimatcheva & Wonderlin 1999).

It seems plausible that this hyperpolarization may provide the adequate electrochemical gradient for the Na-dependent uptake of substrates, such as amino acids, glucose and nucleotides, required for cellular proliferation (Klimatcheva & Wonderlin 1999). Therefore, it is possible that an increase in Na^+/K^+ATPase activity may be linked to a mitogenic action of Ang II. Thus, in order to understand whether Ang II was able to modulate the growth of quiescent breast cancer epithelial cells, we performed cell counts, protein measurements and MTT assay in cells which had been treated with various amounts of Ang II. All assays for cell growth events indicated that Ang II induces a mitogenic response in quiescent MCF-7 cells in a dose-dependent manner which is mediated by AT1 since such effects were blocked by DuP 753, but not by the AT2 antagonist CGP 42112. Such Ang II-mediated proliferation of MCF-7 cells occurs without $[Ca^{2+}]_i$ increase. Even though $[Ca^{2+}]_i$ transients often end up with the activation of the immediate early genes responsible for cell cycle progression, examples of growth control independent of Ca^{2+} have, nevertheless, been reported, which are due to the activity of mitogen-activated protein kinases (MAPKs) (Cao *et al.* 1992). MAPKs can also be activated via G-protein coupled receptors which recruit and activate cytosolic tyrosine kinases (such as Hck, Lyn, JAK, and some members of c-Src tyrosine kinases) in a Ca^{2+}-independent manner (Wang *et al.* 1995) which, in turn, tyrosine-phosphorylate MAPKs. In various cell types Ang II is able to increase $[Ca^{2+}]_i$ and the expression of c-fos, c-Jun and c-myc (Naftilan *et al.* 1989, Taubman *et al.* 1989), and it also stimulates MAPKs (Duff *et al.* 1992, Ishida *et al.* 1992, Molloy *et al.* 1993). Accordingly, it would be reasonable to form the hypothesis that Ang II acts on MCF-7 growth by calcium-independent MAPKs activation.

In summary, we documented the effects of Ang II on Na^+/K^+ATPase activity and on cellular growth in MCF-7 cells, determining that these express an AT1 receptor not coupled to the intracellular Ca^{2+} pathway.

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